Optimisation of GnRH antagonist use in ART

Ouijdane Hamdine

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Optimisation of GnRH antagonist use in ART

Optimaliseren van het gebruik van GnRH antagonisten bij geassisteerde voortplantingstechnieken
(met een samenvatting in het Nederlands)

Proefschrift

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Ouijdane Hamdine
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Promotoren: Prof. dr. F.J.M Broekmans
Prof. dr. B.C.J.M. Fauser

Copromotor: Dr. M.J.C. Eijkemans

Voor mijn ouders

Contents

General introduction

Partly adapted from Hamdine *et al*. Ovarian stimulation for IVF: mild approaches.
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For years, ovarian stimulation has been an integral part of assisted reproductive treatment (ART). It has been applied with the aim of increasing the number of oocytes to compensate for inefficiencies in laboratory procedures enabling the selection of one or more embryos for fresh transfer and the cryopreservation of surplus embryos (1). Currently, a "long" gonadotropin-releasing hormone (GnRH) agonist protocol, either initiated during the luteal phase of the preceding cycle or after oral contraceptive pre-treatment, combined with high doses of exogenous follicle stimulating hormone (FSH) remains the most frequently used stimulation protocol for in vitro fertilisation (IVF) treatment (2). Extensive evidence for the supremacy of the long suppression protocol has led to its widespread use in IVF (2). This conventional approach is complex, expensive, time consuming and may give rise to considerable patient discomfort, substantial drop-out rates and risks for complications such as ovarian hyperstimulation syndrome (OHSS) (2;3). Undesirable effects inherent to the use of GnRH agonists are the incidental formation of ovarian cysts due to the "flare" effect, complaints of estrogen deprivation and the need for increased amounts of exogenous gonadotropins due to ongoing suppression of endogenous gonadotropins (4).

Another complication related to IVF treatment is the risk of multiple pregnancies. Such pregnancies bear an increased risk for late miscarriage, pre-eclampsia, growth retardation, gestational diabetes and preterm delivery, creating enormous health care expenditures. The occurrence of multiple pregnancies after IVF is directly related to the number of embryos transferred. Selection and transfer of more than one embryo is performed to compensate for the low implantation potential of human embryos (1). A high rate of aneuploidy is encountered in embryos arising from IVF which may explain the failure to obtain high implantation rates in IVF (5;6). In the Netherlands, single embryo transfer is performed especially in younger women (< 38 years) to minimise the risk of multiple pregnancies.

Finally, uncertainties remain regarding long-term health risks of IVF. So far, no causative association has been shown between ovarian stimulation with exogenous gonadotropins and increased risk of malignant ovarian disease although there may be an increased risk of borderline tumours (7). In children born after IVF, bone age appeared to be advanced in pubertal girls but not in boys compared with age-matched controls (8). Furthermore, higher systolic and diastolic blood pressure levels and fasting glucose levels as well as an altered body fat composition have been reported in 8-18 year old IVF children (9). These findings emphasise the importance of continued monitoring of IVF-conceived children, as well as the continued quest for further development of minimally invasive assisted reproduction methods.

Improved laboratory performance has reduced the need for large quantities of oocytes (10;11). Supportive evidence regarding a potentially negative effect of supraphysiological estradiol levels on endometrial receptivity (12;13), corpus luteum function (14;15) and oocyte/embryo quality (16-18) indicates that more modest responses to ovarian stimulation might have a beneficial effect upon the implantation potential of the embryo (3;19).

GnRH antagonists

The introduction of GnRH antagonists for the prevention of a premature LH rise has allowed for the development of more patient-friendly protocols. GnRH antagonists compete directly with endogenous GnRH by occupying the GnRH receptor and cause a rapid and immediate, reversible suppression of gonadotropin secretion (20). The immediate suppression and recovery of pituitary function after cessation of administration renders GnRH antagonists particularly suitable for short-term use in IVF (2;4;21). In contrast to GnRH agonists, GnRH antagonists are not associated with an initial stimulation of gonadotropins which avoids the problem of cyst formation. Additionally, because GnRH antagonists are generally started after the initiation of exogenous FSH stimulation, hypo-estrogenaemia is also prevented (3;21). Furthermore, GnRH antagonists are associated with reduced costs, a lower risk of complications such as OHSS (22;23) and a lower burden of treatment (24).

The duration of exogenous FSH administration is generally 1-2 days shorter and slightly fewer follicles are seen at the time of human chorionic gonadotropin (hCG) administration compared with a GnRH agonist. Therefore, the number of retrieved oocytes tends to be lower (25). Initial studies reported significantly lower pregnancy rates following GnRH antagonist treatment in comparison to GnRH agonists (21;26;27). However, the clinical efficacy of GnRH antagonists has recently been further established and non-significant differences in ongoing pregnancy and live birth rates were observed between GnRH antagonists and GnRH agonists (22;23).

Optimisation of GnRH antagonist protocols

Numerous studies have been performed to determine the minimally effective GnRH antagonist dose and treatment schedule in IVF patients. The initiation of recombinant FSH (recFSH) administration in a GnRH antagonist regimen is cycle phase dependent and is usually started on cycle day 2 or 3. Three general approaches for the initiation of the GnRH antagonist have emerged. A single large dose can be administered in the late follicular phase on stimulation day 8 or 9. In the multiple-dose protocol, daily small doses (0.25 mg) are given from stimulation day 5 or 6 onward. Alternatively, in the flexible protocol, daily small doses are initiated depending on the size of the dominant follicle or the estradiol level. The GnRH antagonist is continued until the day that hCG to trigger final oocyte maturation is administered (25;28-30). Luteal phase supplementation has been shown to be mandatory in GnRH antagonist co-treated cycles (14).

A number of treatment regimens have been studied to further optimise GnRH antagonist cycles. Increasing the recFSH (31) or human menopausal gonadotropin (HMG) (32) dose with 75 IU at GnRH antagonist initiation did not result in improved implantation or pregnancy rates. Furthermore, the addition of recombinant LH to recFSH during ovarian stimulation did not affect the live birth rate (33). There was no difference in the incidence of premature LH rises between a fixed or flexible GnRH antagonist, nor was there a difference in clinical outcome

(34;35). Finally, the use of a GnRH agonist trigger to induce final oocyte maturation in GnRH antagonist co-treated cycles with or without cryopreservation of all embryos has been proposed to reduce the risk of OHSS. With adequate luteal phase support no difference was observed in pregnancy rates between hCG triggering or GnRH agonist triggering (36), but with a clear effect on reducing the probability of OHSS occurring.

Currently, there is a growing consensus to support a fixed daily injection protocol starting on day 6 or 7 of the menstrual cycle (i.e. 5-6 days after initiation of stimulation) based on the simplicity of this protocol and the decreased GnRH antagonist consumption (25;28;29;34;35). However, the optimal protocol for routine clinical use has not yet been identified (25).

Profound suppression, elevation and fluctuation of LH has been associated with impaired pregnancy rates (29;37-40). However, others have reported no difference in pregnancy rates (41-45). Additionally, supraphysiological estradiol levels in IVF cycles have been associated with a detrimental effect on both endometrial receptivity and embryo quality (18;39;46), although others have not confirmed this association (47). Finally, elevated progesterone levels during the early or late follicular phase have been shown to negatively affect clinical outcome (48-56).

Previously, commencing GnRH antagonist treatment on stimulation day 1 as compared to day 6 was associated with a lower exposure to LH and estradiol, however, this study lacked the power to assess the impact on pregnancy rates (57). Hence, it can be hypothesized that early GnRH antagonist initiation will result in an improved follicular phase hormonal milieu, with better control of LH and progesterone fluctuations, and a reduction in supraphysiological estradiol levels with a possible beneficial effect on reproductive outcome.

Ovarian response prediction in GnRH antagonists

In this day and age, where cost reduction has become a fundamental issue in healthcare, knowledge of predictive factors of ovarian response and IVF outcome has become increasingly important in order to identify women who may or may not benefit from IVF treatment. Clinicians often use patient characteristics, such as female age, menstrual cycle length, body mass index (BMI) and results from previous IVF cycles to select a treatment protocol (58). As oocyte yield is considered to be an important prognostic variable, it is important to continue modifying IVF treatment protocols in order to optimise outcome in terms of increased live birth rates. Additionally, optimisation of IVF treatment should also result in a lower risk of OHSS, patient burden and costs.

Before one can modify stimulation regimens, it is essential to be able to predict a high ovarian response, as these patients are at increased risk of developing OHSS. Furthermore, by accurate prediction of a low response, women with little chance to conceive due to diminished ovarian reserve could be discouraged from starting IVF, especially when the high costs and burden of treatment are taken into account.

Several parameters, such as FSH, antral follicle count (AFC) and Anti-Müllerian hormone (AMH) have been suggested as predictors of ovarian response and clinical outcome (59). AMH, a member of the transforming growth factor-β family, is produced by granulosa cells of preantral and small antral follicles (60) and has low inter– and intracycle variability (61). The role of AMH has generally been studied in patients treated with a long GnRH agonist protocol. Previously, it has been demonstrated that AMH is an accurate predictor of both high (62) and low ovarian response in GnRH agonist cycles (63), suggesting it would be an ideal marker for the individualisation of ovarian stimulation strategies. Additionally, previous studies utilising AMH to tailor IVF treatment have shown a reduction in the incidence of high and low response as well as improved pregnancy rates compared with non-individualised treatment cycles (64;65).

A limited number of studies have addressed the value of ovarian reserve tests for ovarian response prediction in GnRH antagonist co-treated cycles (66-69). The difference in the accuracy of AMH for the prediction of response found among these studies may be caused by the use of different definitions for ovarian response category. Therefore, the question remains whether AMH is able to correctly predict ovarian response categories in GnRH antagonist co-treated cycles with similar accuracy as in GnRH agonist co-treated cycles.

Aims and outlines of the thesis

The studies presented in this thesis focus on the optimisation of controlled ovarian stimulation for IVF using exogenous FSH and GnRH antagonist co-treatment, by studying the timing of the GnRH antagonist co-medication as well as the role of ovarian reserve markers in optimising ovarian response and reproductive outcome.

The aims of the thesis can be listed as follows:

1. Can the GnRH antagonist co-treatment stimulation protocol for IVF be improved by a change in the timing of the co-treatment, with focus on:
   a. the stimulation phase endocrine profile
   b. the clinical outcome in terms of oocyte yield and pregnancy
2. Does AMH have a consistent role in ovarian response optimisation in GnRH antagonist co-treated stimulation cycles, as expressed by:
   a. its accuracy in ovarian response prediction
   b. its accuracy in the prediction of treatment outcome

Outlines of the thesis

Chapter 2 describes the results of a nested study within a multicentre randomised controlled trial (RCT) which studied the effect of early versus late GnRH antagonist initiation on the stimulation phase endocrine profile.

Chapter 3 describes the results of a multicentre RCT, studying the impact of an early or late start GnRH antagonist protocol on live birth rates.

Chapter 4 concerns the results of a nested study within the RCT and a systematic review on the impact of elevated early follicular progesterone levels on IVF outcome.

Chapter 5 studies the accuracy of AMH and other patient characteristics in the prediction of high and low ovarian response in GnRH antagonist co-treated cycles.

Chapter 6 studies the accuracy of AMH and other patient characteristics in the prediction of ongoing pregnancy rate in GnRH antagonist co-treated cycles.

Chapter 7 describes the results of the conducted studies and puts them in a broader perspective.

Early initiation of GnRH antagonist treatment results in a more stable endocrine milieu during the mid and late follicular phase:
a randomised controlled trial comparing GnRH antagonist initiation on cycle day 2 or 6

The CETRO Trial study group:

O. Hamdine, F.J. Broekmans, M.J.C. Eijkemans, B.J. Cohlen, A. Verhoeff, P.A. van Dop, R.E. Bernardus, C.B. Lambalk, G.J.E. Oosterhuis, C.A.G. Holleboom, G.C. van den Dool - Maasland, H.J. Verburg, P.F.M. van der Heijden, A. Blankhart, B.C.J.M. Fauser, J.S.E. Laven and N.S. Macklon

Fertil Steril. 2013;100(3):867-74

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Abstract

Objective:
To compare the effect of initiating GnRH antagonist on cycle day (CD) 2 vs. CD 6 on LH, estradiol and progesterone levels in the mid and late follicular phase.

Design:
Nested study within a multicentre randomised controlled trial.

Setting:
Reproductive Medicine Centre in an university hospital

Patients:
One hundred and sixty IVF/ intracytoplasmic sperm injection patients.

Interventions:
Recombinant FSH (150-225 IU) was administered daily from CD 2 onward. The study group (CD2) started GnRH antagonist co-treatment on CD 2, whereas the control group (CD6) started on CD 6.

Main Outcome Measure:
The follicular phase endocrine profile.

Results:
LH levels on CD 6 were lower in the CD2 group (0.6 ± 0.4 vs. 1.9 ± 1.4 IU/L). The CD2 group demonstrated both lower estradiol levels on CD 6 (520.1 ± 429.6 vs. 1071.7 ± 654.2 pmol/L) and on the day of hCG administration (3341.4 ± 1535.3 vs. 4573.2 ± 2445.4 pmol/L). Progesterone levels differed neither on CD 6 nor on the day of hCG.

Conclusions:
Early initiation of GnRH antagonist co-treatment results in a more stable endocrine profile, with more physiological levels of estradiol and LH during the follicular phase. The effect on clinical outcomes must be established in larger trials.

Trial registration number:
www.clinicaltrials.gov, no. NCT00866034

Introduction

Currently applied ovarian stimulation regimens for in vitro fertilisation (IVF) are complex, time consuming, expensive, and are associated with the risk of complications such as ovarian hyperstimulation syndrome (OHSS) (2). The availability of GnRH antagonists for the prevention of premature luteinisation, has resulted in the development of simpler, milder and cheaper stimulation protocols. These include the administration of lower doses of recombinant FSH (recFSH) (70) initiated either in the early or midfollicular phase (71), depot versus daily GnRH antagonist injections (29;72) and fixed versus flexible regimens (35). Although there is a growing consensus to support a daily injection protocol (29), and a fixed rather than flexible regimen (34), the optimal protocol for routine clinical use has not yet been identified (25).

Several studies have shown that current GnRH antagonist regimens are associated with variable follicular phase LH levels, and that both profound LH suppression, elevation and fluctuation are associated with an impaired probability of pregnancy (29;37-40). However, the importance of LH levels in this context continues to be debated, as others have reported no difference in pregnancy rates (41-45).

High estradiol (E_2) levels generated by ovarian stimulation with exogenous gonadotropins have been associated with a detrimental effect on both endometrial receptivity and oocyte/embryo quality, resulting in decreased pregnancy rates (18;39;46), although not all data confirm this association (47). While GnRH antagonist co-treatment in IVF is associated with lower peak E_2 concentrations than GnRH agonist co-treatment (73), ovarian stimulation with GnRH antagonists still induces supraphysiological E_2 serum levels that are 3 to 10 times the normal peak concentration reached in a spontaneous cycle (74). Similarly , there is a growing consensus that raised progesterone (P) levels at the end of the follicular phase are detrimental to clinical outcome (49;50;52-56;75).

In current practice, a cycle day (CD) 6 fixed start GnRH antagonist protocol is widely employed (29). However, an early start may result in an improved follicular phase hormonal milieu, with better control of LH and P fluctuations, and a reduction in supraphysiological E_2 levels which arise during ovarian stimulation. In a previous study, commencing GnRH antagonist treatment on stimulation day 1 as compared to day 6 was associated with a lower exposure to LH and E_2 (57). This study was not powered to assess any impact of early GnRH antagonist commencement on pregnancy rates.

It can be hypothesized that earlier initiation of GnRH antagonist treatment will result in a more consistent, and possibly beneficial follicular phase endocrine milieu compared to initiation on CD 6, as is currently advocated. The aim of this study was therefore to prospectively compare the effect of a cycle day 2 versus cycle day 6 fixed start GnRH antagonist protocol on LH, estradiol and progesterone levels in the mid and late follicular phase.

Material and Methods

Patient population

This study was part of a large open-label multicentre randomised controlled trial (RCT) conducted between September 2009 and July 2011 in the Netherlands. The study was approved by our Institutional Review Board, and registered on the Clinical Trial web site (www.clinicaltrials.gov, no. NCT00866034). For the nested study, 200 women undergoing IVF or intracytoplasmic sperm injection (ICSI) were recruited from the IVF outpatient clinic of the Department of Reproductive Medicine and Gynecology of the University Medical Centre Utrecht. Randomisation was performed according to a web based computer-generated randomisation schedule. The allocated treatment was not concealed from the clinicians nor from the patient. Informed consent was obtained from all patients and each patient was enrolled into the study only once. Inclusion criteria were: age ≤ 39 years; body mass index (BMI) ≤ 32 kg/m^2; regular cycle; regular indication for IVF or ICSI; and no more than 2 previous unsuccessful IVF/ICSI cycles. Patients with hypothalamic disease or polycystic ovary syndrome (PCOS) were excluded. Hormonal assessment was performed in 160 women.

Ovarian stimulation

Ovarian stimulation was performed with recFSH (Gonal-f; Merck Serono, the Netherlands). A GnRH antagonist (Cetrotide; Merck Serono, the Netherlands) was used to prevent a premature LH surge. Patients were not pretreated with oral contraceptives. Patients received one of the following treatment protocols; in the early fixed start group (CD2) both recFSH (150-225 IU) and a GnRH antagonist (0.25mg) were commenced on CD 2. In the late fixed start group (CD6) recFSH was administered from CD 2, whereas GnRH antagonist treatment was commenced on CD 6. Final oocyte maturation was induced by administrating 6500 IU of human chorionic gonadotropin (hCG; Ovitrelle; Merck Serono, the Netherlands), when at least one follicle of ≥ 18 mm in diameter and two follicles of ≥ 16 mm in diameter were visualised by ultrasound. Follicle growth was assessed by transvaginal ultrasound, starting from CD 6 and thereafter as necessary in order to ensure that hCG would be administrated when the criteria had been met. Oocyte retrieval was performed 36 hours after hCG administration. Conventional IVF was performed in 66 (41.3%) couples and ICSI in 94 (58.8%) couples. One or two embryos were transferred 3 days after oocyte retrieval. The luteal phase was supplemented with a daily dose of 600mg vaginally administered micronized natural progesterone (Utrogestan; Besins Healthcare, Brussels, Belgium).

Hormonal assessments

Hormonal assessment was performed in 160 patients on CD 2, CD 6 and day of hCG administration in both groups. All blood samples were drawn by venepuncture in the morning before GnRH

antagonist initiation and hCG administration. The results were available at a later stage and therefore could not affect the decisions made by the clinicians. Serum LH and progesterone levels were analysed on the Beckman-Coulter Unicel DXi800 (Woerden, the Netherlands). For LH, functional sensitivity [defined as 20% day-to-day coefficient of variability (CV)] was 0.5 U/L. The day-to-day CV was 7% at 1.4 IU/L, 5.8% at 20 IU/L and 5.2% at 60 U/L. For progesterone, functional sensitivity was 2 nmol/L. The day-to-day CV was 16-19% at 2.8 nmol/L, 8.5% at 32 nmol/L and 8% at 103 nmol/L. Serum estradiol was analysed on the Roche E170 modular immunoanalyser (Almere, the Netherlands). The functional sensitivity for estradiol was 40 pmol/L (with singleton measurements or 20 pmol/L in duplo). The day-to-day CV was 9-11% at 65 pmol/L, 4.5% at 200 pmol/L and 2.8% at 600 pmol/L.

Outcome measures

The primary endpoint of this evaluation was the endocrine profile in the mid and late follicular phase. A premature LH rise was defined as LH ≥ 10 IU/L. A progesterone rise was defined as P > 4.77 nmol/L (> 1.5 ng/mL).

Sample size and data analysis

This study was planned as a nested study in the largest participating centre within a multicentre RCT. A total number of 160 participants was needed to provide 80% power to detect a difference in LH on the day of hCG of 0.45 IU/L between the groups, assuming a standard deviation (SD) of 1 and alpha of 0.05. The expected number of participants in this centre was deemed sufficient to detect this difference. An intention-to-treat analysis was performed. Data for continuous variables are presented as mean values and standard deviation. Between-group statistical comparisons of mean values were performed with *t*-tests on each assessment day. Analysis of covariance (ANCOVA) was used to make comparisons on cycle day 6 and day of hCG. The groups were also compared with respect to the variation, by applying Levene's test for equality of variances. Pearson's correlation coefficient was used to assess the association between hormones. Differences were considered to be statistically significant if *P* value < 0.05.

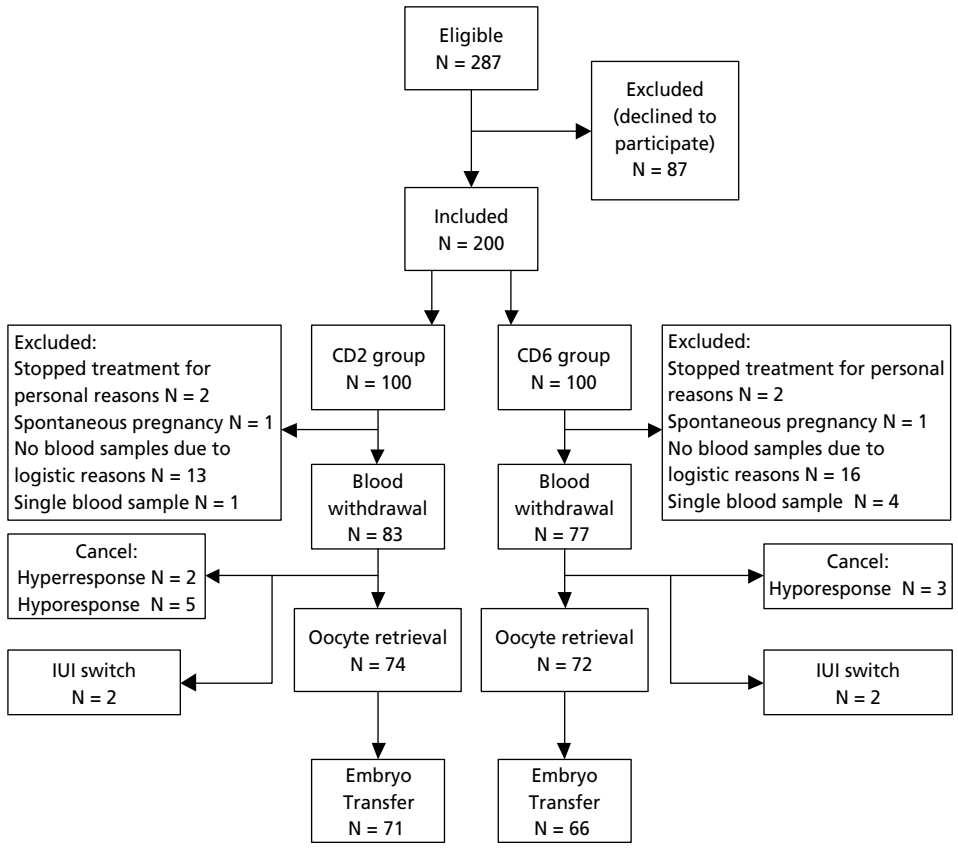
Results

Subjects and ovarian stimulation characteristics

Two hundred patients were included in the study. For various reasons, hormonal sampling was not completed in 40 patients and therefore these patients were excluded in the intention-to-treat analysis (Fig. 1). Protocol violation occurred in 5 cases. In the CD2 group (n = 83), the recFSH dose was increased to 225 IU in 5 patients. Dosage increase did not occur in the CD6

group (n = 77). These patients were all included in the intention-to-treat analysis. In the CD2 group, 9 patients did not undergo oocyte retrieval, compared with 5 patients in the CD6 group (Fig. 1). The number of cancellations due to either hyperresponse (two after CD2 start and none after CD6 start) or hyporesponse (five after CD 2 start and three after CD6 start) did not significantly differ between the two groups.

Figure 1 Flow chart showing the number of participants at each stage of the trial



There were no significant differences between the CD2 and the CD6 group, with regard to age (32.6 ± 3.5 yr. vs. 32.3 ± 4.2 yr., respectively, p = 0.7) and BMI (23.6 ± 3.0 kg/m^2 vs. 23.0 ± 2.7 kg/m^2, respectively, p = 0.2). Stimulation characteristics are shown in Table 1. The duration of stimulation as well as the total dose of recFSH consumed were similar in both groups. The number of follicles ≥ 12 mm on the day of hCG administration and the number of oocytes retrieved were significantly lower in the CD2 group (Table 1). This study was not powered to detect any difference in clinical outcome. No difference was observed between the study and control group with regard to fertilisation rate (58.8 ± 25.8 vs. 55.1 ± 26.0%, respectively, p = 0.7), implantation rate (26.1 ± 43.0 vs. 28.0 ± 43.1, respectively, p = 0.8), and ongoing pregnancy rate per started cycle [25.3% (21/83) vs. 27.3% (21/77), respectively, p = 0.8]. There was no difference between the two groups with regard to the incidence of OHSS. Mild to moderate OHSS occurred in 1 CD2 patient and in 3 CD6 patients, whereas there was only 1 case of severe OHSS which occurred in the CD2 group.

Table 1 Stimulation characteristics

	CD2 group (n = 83)	**CD6 group (n = 77)**	***P* value**
Total dose of recFSH (IU)	1456.6 ± 411.5	1412.3 ± 406.0	0.5
Total duration of stimulation (days)	9.3 ± 2.1	9.0 ± 2.1	0.5
Number of follicles ≥ 12 mm on day of hCG	8.0 ± 3.7	9.5 ± 4.7	**0.037**
Number of oocytes retrieved	7.9 ± 4.4	9.6 ± 5.6	**0.048**

Data are presented as means ± standard deviation.

P value for between-group difference from t-tests. P values in bold are statistically significant.

Endocrinology

The endocrine profiles of both groups are depicted in box and whisker plots (Fig. 2), whereas mean hormonal levels on CD 2, CD 6 and day of hCG for both groups are shown in Table 2.

In neither group were LH rises observed on CD 6 or on the day of hCG administration. LH levels on CD 6 were significantly lower in the CD2 group. The variation in LH levels measured on CD 6 was also much more limited in the CD2 group (SD 0.4 vs. 1.4, $p < 0.001$).

Significantly lower E_2 levels were observed in the CD2 group, on both CD 6 and the day of hCG. Likewise, the range of E_2 levels was much narrower in the CD2 group, both on CD 6 (SD 429.6 vs. 654.2, $p < 0.001$) and the day of hCG (SD 1535.3 vs. 2445.4, $p < 0.001$). Additionally, the mean E_2 level per oocyte was significantly lower in the CD2 group (479.3 ± 210.6 vs. 597.5 ± 413.0, $p = 0.036$). This difference was related to the difference in LH between the groups. After adjustment for the LH level, the difference in E_2 levels per oocyte was no longer significant ($p = 0.6$).

A positive correlation was observed between LH on CD 6 and LH on the day of hCG in a combined group analysis ($r = 0.37$, $p = 0.002$ for the total study group). Likewise, low or high E_2 levels on CD 6 remained low or high on the day of hCG (CD2; $r = 0.32$, $p = 0.007$ and CD6; $r = 0.43$, $p < 0.001$).

P levels did not differ between the two groups on CD 2, CD 6 or on the day of hCG in either mean values or range. Elevated P levels (> 4.77 nmol/L) at initiation of stimulation, were observed in 11 patients in the CD2 group (range 4.9 – 13.0 nmol/L), and in 10 CD6 patients (range 4.8 – 64.0 nmol/L). Elevated P levels on the day of hCG were observed in 9 patients in the CD2 group (range 4.9 – 15.0 nmol/L), compared with 12 patients in the CD6 group (range 4.9 – 7.4 nmol/L). Among those with elevated P levels on the day of hCG, one CD2 and two CD6 patients had already demonstrated an elevated P level at initiation of stimulation.

Figure 2 Box (median values and 25th and 75th percentiles) and whisker (P_5 and P_{95}) plots representing LH (IU/L), estradiol (E_2; pmol/L) and progesterone (P; nmol/L) on cycle day (CD), CD 6 and day of hCG for both the CD2 and CD6 group

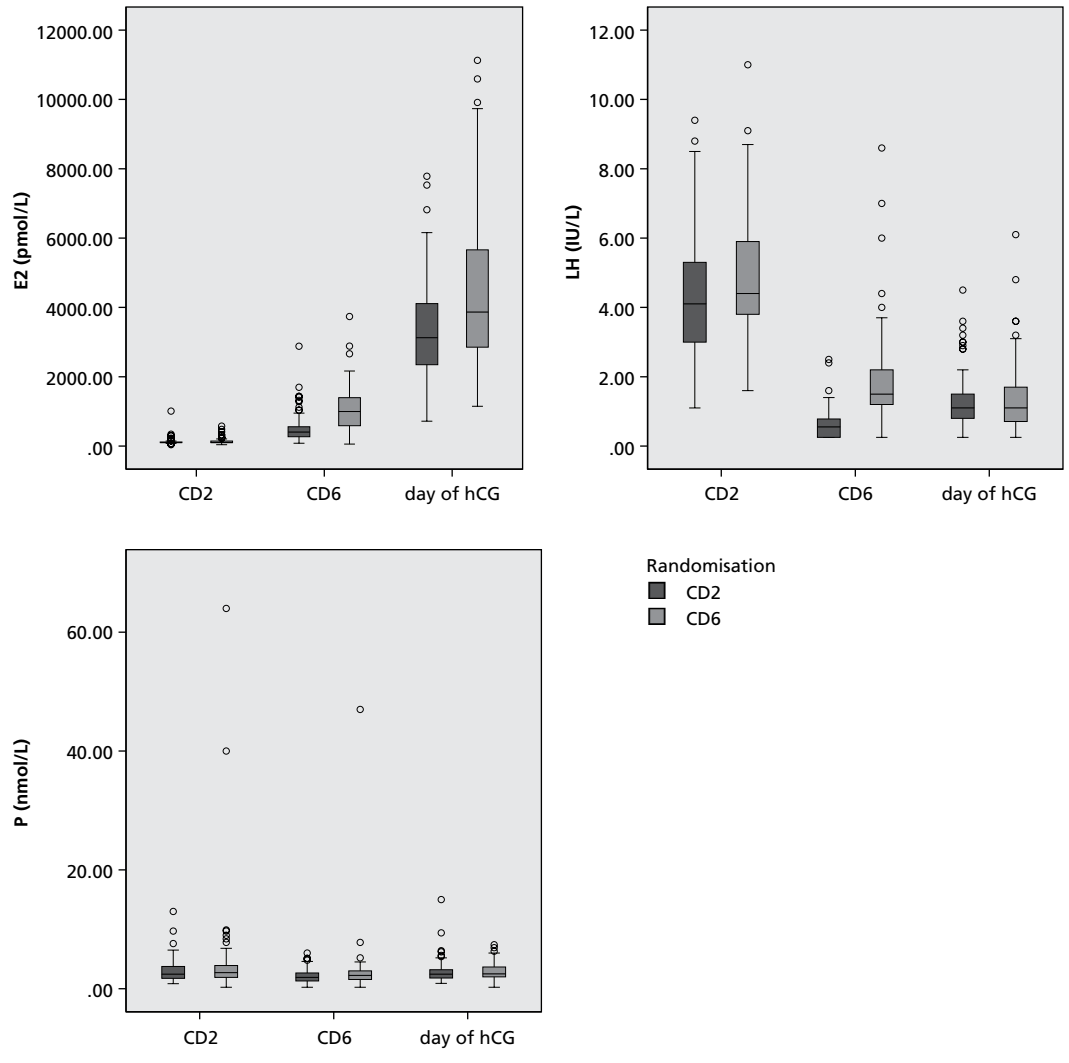


Table 2 Follicular phase endocrine characteristics

	CD2 group (n = 83)	CD6 group (n = 77)	*P* value
LH CD 2 (IU/L)	4.4 ± 1.8	4.9 ± 1.8	0.1
LH CD 6 (IU/L)	0.6 ± 0.4	1.9 ± 1.4	**< 0.001**
LH day hCG (IU/L)	1.3 ± 0.9	1.4 ± 1.1	0.6
E_2 CD 2 (pmol/L)	129.1 ± 112.0	139.5 ± 91.0	0.5
E_2 CD 6 (pmol/L)	520.1 ± 429.6	1071.7 ± 654.2	**< 0.001**
E_2 day hCG (pmol/L)	3341.4 ± 1535.3	4573.2 ± 2445.4	**< 0.001**
P CD 2 (nmol/L)	3.0 ± 2.0	4.4 ± 8.3	0.2
P CD 6 (nmol/L)	2.1 ± 1.2	2.9 ± 5.3	0.2
P day hCG (nmol/L)	3.0 ± 2.1	3.0 ± 1.6	0.9

Data are presented as means ± standard deviation.

P value for between-group difference from t-tests. P values in bold are statistically significant.

LH, luteinizing hormone; E_2, estradiol; P, progesterone

Discussion

This study demonstrates that early initiation of GnRH antagonist co-treatment for ovarian stimulation, compared with standard initiation on CD 6, results in lower and less variable midfollicular levels of LH and of E_2 during the mid and late follicular phase, without any significant effect on follicular P levels. These findings indicate a possible improvement of the hormonal milieu in ovarian stimulation for IVF, where over-exposure to gonadotropins and steroids becomes limited.

More stable and reduced LH levels after early initiation are a direct effect of GnRH antagonist co-treatment. Previous studies on pharmacokinetic and pharmacodynamic characteristics of the GnRH antagonists Ganirelix and Cetrorelix, have demonstrated an initial decrease in LH, FSH and E_2 levels 24 hours after the first injection, followed by a gradual increase during the rest of the treatment period. LH appeared suppressed to a larger extent than FSH and E_2 (37;72;76).

The lower E_2 output per oocyte, observed in the CD2 group, is likely to reflect reduced E_2 biosynthesis. The 'two cell, two gonadotropin' concept indicates that the more profound suppression of endogenous LH by early initiation of GnRH antagonist results in reduced stimulation of the theca cells, and hence a reduced presence of androgen substrate for FSH modulated conversion by aromatase to E_2 in granulosa cells (77).

The LH and E_2 levels observed after initiation of GnRH antagonist on CD 2 are consistent with those reported by Kolibianakis *et al.* (2003), who demonstrated lower LH and E_2 levels in the midfollicular phase as well as lower E_2 levels on the day of hCG in the CD2 starting arm (57). In the present study, LH on CD 6 was positively correlated to LH on the day of hCG. The same observation was noticed for E_2 on CD 6 and E_2 on the day of hCG. This may mean that in case of elevated midfollicular LH levels, standard initiation of the GnRH antagonist on CD 6 results in an earlier increase in E_2 levels, as LH levels may drive the steroid biosynthesis more intensely, as predicted by the 'two cell, two gonadotropin' concept. Early and rapid E_2 rises may by itself elicit more frequent LH rises in these cases, an event that will be largely prevented when the GnRH antagonist has been initiated early in the cycle. Conversely, a high exposure to both LH and E_2 in the early follicular phase has been associated with a reduced chance of pregnancy (39).

In the present study, spontaneous LH surges on CD 6 or on the day of hCG were observed in neither arm of the study. Premature LH surges occasionally occur prior or after GnRH antagonist initiation. The incidence varies and a wide range from 1.4 – 35% has been reported (29;35;78). It is well known that GnRH antagonist action is characterised by an immediate, reversible suppression of pituitary gonadotropin secretion by competitive occupancy of the GnRH receptor (73). Still, LH peak suppression in general may be effected by other mechanisms than the action of the GnRH antagonist alone, such as release of high amounts of E_2 or gonadotropin surge inhibitors from the ovaries. It is also possible that in the period between the GnRH antagonist injections, the pituitary is not continuously protected against the feedback effects of E_2, resulting in activation of intracellular mechanisms that enhance gonadotropin secretion (79). In turn, this could lead to premature luteinisation and early rises in progesterone.

Early GnRH antagonist initiation did not affect P levels on day 6 and day of hCG, despite a lower number of follicles and lower levels of both LH and E_2. The mechanisms responsible as well as the impact of elevated P levels on clinical outcome remains a subject of much debate. Little information is available concerning the association between elevated P levels at initiation of stimulation and IVF outcome in terms of ongoing pregnancy. The incidence of abnormal P levels on CD 2 has been reported to be between 4.9% (51) and 6.2% (48). In our study, which involved a smaller patient population, elevated P levels at initiation of stimulation were present in 13.1% of patients. Kolibianakis *et al.* (2004) demonstrated decreased pregnancy rates in case of elevated P levels on day 2 of the cycle, and proposed that high P levels on day 2 might be attributed to residual corpus luteum activity, resulting in advanced or disrupted endometrial development (51). However, while administration of GnRH antagonist during 3 consecutive days prior to initiation of stimulation resulted in normalisation of P levels, this was not associated with improved pregnancy rates (48).

Raised P levels at the end of the follicular phase have been reported in up to 38% of GnRH antagonist cycles (49;55;56). In the present study the overall incidence was 13.8%. Several

studies have suggested a negative impact on IVF outcome (49;50;52-56;75). It remains unclear whether this is caused by an adverse effect on the endometrium or because of a possibly negative effect on oocyte/embryo quality. P rises might be attributed to an excessive number of follicles with each one producing a normal amount of P, rather than to premature luteinisation (55). Moreover, high P levels on the day of hCG have been associated with the administration of higher doses of FSH and a longer duration of stimulation. This phenomenon has been attributed to increased granulosa cell steroidogenic activity caused by intense FSH stimulation rather than to excessive LH activity (49).

A weakness of the present study is the lack of power to assess the impact of the two studied protocols on pregnancy rates. Previously, a meta-analysis comparing a fixed (day 6) with a flexible (according to leading follicle size) approach of GnRH antagonist initiation (34), revealed a trend towards a higher pregnancy rate in favour of the fixed protocol. However, flexible GnRH antagonist administration started before day 6 resulted in higher ongoing pregnancy rates (80). This was possibly due to reduced exposure to LH and E_2.

A further limitation of this study is the frequency of endocrine measurements made. These were performed 3 times during the stimulation period, whereas more repeated measurements during the follicular phase would have resulted in a better representation of the endocrine profile. In this study FSH levels were not measured. This was decided because a previous study reported no difference in serum FSH levels on day 6 of stimulation and day of hCG, nor in FSH exposure following early versus midfollicular phase commencement of GnRH antagonist treatment (57). Therefore, a lower exposure to endogenous FSH as an explanation for the observed effects on LH and E_2 secretion seems unlikely. Similarly, there is no evidence to suggest a deeper endogenous FSH suppression in the early start group as the number of cycle cancellations due to a hyporesponse did not differ significantly between the two groups.

The strength of this study is the randomised controlled design. The study protocol required that the dose of recFSH remain fixed throughout the entire stimulation period. However, the dose was adjusted according to ovarian response in 5 CD2 patients. These patients were not excluded, as variations in usage of recFSH were deemed not to influence the endocrine outcomes, which were the focus of the present study, since it has been demonstrated that FSH dosages over 150 IU daily will not alter the stimulation or response level of the ovaries (70;81). The difference in the need for dose adjustment therefore might be caused by the presence of a small or slowly developing follicle cohort.

Furthermore, a separate per protocol data analysis (data not shown) revealed that the differences observed in hormonal levels between the two groups were similar compared to the intention-to-treat analysis. Therefore, these differences were not biased by different dosage levels of gonadotropins used during stimulation.

The observations in this study may have relevance for the optimisation of GnRH antagonist

stimulation cycles for IVF. Supraphysiological E_2 levels have been associated with an adverse effect on endometrial receptivity as well as on oocyte/embryo quality. An early start with a GnRH antagonist could be beneficial for both endometrial and oocyte quality, as E_2 levels appear to be better controlled. Previously, it has been demonstrated that midfollicular administration of GnRH antagonist may induce a transient follicular arrest without triggering new folliculogenesis, depending on the magnitude and duration of gonadotropin suppression (82). Additionally, early initiation of the GnRH antagonist might result in decreased follicular recruitment. Indeed, a small but statistically significant effect on the number of follicles and oocytes obtained was observed in the present study (Table 1). If this effect is confirmed in larger trials without a negative impact on pregnancy rates, it may aid in the normalisation of ovarian response and in the reduction of the risk of OHSS in predicted high responders. Furthermore, it may be of importance in the development of milder stimulation regimens (11).

In summary, this study shows that early initiation of GnRH antagonists results in an endocrine milieu that is more stable and closer to the normal cycle conditions, with lower levels of LH and E_2 during the mid and late follicular phase. The effect of early GnRH antagonist initiation on pregnancy achievement and its place with regard to the optimisation of GnRH antagonist protocols remains to be established in larger clinical trials focussing on clinical outcome.

Comparison of early vs. late initiation of GnRH antagonist co-treatment for controlled ovarian stimulation in IVF: a randomised controlled trial

The CETRO Trial study group:

O. Hamdine, F.J. Broekmans, M.J.C. Eijkemans, B.J. Cohlen, A. Verhoeff, P.A. van Dop, R.E. Bernardus, C.B. Lambalk, G.J.E. Oosterhuis, C.A.G. Holleboom, G.C. van den Dool - Maasland, H.J. Verburg, P.F.M. van der Heijden, A. Blankhart, B.C.J.M. Fauser, J.S.E. Laven and N.S. Macklon

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3

Abstract

Study question: What is the impact of initiating GnRH antagonist co-treatment for in vitro fertilisation (IVF) on cycle day (CD) 2 on live birth rate per started cycle (LBR) and on the cumulative live birth rate (CLBR) compared to initiation on CD 6?

Summary answer: Early initiation of GnRH antagonist does not appear to improve clinical outcomes of IVF compared with midfollicular initiation.

What is known already: During ovarian stimulation for IVF, GnRH antagonist co-treatment is usually administered from the midfollicular phase onwards. Earlier initiation may improve the follicular phase hormonal milieu and therefore overall clinical outcomes.

Study design, size, duration: Open-label, multicentre randomised controlled trial, conducted between September 2009 and July 2011. A web based program was used for randomisation. 617 IVF-intracytoplasmic sperm injection (ICSI) patients were included.

Participants/materials, setting, methods: Recombinant FSH (150-225 IU) was administered daily from CD 2 onwards in both groups. The study group (CD2; n = 308) started GnRH antagonist co-treatment on CD 2, whereas the control group (CD6; n = 309) started on CD 6.

Main results and the role of chance: There were no significant differences in clinical outcomes between the two groups. A non-significant trend towards a higher live birth rate per started cycle and cumulative live birth rate was observed in the CD6 group compared with the CD2 group (LBR: 24.0% vs. 21.5%, $p = 0.5$; CLBR: 29.9% vs. 26.7%, $p = 0.6$).

Limitations, reasons for caution: The study was terminated prematurely because no significant difference was observed in clinical outcomes after 617 inclusions. A much larger study population would be needed to detect a small significant difference in favour of either study arm, which raises the question whether this would be relevant for clinical practice.

Wider implications of the findings: The present study shows that the additional treatment burden and costs of starting GnRH antagonist on CD 2 versus CD 6 are not justified, as early initiation of GnRH antagonist does not improve live birth rates.

Trial registration number: www.clinicaltrials.gov, no. NCT00866034

Introduction

The clinical efficacy of GnRH antagonist co-treatment for the prevention of premature luteinisation during ovarian hyperstimulation in in vitro fertilisation (IVF), has recently been established. GnRH antagonists have been shown to offer increased safety and reduced costs compared with GnRH agonist cycles, with no clear significant difference in ongoing pregnancy rate and live birth rate (22). Currently, there is a growing consensus to support a fixed daily injection protocol starting on day 6 or 7 of the menstrual cycle (i.e. 5-6 days after initiation of stimulation) (28;29;34). However, the optimal protocol for routine clinical use has not yet been identified (25).

Starting GnRH antagonist co-treatment in the midfollicular phase may be too late in some patients. Several studies have demonstrated the negative impact of hormonal fluctuations during the follicular phase on IVF outcomes. Previous research has indicated that prevention of high LH levels during the follicular phase may improve endometrial receptivity and hence pregnancy rates (37;39;40;57). However, others have reported no impact on pregnancy rates (41-43;45). Additionally, both high estradiol (E_2) levels and progesterone (P) levels have been associated with impaired endometrial receptivity and oocyte/embryo quality (18;39;46;49;50;52-54). Furthermore, an excessive ovarian response has been shown to markedly reduce implantation rates in both mild stimulation and GnRH agonist cycles (11).

The possible benefits of starting GnRH antagonist treatment at the initiation of stimulation is indicated by two studies that showed this approach to result in more physiological levels of both LH and E_2 during the follicular phase (57;98). This protocol may improve the chance of achieving in-phase endometrial maturation at the time of embryo transfer, thereby improving clinical outcomes. Early initiation of the GnRH antagonist may also decrease the incidence of premature LH surges and hence premature luteinisation. Moreover, early initiation of GnRH antagonist may also moderate ovarian response by early suppression of endogenous FSH and subsequently reduce endometrial exposure to E_2 (98), with consequent beneficial effects on endometrial receptivity. Consequently, a moderate ovarian response could reduce the risk of the potentially life-threatening ovarian hyperstimulation syndrome (OHSS).

For these reasons, it could be hypothesized that in many women, an early start of GnRH antagonist treatment would improve live birth rates. The aim of this study was therefore to prospectively compare the effect of an early fixed start [cycle day (CD) 2] versus a late fixed start (CD 6) GnRH antagonist protocol on single cycle and cumulative live birth rates, and on adverse events such as OHSS.

Subjects and Methods

Patient population

This open label, multicentre randomised controlled trial was conducted between September 2009 and July 2011 in the Netherlands. The study was approved by the Institutional Review Board (IRB) of each participating centre, and registered on the Clinical Trial web site (www.clinicaltrials.gov, no. NCT00866034). Six hundred and seventeen women undergoing IVF or intracytoplasmic sperm injection (ICSI) were recruited from the IVF outpatient clinics of 13 fertility centres. Randomisation was performed according to a web based computer-generated randomisation schedule. The allocated treatment was concealed neither from the clinicians nor from the patient. Informed consent was obtained from all patients and each patient was enrolled into the study only once. Inclusion criteria were: age ≤ 39 years; body mass index (BMI) ≤ 32 kg/m^2; regular cycle; regular indication for IVF or ICSI; and no more than 2 previous unsuccessful IVF/ICSI cycles. Patients with World Health Organization (WHO) class 1 and 2 anovulation were excluded.

Ovarian stimulation

Ovarian stimulation was performed with recombinant FSH (recFSH, Gonal-f; Merck Serono, the Netherlands, or Puregon; MSD, the Netherlands). A GnRH antagonist (Cetrotide; Merck Serono, the Netherlands, or Orgalutran; MSD, the Netherlands) was used to prevent a premature LH surge. Two centres used oral contraceptive pretreatment (OCP) to minimise weekend oocyte retrievals. Patients were randomised to receive one of the following treatment protocols: in the early fixed start group (CD2) both recFSH (150-225 IU) and a GnRH antagonist (0.25 mg) were administrated from CD 2 onward. In the late fixed start group (CD6), recFSH (150-225 IU) was administered from CD 2, and GnRH antagonist treatment was started on CD 6. Dose adjustment according to ovarian response was regarded as a protocol violation. RecFSH was administered up to, but not including, the day of hCG administration, whereas GnRH antagonist treatment continued to include the day of human chorionic gonadotropin (hCG) administration. Final oocyte maturation was induced by administering 6500 IU of hCG (Ovitrelle; Merck Serono, the Netherlands), when at least one follicle of ≥ 18 mm in diameter and two follicles of ≥ 16 mm in diameter were visualised by ultrasound. Follicle growth was assessed by transvaginal ultrasound, starting from CD 6 and thereafter as often as necessary in order to ensure that hCG would be administered when the criteria had been met, with the possibility of postponing hCG triggering by maximally one day. Oocyte retrieval was performed 36 hours after hCG administration. One or two embryos were transferred 3 or 4 days after oocyte retrieval according to local protocols. The luteal phase was supplemented with a daily dose of 600 mg vaginally administered micronized natural progesterone (Utrogestan; Besins Healthcare, Brussels, Belgium). All patients underwent one treatment cycle as part of this protocol. An outline of the two treatment regimens applied is depicted in appendix 1.

Outcome measures

The primary outcomes of this study were live birth rate per started cycle (LBR), and cumulative live birth (CLBR) from fresh and/or cryopreserved embryos originating from, and transferred within six months of the initial treatment cycle. Secondary outcomes included the duration of stimulation; total cumulative dose of recFSH consumed; number of oocytes retrieved; fertilisation rate; number of suitable embryos; implantation rate; biochemical, clinical and ongoing pregnancy rate.

The occurrence of OHSS, cycle cancellation due to a risk of OHSS and poor response were evaluated as safety endpoints.

Power analysis

Based on a power of 80% and an alpha of 5% a study population of 1105 patients per arm was required to demonstrate an increase in live birth rate in a fresh cycle from 20% to 25% in the early fixed start group. Taking a ~9% loss of patients into account, an inclusion of 1215 patients per arm was deemed necessary. The total sample size of the study was therefore 2430 patients. An interim analysis was planned after half of the inclusions had been reached, to evaluate the efficacy of both protocols and to reduce the number of patients needed to include or discontinue the trial for safety, ethical, compliance or efficacy reasons. However, due to a limited rate of patient inclusion during the first two years of execution, the interim analysis was performed after the inclusion of 617 patients. Data from 484 patients with a completed fresh IVF treatment cycle were available for the interim analysis, which was performed on ongoing pregnancy rate per started fresh cycle, because the primary endpoint live birth was not yet available for all patients. There appeared to be no significant difference in ongoing pregnancy rates between the two groups, although a trend towards higher ongoing pregnancy rates was observed in the CD6 group [21.9% vs. 20.8%, difference 1.1%, 95% confidence interval (CI) -8.6 – 6.4]. Since the effect of the different protocols was counter to that hypothesized, a new power calculation was performed. This calculation revealed that, in order to show this difference at interim analysis to be significant with an alpha of 5% and a power of 80%, a study population of 2415 instead of 1215 patients per arm would have been required. Since confirming superiority of the standard treatment over the experimental protocol was considered to be of insufficient clinical value, the study was terminated after IRB approval.

Statistical analysis

Data for continuous variables are presented as mean values and standard deviation (SD). Between-group statistical comparisons of mean values were performed with *t*-tests. Chi squared tests and Fisher's Exact tests were used for count data. Logistic regression tests were used to check for differences between the centres. Differences were considered to be statistically significant if P value < 0.05. An intention-to-treat (ITT) analysis as well as a per-protocol (PP) analysis were performed. Both are shown in the results tables. Because no difference was observed between the two analyses, the ITT analysis will be mainly discussed.

Results

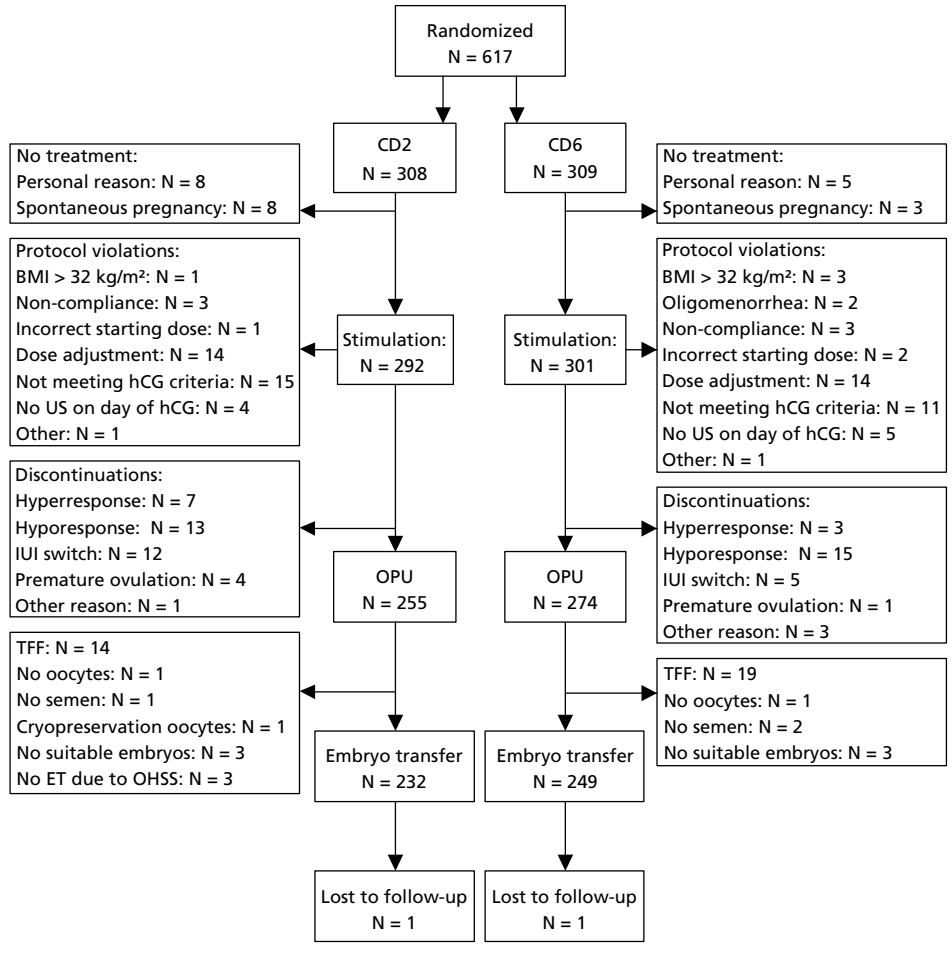
Study progression

A total of 617 patients consented to participate in the study. IVF was performed in 296 (48.0%) couples and ICSI in 297 (48.1%) couples. Figure 1 charts the flow of both groups through each stage of the trial. Twenty four patients withdrew prior to the start of treatment for personal reasons or as a result of conceiving naturally. A total of 593 patients started stimulation in one of the two treatment arms. Oocyte retrieval was performed in 529 patients, of whom 481 proceeded to fresh embryo transfer. Protocol violations occurred in a total of 80 cases, however these patients were all included in the ITT analysis. In the CD2 group, the recFSH dose was increased to 225 IU in 13 patients. The dose was reduced to 112.5 IU in one patient. In the CD6 group, the dose was increased to 225 IU in 7 patients and to 300 IU in 4 patients. Dosage reduction to 112.5 IU occurred twice, and to 75 IU once. In each group one patient was lost to follow up. Subject demographics as well as fertility characteristics are shown in Table 1.

Clinical outcome

The treatment characteristics are depicted in Table 2. Logistic regression showed no difference in IVF outcomes between the 13 fertility centres. Additionally, pregnancy rates did not appear to be affected by OCP pretreatment. Premature ovulation occurred 4 times in the CD2 group (1.3%), and once in the CD6 group (0.3%). The total dose of recFSH used, as well as the total duration of stimulation, did not differ between the two groups. Furthermore, no differences were observed with regard to number of oocytes retrieved, fertilisation rate, number of embryos suitable for transfer and number of embryos transferred or cryopreserved.

Figure 1 Flowchart showing the numbers of participants at each stage of the trial. Protocol violations (n = 80) and patients who discontinued prior to the start of treatment (n = 24) were included in the intention-to-treat analysis and excluded in the per-protocol analysis.



US, ultrasound; IUI, intra-uterine insemination; OPU, oocyte pick-up; TFF, total fertilisation failure; ET, embryo transfer; OHSS, ovarian hyperstimulation syndrome

Table 1 Demographics and fertility characteristics per treatment group

	Intention-to-treat			Per protocol		
	CD2 group (n = 308)	**CD6 group (n = 309)**	***P* value**	**CD2 group (n = 253)**	**CD6 group (n = 260)**	***P* value**
Demographics						
Age (years)	32.1 ± 3.9	32.2 ± 4.2	0.9	32.0 ± 4.0	32.2 ± 4.1	0.6
Height (cm)	170.3 ± 7.1	170.7 ± 7.1	0.5	170.6 ± 7.0	170.8 ± 7.1	0.8
Weight (kg)	68.5 ± 11.1	69.9 ± 11.7	0.1	68.3 ± 10.5	69.6 ± 10.8	0.2
BMI (kg/m²)	23.6 ± 3.5	24.0 ± 3.5	0.2	23.5 ± 3.4	23.8 ± 3.2	0.2
Fertility characteristics						
Primary infertility, n (%)	196 (63.6)	215 (69.8)	0.1[a]	167 (66.0)	181 (69.6)	0.4[a]
Duration of infertility (years)	2.7 ± 1.9	2.8 ± 1.9	0.6	2.7 ± 1.9	2.8 ± 1.8	0.9
Cause of infertility, n (%)						
Male factor	182 (59.1)	190 (61.7)		158 (62.5)	163 (62.7)	
Unexplained	83 (26.9)	79 (25.6)		66 (26.1)	63 (24.2)	
Tubal factor	34 (11.0)	27 (8.8)	0.7[a]	24 (9.5)	24 (9.2)	0.8[a]
Endometriosis	8 (2.6)	8 (2.6)		4 (1.6)	7 (2.7)	
Other	1 (0.3)	4 (1.3)	0.4[b]	1 (0.4)	3 (1.2)	0.6[b]

Data are presented as means ± standard deviation (SD) unless otherwise stated.

P value for between-group difference from t-tests unless otherwise stated.

[a] *P value for between-group difference from Chi squared tests.*

[b] *P value for between-group difference from Fisher's Exact tests.*

Table 2 Clinical parameters from the stimulation phase up to the embryo transfer per started cycle

	Intention-to-treat			Per protocol		
	CD2 group (n = 308)	**CD6 group (n = 309)**	***P* value**	**CD2 group (n = 253)**	**CD6 group (n = 260)**	***P* value**
Stimulation characteristics						
Pretreatment with OCP, n (%)	19 (6.4)	16 (5.3)	0.6[a]	17 (6.7)	15 (5.8)	0.7[a]
Total dose of rec-FSH (IU)	1526 ± 439	1527 ± 473	1.0	1491 ± 393	1481± 413.9	0.8
Total duration of stimulation (days)	9.7 ± 2.3	9.5 ± 2.3	0.3	9.5 ± 2.0	9.2 ± 2.0	0.2
Clinical outcome per started cycle						
Oocytes retrieved	9.1 ± 5.8	9.2 ± 5.7	0.8	9.5 ± 6.0	9.4 ± 5.6	0.8
2PN oocytes	4.7 ± 3.7	4.7 ± 4.0	0.9	4.9 ± 3.9	4.9 ± 4.0	1.0
Fertilisation rate (%)	54.6 ± 27.7	52.7 ± 28.0	0.4	54.2 ± 28.1	53.1 ± 27.6	0.7
Embryos suitable for transfer	4.4 ± 3.6	4.5 ± 3.9	0.7	4.6 ± 3.7	4.7 ± 3.9	0.8
Embryos cryopreserved	1.4 ± 2.1	1.6 ± 2.3	0.3	1.5 ± 2.1	1.7 ± 2.4	0.3
Single embryo transfer (%)	60.1	65.7	0.3[a]	64.0	70.4	0.2[a]
Double embryo transfer (%)	15.3	14.9		15.8	15.0	

Data are presented as means ± standard deviation (SD) unless otherwise stated.

P value for between-group difference from t-tests unless otherwise stated.

[a] *P value for between-group difference from Chi squared tests.*

Table 3 demonstrates the clinical efficacy outcomes per started cycle. There were no significant differences in clinical outcomes between the two groups. However, the CD6 group showed a trend towards higher implantation rates and ongoing pregnancy rates (Table 3). The difference between the two groups was much smaller in the freeze-thaw cycles (0.6%, $p = 0.6$). No differences were observed in the biochemical pregnancy or miscarriage rate ($p = 0.5$). Furthermore, the CD6 group showed a non-significant trend towards higher live birth rates per started cycle as well as higher cumulative live birth rates, compared with the CD2 group. Figure 2 depicts a Kaplan Meier plot showing the cumulative live birth rate in both treatment arms. There is a gradual increase in the rate of accumulation in favour of the CD6 group. The difference after the first treatment cycle is maintained during the time period in which pregnancies accumulate from the freeze-thaw cycles. Due to either late miscarriage or premature birth, live birth was not achieved in two CD2 and four CD6 patients.

Table 3 Clinical efficacy outcomes per fresh started cycle

	Intention-to-treat			Per protocol		
	CD2 group (n = 308)	**CD6 group (n = 309)**	***P* value**	**CD2 group (n = 253)**	**CD6 group (n = 260)**	***P* value**
Pregnancy rate per started cycle						
Positive hCG, n (%)	94 (30.5)	98 (31.7)	0.7	79 (31.2)	80 (30.8)	0.9
Ongoing implantation rate per embryo (%)	25.4 ± 43.6	28.1 ± 43.5	0.5[a]	27.0 ± 44.6	26.6 ± 42.7	0.9[a]
Ongoing pregnancy rate, n (%)	69 (22.4)	79 (25.6)	0.4	59 (23.3)	65 (25.0)	0.7
per OPU, n (%)	62 (24.1)	77 (28.1)	0.3	57 (25.6)	65 (26.9)	0.7
per ET, n (%)	62 (26.7)	77 (30.9)	0.3	57 (28.2)	65 (29.3)	0.8
Live birth rate, n (%)	66 (21.5)	74 (24.0)	0.5	57 (22.5)	61 (23.5)	0.8
Pregnancy outcome for patients with positive hCG						
Biochemical pregnancy, % (n)	10.6 (10/94)	5.1 (5/98)	0.5	12.7 (10/79)	5.0 (4/80)	0.4
Early miscarriage, % (n)	13.8 (13/94)	13.3 (13/98)	0.5	11.4 (9/79)	12.5 (10/80)	0.4
Ectopic pregnancy, % (n)	2.1 (2/94))	1.0 (1/98)	0.6[b]	1.3 (1/79)	1.3 (1/80)	1.0[b]
Ongoing pregnancy, % (n)	73.4 (69/94)	80.6 (79/98)	0.5	74.7 (59/79)	81.3 (65/80)	0.6
Multiple pregnancy,% (n)	6.4 (6/94)	5.1 (5/98)	0.7[b]	7.6 (6/79)	5.0 (4/80)	0.5[b]
Live birth, % (n)	70.2 (66/94)	75.5 (74/98)	0.4	72.3 (57/79)	76.3 (61/80)	0.6
Cumulative ongoing pregnancy rate						
OPR in cryo-thaw cycle only, n (%)	16 (5.2)	18 (5.8)	0.6	15 (5.9)	16 (6.2)	0.9
OPR in fresh and cryo-thaw cycle per started cycle, n (%)	85 (27.6)	97 (31.4)	0.3	74 (29.2)	81 (31.2)	0.6
Cumulative live birth rate						
LBR in Cryo-thaw cycle only, n (%)	16 (5.2)	18 (5.8)	0.6	15 (5.9)	16 (6.2)	0.9
LBR in fresh and cryo-thaw cycle, per started cycle n (%)	82 (26.7)	92 (29.9)	0.6	72 (28.5)	77 (29.6)	0.9

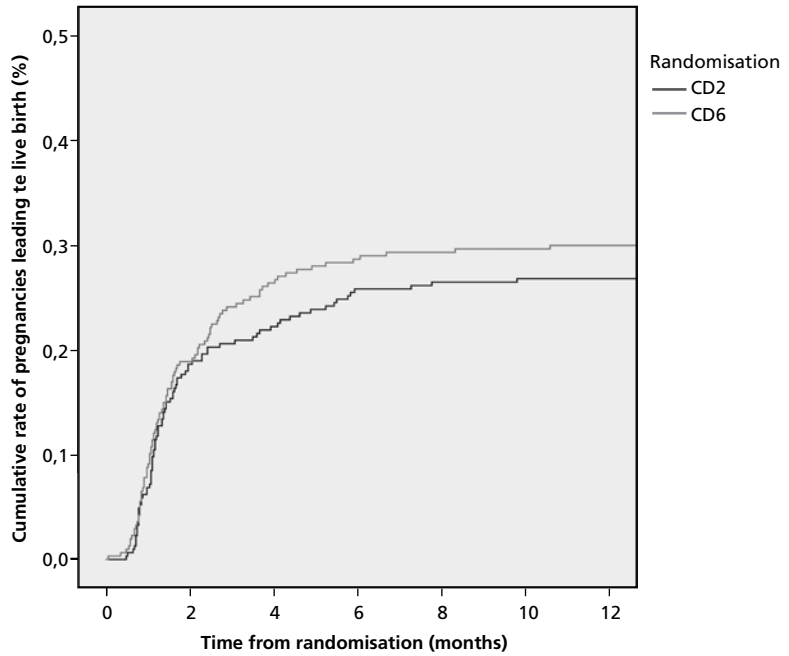
OPR; ongoing pregnancy rate, LBR; live birth rate

P values for between-group difference from Chi squared tests unless otherwise stated.

[a]*P values for between-group difference from t-tests.*

[b]*P value for between-group difference from Fisher's Exact tests.*

Figure 2 Kaplan Meier plot showing the cumulative rate of pregnancies leading to live birth in both treatment arms



Safety

The total cancellation rate per started cycle was 10.4% in the CD2 group and 7.4% in the CD6 group (p = 0.2). Cancellation due to poor ovarian response occurred in 13 CD2 patients and in 15 CD6 patients, whereas IVF treatment was converted into an intra-uterine insemination in 12 and 5 cases respectively (Fig. 1). Due to a risk of OHSS, 7 patients in the CD2 group and 3 patients in the CD6 group did not receive hCG. The overall incidence of OHSS was low (1.9%). Mild-to-moderate OHSS was observed in 10 patients (CD2; 3 and CD6; 7, p = 0.3), whereas one case of severe OHSS was observed in either group.

Discussion

To our knowledge, the current study represents the largest randomised controlled trial investigating the impact of early initiation of a GnRH antagonist on live birth rates after IVF/ICSI treatment. Early initiation of GnRH antagonist treatment showed no beneficial effect on cumulative live birth rates. Indeed, a trend towards better outcomes was observed when using the currently established, midfollicular phase fixed start regimen.

The expected benefit of early initiation of GnRH antagonist treatment on IVF/ICSI outcomes was based on three assumptions: moderation of the ovarian response related to early suppression of endogenous FSH by the GnRH antagonist and the mitigated subsequent exposure to E_2 levels; tighter prevention of untimely LH surges, with reduction of premature ovulation rates; and a more consistent control on early and late follicular phase P levels, enabling improved conditions for endometrial receptivity.

The non-physiological endocrine milieu associated with ovarian stimulation is thought to be the cause for suboptimal endometrial receptivity (2). Moreover, endometrial advancement of more than 3 days on the day of oocyte retrieval has been associated with a decreased chance of pregnancy, especially in cases with high LH levels at initiation of stimulation and with a prolonged duration of stimulation before starting GnRH antagonist treatment (38). Additionally, a high exposure of the endometrium to untimely elevated E_2, as well as elevated P levels during the follicular phase has been associated with a reduced chance of pregnancy, possibly due to endometrial advancement (39;49;50;52-56).

Compared with midfollicular initiation of GnRH antagonist treatment, early initiation has resulted in more profoundly suppressed LH and E_2 levels on day 6 of the cycle (57;98). Furthermore, in a single centre nested study of subjects recruited to this RCT, a lower oocyte yield was observed in the CD2 group compared to the CD6 group (p = 0.048), indicating a possible benefit for this approach in preventing overexposure of the endometrium to estradiol (98). The lack of confirmation of this result in the present study might indicate a type 1 error, or reflect subtle differences in application of the protocol in the centre which performed the nested study. The present findings are however consistent with those of a small pilot study, where number and classes of growing follicles appeared comparable between the two study groups (57).

Conflicting data exists regarding the impact of low endogenous LH levels on reproductive outcomes in GnRH antagonist cycles (40;45;83). However, it has been shown that the addition of LH to recFSH does not affect live birth rates (33). Additionally, the present study shows a similar fertilisation rate as well as number of embryos obtained, indicating that profound suppression of endogenous LH and E_2 does not interfere with normal folliculogenesis, oocyte maturation and fertilisation processes, and as such may not be an explanation for the trend towards lower pregnancy rates in the CD2 group.

In this study, the overall incidence of premature ovulation was 0.8%, which is lower than what has been reported in the literature to date. A number of studies have demonstrated that LH rises may occur in 1.4 – 35% of GnRH antagonist stimulation cycles (29;35;78;84). Since the GnRH antagonist is a competitive GnRH receptor blocker, trigger signals such as a fast rising E_2, may lead to endogenous GnRH surges that may overcome the competitive blockage. From the earlier endocrine studies, the positive effect in terms of a tighter control of LH, specifically in the first phase of the stimulation cycle, has been demonstrated (37;39;40;57). In our study, however, a benefit in terms of improved clinical outcomes could not be claimed, and a late GnRH antagonist start regimen still offers the best clinical outcome profile.

Finally, the role for elevated early follicular phase P levels in affecting IVF outcome has become apparent from two studies (48;51). Early initiation of the GnRH antagonist could aid in swift suppression of any residual corpus luteum function, by reducing LH exposure. Although the mechanism of action of elevated P in jeopardising clinical outcomes has not been elucidated, normalisation of P levels may be beneficial. So far, studies have not confirmed that early GnRH antagonist exposure will completely neutralise the negative effects of elevated early follicular P levels (48). Although the incidence of elevated early follicular phase P levels in GnRH antagonist cycles is low [4.9-6.2%,(48;51)], we assume that a small percentage of our study population has elevated P levels. If the effect of early GnRH antagonist initiation were highly relevant in this regard, a possible trend towards higher pregnancy rates in the early start arm might be expected, but was not observed. Moreover, in the nested endocrine study, no differences in P levels between the two arms could be identified, neither on day 6 of the cycle nor on the day of hCG (98).

Contrary to expectations, this study, which involved a large patient population, showed a trend towards higher pregnancy rates in favour of the CD6 group. This may mean that a more stable endocrine profile does not necessarily result in improved endometrial receptivity and hence higher pregnancy rates. While a possible negative effect of GnRH antagonists on oocyte/embryo quality has been suggested in the past (29) this has not since been substantiated. Moreover, no adverse effect was observed on the performance of the freeze-thaw embryos in subsequent replacement cycles (85). Previously, Raga *et al.* (1998) have detected GnRH receptors in the endometrium (86). However, gene expression studies so far do not support a detrimental effect of GnRH antagonists on endometrial receptivity (87). Our data support the contention that embryo quality is not negatively affected by GnRH antagonist exposure, since the ongoing pregnancy rates in the freeze-thaw cycles were similar, in spite of different exposure time in the two arms.

The strength of this study is the multicentre randomised controlled design. The heterogeneous patient population and ITT analysis reflects daily practice and makes extrapolation of the results to the general IVF population possible. Moreover, using live birth as an endpoint in clinical trials in reproductive medicine is well recognised and leads to a better understanding of their implication for clinical practice (23).

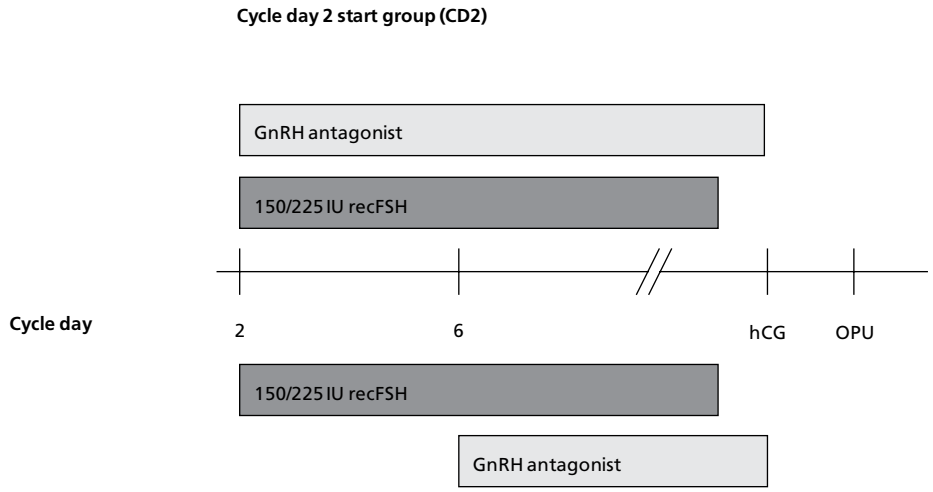
The study was terminated prematurely because no significant difference was observed in clinical outcomes after 617 inclusions. A much larger population would be needed to detect a small difference in favour of either study arm, which raises the question whether this would be relevant for clinical practice. A further limitation of the study is the lack of endocrine data to confirm our previous findings with regard to the endocrine profile in the follicular phase (98). The reasons we decided not to perform hormonal measurements in all subjects were related to the logistic challenges associated with the multicentre design, and possible discrepancies between the participating centres in, for example, the assays used.

The study protocol required that the dose of recFSH remain fixed throughout the entire stimulation period. However, the dose was adjusted according to ovarian response in a small number of patients (n = 28). These patients were not excluded from the ITT analysis, as variations in usage of recFSH were deemed not to influence the clinical outcomes. Moreover, it has been demonstrated that FSH dosages over 150 IU daily will not alter the stimulation or response level of the ovaries (70;81). The per-protocol data analysis revealed that the differences observed between the two groups were similar to those in the ITT analysis.

The observations in this study may have relevance for the optimisation of GnRH antagonist stimulation cycles for IVF. The per-protocol analysis revealed that the observed non-significant differences with regard to live birth and cumulative live birth rate were slightly smaller compared with the ITT analysis. Because the per-protocol analysis demonstrated the direct effect of the different treatment protocols, the clinical importance of this finding is probably negligible. Additionally, increasing the number of daily injections by four may increase patient discomfort and imposes the risk losing some of the benefits of a GnRH antagonist protocol. Furthermore, adding four extra injections will slightly increase treatment costs by € 159,38 per patient per cycle. This study shows clearly that the additional treatment burden and costs are not justified as early initiation of GnRH antagonists does not improve live birth rates. In the efforts to improve GnRH antagonist cycles, the focus may be put on individualised recFSH regimens based on ovarian reserve tests such as antral follicle count (AFC) and Anti-Müllerian hormone (AMH). Such an approach could aid in improving IVF outcome, as well as in reducing the risk of OHSS or cancellation due to poor response. Previous systematic reviews by our group have shown that both AFC and AMH can predict ovarian response, and hence allow for individualisation of recFSH dosage (88;89). Whether response prediction and individualised dosing will improve clinical outcome needs to be elucidated once accurate response prediction has become established for GnRH antagonist cycles (66;68).

In conclusion, this large study has demonstrated that early initiation of GnRH antagonist treatment does not improve clinical outcomes of IVF treatment compared with midfollicular initiation of GnRH antagonist. The currently used fixed start GnRH antagonist protocol starting on CD 6 remains the best choice at present.

Appendix 1 An outline of the two treatment regimens applied. The upper panel depicts the investigational group (start GnRH antagonist on cycle day 2). The lower panel depicts the control group (start GnRH antagonist on cycle day 6).



RecFSH; recombinant FSH, hCG; human chorionic gonadotropin, OPU; oocyte pick-up

Elevated early follicular progesterone levels and IVF outcomes:
a prospective intervention study and meta-analysis

The CETRO Trial study group:

O. Hamdine, F.J. Broekmans, M.J.C. Eijkemans, B.J. Cohlen; A. Verhoeff, P.A. van Dop, R.E. Bernardus, C.B. Lambalk, G.J.E. Oosterhuis, C.A.G. Holleboom, G.C. van den Dool - Maasland, H.J. Verburg, P.F.M. van der Heijden, A. Blankhart, B.C.J.M. Fauser, J.S.E. Laven and N.S. Macklon

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4

Abstract

Objective: To assess the impact of elevated early follicular progesterone (P) levels in GnRH antagonist cycles on clinical outcome using prospective data in combination with a systematic review and meta-analysis.

Design: Nested study within a multicentre randomised controlled trial and a systematic review and meta-analysis.

Setting: Reproductive Medicine centre in an University Hospital

Patients: 158 IVF/intracytoplasmic sperm injection patients.

Interventions: Recombinant FSH (150-225 IU) was administered daily from cycle day (CD) 2 onward. GnRH antagonist treatment was randomly started on CD 2 or 6. These women were divided into two groups according to their P level on CD2; normal or elevated (> 4.77 nmol/L or > 1.5 ng/mL). A systematic search of MEDLINE and EMBASE from 1972 – 2013 was performed to identify studies analysing elevated early P levels in GnRH antagonists.

Main Outcome Measure: Ongoing pregnancy rate (OPR) per started cycle.

Results: The incidence of elevated P was 13.3%. A non-significant difference in OPR was present between the normal and elevated P group (27.0% vs. 19.0%). No differential impact of early or late GnRH antagonist initiation on the effect of elevated or normal P on OPR was observed. The meta-analysis (n=1052) demonstrated that elevated P levels significantly decreased the OPR with 15% (95% CI -23, -7%). Heterogeneity across the studies, presumably based on varying protocols, may have modulated the effect of elevated P.

Conclusion: From the present meta-analysis it appears that early elevated P levels are associated with a lower OPR in GnRH antagonists. The incidence of such a condition, however, is low.

Trial registration number: www.clinicaltrials.gov, no. NCT00866034

Introduction

The end of the menstrual cycle is characterised by regression of the corpus luteum and reduced progesterone (P) production, which reaches its nadir at menstruation. This process is known as functional luteolysis (90) and is followed by structural regression of the corpus luteum which occurs after a decrease in P synthesis (91). The reduced production of P is associated with a decline in steroidogenic acute regulatory (StAR) gene and protein expression. Additionally, several molecules such as tumour necrosis factor-α and interleukin-1β, a reduced luteal perfusion and apoptosis are thought to contribute to functional and structural luteolysis (91).
In in vitro fertilisation (IVF) cycles co-treated with GnRH antagonist to prevent premature luteinisation, the timing of commencing ovarian stimulation is related to the onset of menses. However, what is reported as the onset of menses may in fact sometimes be a breakthrough bleeding, possibly as a result of inefficient luteolysis.
The causes for disturbed luteolysis remain unclear. Perhaps the mechanisms underlying functional luteal regression play a certain role. It is also possible that ovarian aging plays a role in creating a disturbed luteal endocrine milieu. Significantly higher P levels in the early follicular phase of a spontaneous cycle have been demonstrated in women with a poor response during a previous IVF treatment and are possibly caused by continued production by the corpus luteum. These women tended to have a higher median age (92). The authors suggested that the follicular phase characteristics of these poor responders indicated ovarian aging.
The presence of elevated serum P levels on day 2 of the cycle has been associated with a decreased chance of pregnancy, which might be elicited by advanced or disrupted endometrial receptivity (51). Furthermore, the presence of a still functioning corpus luteum, may provide a suboptimal endocrine milieu for new follicular growth and subsequently affect pregnancy rates (93;94). Little information is available concerning the association of elevated P levels at initiation of ovarian stimulation with IVF outcome. In long GnRH agonist cycles, suppression of gonadotropins results in basal levels of steroid hormones at initiation of stimulation and therefore consistently normal P levels (95). However, elevated baseline P levels have been reported in short GnRH agonist cycles (96;97) and GnRH antagonist cycles (48;51;98). The incidence of high P levels on cycle day (CD) 2 in GnRH antagonist cycles has shown to be between 4.9% and 13.3% (48;51;98). Delaying the administration of gonadotropins in GnRH antagonist cycles, could result in normalisation of P levels (51). Recently, it has been suggested in an uncontrolled study that pre-treatment with a GnRH antagonist during 3 consecutive days prior to ovarian stimulation leads to normalisation of P levels, resulting in adequate ovarian stimulation and acceptable pregnancy rates (48).
It can therefore be postulated that starting GnRH antagonist co-treatment on day 2 of the cycle may suppress elevated early follicular P levels, thereby improving the chance of achieving

in-phase endometrial maturation at the time of embryo transfer. The aim of this study was to assess the impact of elevated early follicular P levels (> 4.77 nmol/L or > 1.5 ng/mL) on ongoing pregnancy rates in GnRH antagonist cycles. For this we used previously unutilised prospective data derived from patients who had participated in a recently published randomised study comparing an early (CD 2) or late (CD 6) start GnRH antagonist protocol. These data were then analysed as part of a systematic literature review and meta-analysis.

Subjects and Methods

Prospective data

Patient population

The present study was derived from a nested study (98) as part of a large open-label multicentre randomised controlled trial (RCT) on the timing of GnRH antagonist initiation, conducted between March 2009 and July 2011 in the Netherlands (99). The study was approved by our Institutional Review Board, and registered on the Clinical Trial web site (www.clinicaltrials.gov, no. NCT00866034). Two hundred women undergoing IVF or intracytoplasmic sperm injection (ICSI) were recruited from the IVF outpatient clinic of the Department of Reproductive Medicine and Gynecology of the University Medical centre Utrecht. A web-based computer-generated randomisation schedule was used for randomisation. The participants and clinicians were not blinded to group allocation. Informed consent was obtained from all patients and each patient was enrolled into the study only once. Inclusion criteria were: age ≤ 39 years; body mass index (BMI) ≤ 32 kg/m^2; regular cycle; and no more than 2 previous unsuccessful IVF/ICSI cycles. Patients diagnosed with World Health Organization class 1 and 2 were excluded.

Ovarian stimulation

Ovarian stimulation was performed with recombinant FSH (recFSH, Gonal-f; Merck Serono, the Netherlands) and a GnRH antagonist (Cetrotide; Merck Serono, the Netherlands) was used to prevent a premature LH surge. None of the patients had received hormonal treatment in the cycle preceding treatment, nor were they pre-treated with oral contraceptives. In both treatment arms recFSH (150-225 IU) was administered daily from CD 2 onward. The study group (CD2) started GnRH antagonist co-treatment (0.25 mg) on CD 2, whereas the control group (CD6) started on CD 6. Final oocyte maturation was induced by administrating 6500 IU of human chorionic gonadotropin (hCG; Ovitrelle; Merck Serono, the Netherlands), when at least one follicle of ≥ 18 mm in diameter and two follicles of ≥ 16 mm in diameter were visualised by ultrasound. Follicle growth was assessed by transvaginal ultrasound. Oocyte retrieval was performed 36 hours after hCG administration. One or two embryos were transferred 3 days after oocyte retrieval. The luteal phase was supplemented with a daily dose of 600 mg vaginally administered micronized natural progesterone (Utrogestan; Besins Healthcare, Brussels, Belgium). Hormonal assessment was performed in 158 patients on CD 2 of the treatment cycle in both groups. To assess the overall clinical impact of elevated P levels, both treatment arms were collapsed and these women were divided into two groups according to their P level on CD 2; normal or elevated P. This seemed justified as the incidence of elevated P was similar in both treatment groups of the RCT, and previously no significant difference in pregnancy rates had been observed between the randomised treatment arms (98). The cycle was not cancelled nor postponed in cases with elevated P levels at initiation of stimulation, as the results of testing were not made available to clinicians.

Hormonal assessments

Serum P samples were drawn by venepuncture in the morning before initiation of ovarian stimulation (CD 2). P was analysed on the Beckman-Coulter Unicel DXi800 (Woerden, the Netherlands). Functional sensitivity [defined as 20% day-to-day coefficient of variability (CV)] was 2 nmol/L (0.6 ng/mL). The day-to-day CV was 16-19% at 2.8 nmol/L (0.9 ng/mL), 8.5% at 32 nmol/L (10.1 ng/mL) and 8% at 103 nmol/L (32.4 ng/mL). Elevated P was defined as *P* > 4.77 nmol/L (> 1.5 ng/mL), which is a common cut-off used in the literature. Serum estradiol samples were collected on CD 2, 6 and the day of hCG. Estradiol was analysed on the Roche E170 modular immunoanalyser (Almere, the Netherlands). The functional sensitivity for estradiol was 40 pmol/L (with singleton measurements or 20 pmol/L in duplo). The day-to-day CV was 9-11% at 65 pmol/L, 4.5% at 200 pmol/L and 2.8% at 600 pmol/L. Serum samples routinely collected prior to the start of stimulation were used to determine serum anti-mullerian hormone (AMH). AMH was determined in a sandwich ELISA (AMH Gen II ELISA, A79765, Beckman Coulter; Inc., USA). The lower limit of detection was 0,16 µg/L. Inter-assay variation was 10% at 0,27 µg/L and 4,7% at 3,9 µg/L (n = 18).

Literature search

A literature search in MEDLINE and EMBASE electronic databases from 1972 – 2013 was performed to identify relevant studies assessing the impact of elevated early follicular P levels in GnRH antagonist cycles. The following Medical Subject Headings (MeSH) search terms were used: 'IVF', 'ICSI', 'GnRH antagonist', 'ART', 'ovarian stimulation' and 'progesterone'.

Outcome measures

The primary endpoint of both the prospective study and the meta-analysis was the ongoing pregnancy rate per started cycle. In the prospective study, ongoing pregnancy rate per started cycle was defined as the presence of at least one vital fetus beyond 10 weeks of gestation.

Secondary endpoints of the prospective study included the duration of stimulation; total cumulative dose of recFSH consumed; number of oocytes retrieved; fertilisation rate; number of suitable embryos; implantation rate; clinical pregnancy rate; live birth rate per started cycle and cumulative live birth rate from fresh and cryopreserved embryos originating from, and transferred within 6 months of the initial treatment cycle.

Data analysis

This study was planned as a nested group analysis within a larger RCT. An intention-to-treat analysis was performed. Data for continuous variables are presented as mean values and standard deviation. Between-group statistical comparisons of mean values were performed with *t*-tests. Chi squared tests and Fisher's exact tests were used for categorical values. Pearson's correlation coefficient was used to assess the association between P levels on CD 2 and estradiol levels during the follicular phase as well as stimulation characteristics. Logistic regression was used to determine the impact of early or late GnRH antagonist initiation on the effect of normal/high baseline P levels on ongoing pregnancy rates. Differences were considered to be statistically significant if *P* value < 0.05. Our group has previously demonstrated no significant difference in IVF outcome between early or late GnRH antagonist initiation (98). Furthermore, as the low occurrence of elevated early follicular P levels was similar in both groups and an as large as possible number of patients was expected to be necessary to detect a significant difference, the CD2 and CD6 group were collapsed. In a second stage, a systematic literature search and meta-analysis was performed to assess the impact of elevated P levels on ongoing pregnancy rates in GnRH antagonist cycles with sufficient power. For the meta-analysis the differences in ongoing pregnancy rates were pooled across the studies, resulting in a weighted risk difference, using the fixed effects approach. The 95% confidence interval (CI) was calculated for this risk difference. Heterogeneity was assessed by the I^2 measure. The analysis was performed in Review Manager 5.

Results

Prospective data

Two hundred patients were included in this prospective study. For various reasons, hormonal sampling on CD 2 was not completed in 42 patients. Supplemental Figure 1 shows the number of participants at each stage of the trial. Conventional IVF was performed in 63 (43.8%) couples and ICSI in 81 (56.3%) couples. Elevated P levels at initiation of stimulation were present in 21 out of 158 patients (13.3%, range 4.8 – 64.0 nmol/L or 1.5 – 20.1 ng/mL). There were no significant differences between the normal and high P group with regard to patient characteristics (Table 1). In the normal P group, GnRH antagonist treatment was started on CD 2 in 70 patients and on CD 6 in 67 patients. In the high P group, a GnRH antagonist was initiated on day 2 in 11 patients and on day 6 in 10 patients. Stimulation characteristics were similar in the two groups (Table 2). In the normal P group, cycle cancellation due to the risk of ovarian hyperstimulation syndrome (OHSS) occurred twice, whereas cycle cancellation or conversion into intra-uterine insemination (IUI) due to poor ovarian response occurred in 8 (5.8%) and 3 (2.2%) patients, respectively. Eight patients had no embryo transfer due to fertilisation failure (n = 3), no available semen (n = 3) and poor embryo quality (n = 2). In the high P group, one patient underwent an IUI due to poor ovarian response. One patient had no embryo transfer due to fertilisation failure.

The normal P group had significantly higher mean estradiol levels on CD 6 (834.9 ± 641.5 vs. 521.0 ± 286.6 pmol/L, $p < 0.001$). No difference in estradiol levels was observed on day 2 nor on the day of hCG. A positive correlation was observed between P levels on CD 2 and estradiol levels on CD 2 ($r = 0.4$, $p < 0.001$), the total stimulation period ($r = 0.2$, $p = 0.003$) as well as the total dose of recFSH consumed ($r = 0.2$, $p = 0.03$). There was neither a correlation with female age ($p = 0.4$), nor with the number of oocytes retrieved ($p = 0.7$). Logistic regression demonstrated no significant differential impact of early or late GnRH antagonist initiation on the effect of elevated or normal P levels on ongoing pregnancy rates (test for interaction: $p = 0.3$). The same applied to a potential differential impact of age (test for interaction: $p = 0.8$). Table 3 shows the clinical outcomes per started cycle. There were differences between the normal and high P group with regard to implantation rate, clinical and ongoing pregnancy rate and (cumulative) live birth rate, but none of these differences reached significance at statistical comparison.

Table 1 Demographics and fertility characteristics

	Normal P group N = 137	High P group N = 21	*P* value
Demographics			
Age (years)	32.2 ± 4.0	33.6 ± 3.0	0.1
BMI (kg/m²)	23.4 ± 2.9	22.7 ± 3.1	0.3
Smoking, n (%)	25 (18.2)	2 (9.5)	0.5[a]
AMH (µg/L)	2.8 ± 2.6	2.6 ± 1.9	0.6
Fertility characteristics			
Primary infertility, n (%)	104 (75.9)	17 (81.0)	0.6[b]
Duration of infertility (years)	2.6 ± 2.0	2.5 ± 1.0	0.8
Cause of infertility, n (%)			
Male factor	87 (63.5)	15 (71.4)	0.8[b]
Unexplained	33 (24.1)	4 (19.0)	
Tubal factor	14 (10.2)	2 (9.5)	
Endometriosis	3 (2.2)	0 (0.0)	

Data are presented as means ± standard deviation (SD) unless otherwise stated.
P value for between-group difference from t-tests unless otherwise stated.
[a]P value for between-group difference from Fisher's exact test
[b]P value for between-group difference from Chi squared tests.

Table 2 Stimulation characteristics in the normal progesterone (Normal P) and high progesterone (High P) group

	Normal P group N = 137	High P group N = 21	*P* value
Total dose of recFSH (IU)	1420 ± 411	1543 ± 412	0.2
Total duration of stimulation (days)	9.1 ± 2.1	9.6 ± 2.2	0.3
Oocytes retrieved	8.7 ± 5.0	9.5 ± 5.7	0.5
2PN oocytes	4.7 ± 3.2	4.2 ± 2.7	0.5
Low response (< 4 oocytes), n (%)	14 (11.3)	4 (20.0)	0.3[a]
High response (> 15 oocytes), n (%)	14 (11.3)	4 (20.0)	0.3[a]
Fertilisation rate (%)	58.3 ± 25.0	49.1 ± 29.6	0.1
Embryos suitable for transfer	4.3 ± 3.0	4.0 ± 2.6	0.7
Embryos cryopreserved	1.7 ± 2.4	1.5 ± 1.8	0.8
Single embryo transfer (%)	75.6	75.0	1.0[a]

Data are presented as means ± standard deviation (SD) unless otherwise stated.
P value for between-group difference from t-tests unless otherwise stated.
[a]P value for between-group difference from Chi squared tests.

Table 3 Clinical efficacy outcomes per fresh started cycle

	Normal P group N = 137	High P group N = 21	*P* value
Pregnancy outcome per started fresh cycle			
Positive hCG test, % (n)	35.8 (49/137)	19.0 (4/21)	0.1
Clinical pregnancy, % (n)	32.8 (45/137)	19.0 (4/21)	0.3
Ongoing implantation rate (%)	28.4 ± 43.4	15.8 ± 37.5	0.2[a]
Ongoing pregnancy rate, % (n)	27.0 (37/137)	19.0 (4/21)	0.4
Live birth rate, % (n)	26.3 (36/137)	19.0 (4/21)	0.6
Cumulative ongoing pregnancy rate			
Cryo-thaw cycle only, n (%)	11 (8.0)	0 (0.0)	0.4
Fresh cycle and cryo-thaw cycle per started cycle, n (%)	48 (35.0)	4 (19.0)	0.2
Cumulative live birth rate			
Fresh cycle and cryo-thaw cycle per started cycle, n (%)	47 (34.3)	4 (19.0)	0.2

P value for between-group difference from Chi squared tests, unless otherwise stated.
[a] Mean ± standard deviation (SD), P value for between-group difference from t-tests.

Meta-analysis

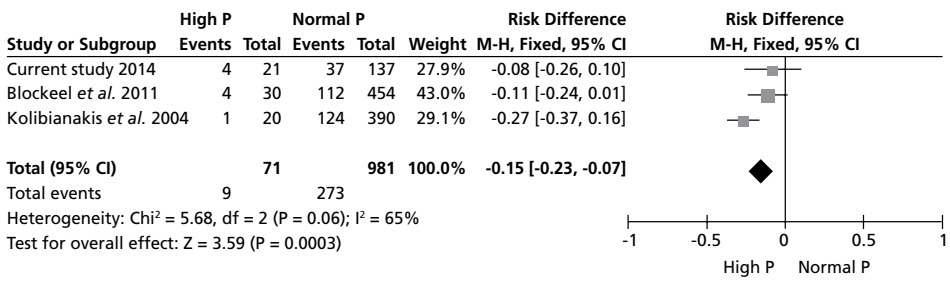
The MeSH strategy yielded 685 publications of which 661 were excluded because they did not fulfill the selection criteria based on the title. Twenty-two articles were excluded based on the abstracts. Only two studies could be identified from our systematic literature search that compared ongoing pregnancy rates in patients with normal and elevated P levels on day 2 of the treatment cycle in a GnRH antagonist protocol (48;51). The bibliographies of these studies were hand searched, however, no more relevant studies were found.

Both studies included an element of intervention, although the efficacy of these interventions has not been established. Kolibianakis *et al.* (2004) prospectively assessed the impact of elevated early P levels on pregnancy rates. In case of elevated P (20 out of 410 patients), initiation of stimulation was postponed for 1 or 2 days and was started if P levels normalised. Despite this intervention, a significantly lower ongoing pregnancy rate per started cycle (5.0% vs. 31.8%, $p = 0.01$) and per embryo transfer (6.3% vs. 36.9%, $p = 0.01$) was still observed in the high P group compared with the normal P group.

Blockeel *et al.* (2011) prospectively compared ongoing pregnancy rates in patients with normal and elevated early follicular P levels. In the presence of elevated P levels (30 out of 484 patients), a GnRH antagonist was administered during 3 consecutive days which resulted in the normalisation of P levels in all patients. The GnRH antagonist was then discontinued after which ovarian stimulation was started. However, there was no significant difference in pregnancy rate between the two groups, although a trend towards lower ongoing pregnancy rates was observed in the high P group.

Pooling the differences in ongoing pregnancy rates in these two studies with the current study (n = 1052), the risk difference was demonstrated to be – 0.15 [95% CI (– 0.23, – 0.07), $p = 0.0003$] (Figure 1). This implies that the chance to achieve an ongoing pregnancy is decreased with 15% in case of elevated P levels prior to starting ovarian stimulation with GnRH antagonist co-treatment. The results showed a certain degree of heterogeneity, as is evident from the I^2 value of 65% ($p = 0.06$).

Figure 1 Forest plot representing a meta-analysis on the available literature with regard to the impact of early elevated P levels on ongoing pregnancy rate per started cycle in GnRH antagonist cycles



Discussion

The prospective study demonstrated that elevated P levels on CD 2 may affect ongoing pregnancy rates in GnRH antagonist cycles. However, due to limited numbers the observed differences did not reach statistical significance. Results of a combined analysis of the current and previously published data in a formal meta-analysis revealed that elevated P levels are associated with reduced chances of pregnancy and this finding may urge the development of a solution strategy.

Elevated early follicular P levels are observed in a proportion of menstrual cycles and are probably caused by inefficient or incomplete luteolysis. In a group of 316 infertile patients, the incidence of elevated P levels on day 4-5 of the cycle was 11.4% (100). It remains unclear why this phenomenon occurs in certain cycles, and also the recurrence rate from cycle to cycle is unknown.

The patients in our cohort were relatively young; 32.2 ± 4.0 years in the normal P group compared with 33.6 ± 3.0 years in the high P group ($p = 0.1$). Although we did not observe a differential impact of age on the effect of normal or high P levels, it is possible that reproductive aging plays a role in the process of inefficient luteolysis. Ovarian aging has been associated with a shorter follicular phase (94) and abnormalities in luteal phase function (101). Deficiencies in both luteal phase estradiol and progesterone metabolites have been identified in women of late reproductive age (93). Advanced follicular growth in the presence of a poorly functioning corpus luteum has previously been demonstrated, which may provide a suboptimal hormonal milieu for new follicular growth (94). This is considered a sign of advanced ovarian ageing. Endometrial development is not likely to be affected by the ageing process, exemplified by the fact that in oocyte donation programs pregnancy rates depend mainly on the age of the donor (102).

The origin and regulation of progesterone secretion throughout the follicular phase of the natural menstrual cycle remain poorly understood. Other potential sources of elevated serum follicular phase progesterone, aside from the corpus luteum, have been suggested. The developing new dominant follicle and the cortex of the adrenal gland are able to produce progesterone as well (100;103-105). The adrenals are possibly the main source of circulating P during the early follicular phase, whereas the ovaries contribute mainly during the late follicular phase (103). However, a direct link between the gonadal axis and the adrenal axis has not been established. Therefore, the question remains why certain women have elevated baseline P levels. Furthermore, if what is reported as the onset of menses, is in fact a breakthrough bleeding before the actual menstruation, then the observed elevated P levels could be considered as normal late luteal phase P levels.

Despite the intervention strategies used by Kolibianakis *et al.* (2004) and Blockeel *et al.* (2011) to treat patients with elevated early follicular P levels, pregnancy rates remained lower in the high P groups compared to the normal P groups. A similar trend towards lower pregnancy rates in the high P group was also observed in the present prospective study, even though we did not intervene in case of elevated P levels. Pooling these 3 studies in a meta-analysis

demonstrated that in GnRH antagonist cycles, elevated early follicular P levels were indeed significantly associated with lower pregnancy rates.

The current prospective study demonstrated no differences between the two groups in terms of age, AMH, BMI, smoking or stimulation characteristics which is consistent with previously published studies (48;51). A positive correlation was observed between baseline P levels and baseline estradiol levels. Higher mean estradiol levels were observed in the normal P group on CD 6 only. Others have reported lower estradiol levels during the mid and late follicular phase of patients with elevated P levels on CD 2, which were attributed to a slightly increased number of follicles in the normal P group (75). Additionally, the prospective study demonstrated that the total duration of stimulation as well as the total gonadotropin requirement were positively correlated to baseline P levels. Moreover, lower pregnancy rates have been demonstrated in case of prolongation of the follicular phase in GnRH antagonist cycles (75). This all may point in the direction of affected ovarian reserve as a potential source for the reduced pregnancy rates, but final proof for such explanation is still to be delivered.

A strength of the prospective study is that it has been part of a randomised controlled design without any subjection to verification bias. Moreover, the prospective design implies a high degree of rigour in the data collection. Furthermore, the fact that the treatment was cancelled nor delayed made it possible to determine the effect of early initiation of GnRH antagonist on basal P levels and their impact on pregnancy rates. A post hoc power analysis revealed that, using the ongoing pregnancy rate in the normal P group of 27%, of 19% in the high P group and the incidence of elevated P of 21/137 = 15% as observed in our study, it would require a study with 1700 normal P and 261 high P patients to achieve a power of 80%. In view of these calculations the meta-analysis of the existing literature was justified, with the possibility that if the difference in ongoing pregnancy rates would be greater, a smaller sample size would suffice.

A limitation of the current prospective study is that, even after collapsing the two treatment arms, the source of the data implied a limited number of cases that was too low to demonstrate a true difference in clinical outcome between the normal and high P group. This is confirmed by the post hoc power analysis. The current study demonstrated a non-significant trend towards lower pregnancy rates in the presence of elevated early follicular P levels. However this may reflect a type 2 error. Furthermore, due to logistic or personal reasons blood samples were not available in 42 patients (Supplemental Fig. 1). Their baseline characteristics, however, were similar to the remaining study population and therefore this loss in cases is not likely to have influenced the results.

A possible limitation of the meta-analysis is the fact that the included studies incorporated different treatment strategies. Kolibianakis *et al.* (2004) and Blockeel *et al.* (2011) intervened in different ways when elevated P levels were encountered, and the relative efficacy of these interventions has not been established. Both studies commenced GnRH antagonist treatment

on day 6 of stimulation. The current prospective study did not intervene in case of elevated P levels but treated all patients with either an early (CD 2) or late (CD 6) start GnRH antagonist protocol. The observed heterogeneity was not significant and may have been caused by the different treatment regimens applied in order to treat the high P groups. However, it is not likely that delaying initiation of ovarian stimulation for 1-2 days or the administration of a GnRH antagonist prior to stimulation would have negatively affected IVF outcome. Additionally, no difference in clinical outcome has been observed between early or late GnRH antagonist initiation (98). So, even if the applied variations in stimulation approach across the studies would have had some influence on the prospects for patients with elevated P levels, the observed negative effect in the meta-analysis may then be only an underestimation.

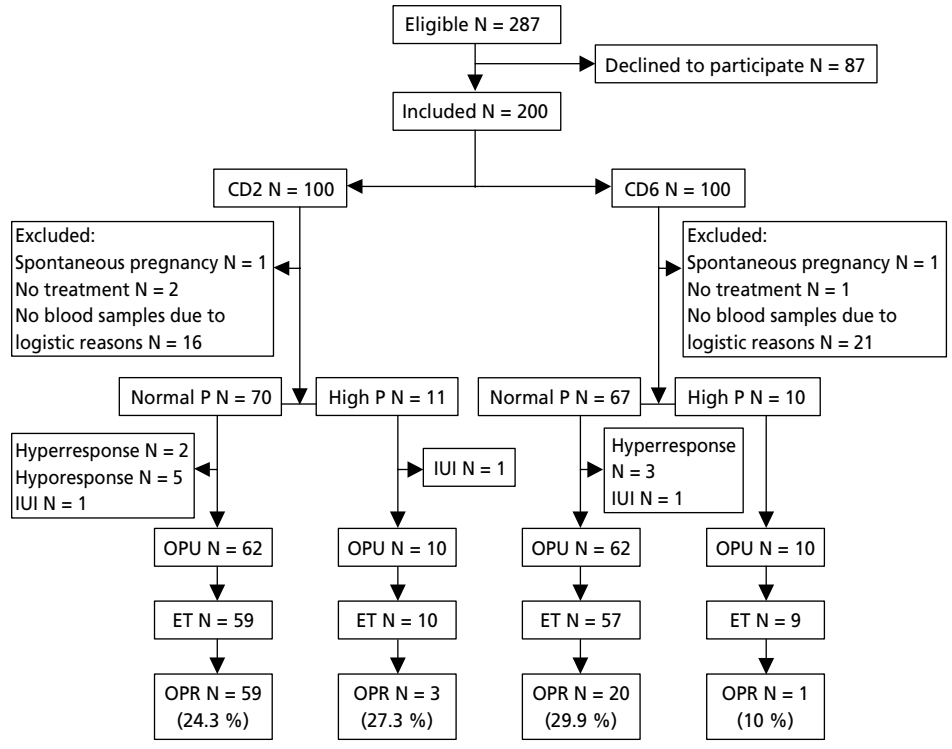
The true impact of elevated P levels prior to the start of ovarian stimulation with GnRH antagonist co-treatment on clinical outcome remains a subject of debate. However, the results of the meta-analysis should be treated with caution since substantial heterogeneity was observed. Although a number of treatment options have been proposed, high quality evidence regarding the management of women with elevated baseline P levels in order to optimise or normalise their prospects for successful IVF outcome, is still lacking. In GnRH antagonist cycles, delaying initiation of ovarian stimulation by 1 or 2 days resulted in normalisation of P levels in a majority of the patients, however, pregnancy rates were still significantly lower in the high P group (51). Furthermore, normalisation of baseline P levels by administering a GnRH antagonist during 3 consecutive days has previously been demonstrated (48). With this approach, a non-significant difference in pregnancy rates between the normal and high P group was subsequently reported. Early or late GnRH antagonist initiation had no significant differential impact on the effect of high or normal progesterone on ongoing pregnancy rates, suggesting that management of patients with elevated P levels at initiation of stimulation cannot be achieved by early commencement of GnRH antagonist treatment. Finally, adrenal suppression by glucocorticoids has resulted in decreased P levels but the effect on pregnancy rates is debatable (100;103). Data from a well powered RCT is required to address this.

The meta-analysis demonstrated that the chance to achieve pregnancy is decreased with 15% in case of elevated P levels prior to starting ovarian stimulation with GnRH antagonist co-treatment. If the incidence of elevated P levels in the current meta-analysis (6.7%), the suggested reduction in pregnancy rate and the presence of an effective strategy to normalise the condition are taken into account, the number of women needed to be screened and treated in an adjusted fashion in order to achieve one additional pregnancy would be 100. Currently in our hospital, laboratory costs for P assessments are € 7,66 per sample which results in € 766 of additional costs per pregnancy, if all patients were screened for elevated P at the start of gonadotropin treatment. These costs are only justified if a randomised controlled trial demonstrates that patients with elevated baseline P levels can indeed be treated differently

in order to optimise IVF outcome. Judging from the low incidence in our meta-analysis, a total number of 826 participants would be required to provide sufficient power to detect a significant difference in ongoing pregnancy rates of 15% (going from 13% to 28%) from any proposed therapeutic approach. The question remains whether the estimated costs of such a large RCT are justified, especially in an era where cost reduction has become an important issue. However, if elevated early follicular P levels would appear to be related to ageing of the ovaries (92) then an obvious strategy using the woman's own oocytes may be not be found.

In conclusion, based on the systematic review and meta-analysis, elevated P levels on day 2 of the cycle affect ongoing pregnancy rates in GnRH antagonist cycles in a negative way. However, in view of the relatively low incidence of this condition and the absence of a proven effective treatment strategy, routine screening for P is not recommended.

Supplemental Figure 1 Flowchart showing the numbers of participants at each stage of the trial



CD; cycle day, P; progesterone, IUI; intra uterine insemination, OPU; oocyte pick-up, ET; embryo transfer, OPR; ongoing pregnancy rate per started cycle

Ovarian response prediction in GnRH antagonist cycles using Anti-Müllerian hormone: a prospective cohort study

O. Hamdine, M.J.C. Eijkemans, E.W.G. Lentjes, H.L. Torrance, N.S. Macklon, B.C.J.M. Fauser and F.J. Broekmans

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Abstract

Study question: What is the clinical value of Anti-Müllerian hormone (AMH) for the prediction of high or low ovarian response in controlled ovarian stimulation for in vitro fertilisation (IVF) using GnRH antagonist co-treatment?

Summary answer: AMH as a single test has substantial accuracy in the prediction of high and low ovarian response in GnRH antagonist cycles for IVF.

What is known already: The role of AMH and other patient characteristics in ovarian response prediction has been studied extensively in long GnRH agonist protocols, however, little information is available regarding the clinical value in GnRH antagonist cycles.

Study design, size, duration: Prospective cohort study at the University Medical Centre Utrecht, the Netherlands. A total of 487 patients scheduled for IVF/intracytoplasmic sperm injection (ICSI) between 2006 and 2011 were included in the study.

Participants/materials, setting, methods: Patients with a regular cycle who underwent their 1st IVF/ICSI cycle with GnRH antagonist co-treatment while receiving a starting dose of 150 or 225 IU recombinant FSH were included in the study. Patients were divided into 3 subgroups according to the following ovarian response categories; high (>15 oocytes or cycle cancellation), normal (4-15 oocytes) and low (< 4 oocytes or cycle cancellation). Serum samples collected prior to IVF treatment were used to determine serum AMH levels.

Main results and the role of chance: According to the predefined ovarian response categories, 58 patients were classified as high, 326 as normal and 101 as low responder. Ongoing pregnancy rates did not clearly differ among these three response groups (19.0%, 22.1% and 16.8%, respectively, $p = 0.9$). For the prediction of high response, AMH had an area under the receiver-operating characteristic curve (AUC) of 0.87. Both female age and BMI had lower accuracy (AUC 0.66 and 0.58, respectively). For low response prediction, again, AMH had a better accuracy (AUC 0.79) than female age and BMI (AUC 0.59 and 0.56, respectively). In a multivariate model, including the factors age, AMH, BMI, smoking, type and duration of subfertility, only BMI added some predictive value to AMH for both high and low response prediction. Clinical test characteristics demonstrated that, using a specificity of ~90%, the detection rate of AMH for high and low response, corresponding with a test cut-off of 4.5 µg/L and 0.8 µg/L, was ~60% and ~45%, respectively.

Limitations, reasons for caution: The impact of the antral follicle count (AFC) on ovarian response prediction in GnRH antagonists was not assessed. However, it has previously been demonstrated that in GnRH antagonist cycles AMH has a better accuracy for the prediction of ovarian response than the AFC.

Wider implications of the findings: The current study demonstrates that AMH is an adequate predictor for both high and low response in GnRH antagonist cycles showing similar accuracy as reported in previous studies on GnRH agonists. The optimisation and individualisation of GnRH antagonist protocols may be improved by using an AMH-tailored approach.

Trial registration number: www.clinicaltrials.gov, Protocol ID 13-109

Introduction

The optimisation and individualisation of controlled ovarian stimulation for in vitro fertilisation (IVF) has become increasingly important. Clinicians often use patient characteristics, such as female age, menstrual cycle length, body mass index (BMI) and results from previous IVF cycles to select a treatment protocol (58). Treatment individualisation has been hampered by disagreement as to which ovarian marker provides an accurate estimation of potential success for patients prior to IVF treatment. Several ovarian markers, including basal follicle stimulating hormone levels (FSH), antral follicle count (AFC) and Anti-Müllerian hormone (AMH) have been suggested as predictors of ovarian response and clinical outcome (59). The role of these ovarian response tests (ORTs), has generally been studied in patients treated with a long GnRH agonist protocol. It has been demonstrated that AMH is an accurate predictor of both high (62) and low ovarian response in GnRH agonist cycles (63), suggesting it would be an ideal marker for the individualisation of controlled ovarian stimulation strategies. Indeed, the use of an AMH tailored approach has previously been suggested by several investigators (64;65;106).

These days, many clinicians choose to use GnRH antagonists as a tool for LH surge suppression, as the availability of GnRH antagonists has enabled a reduction in complexity and costs, and has led to improved safety compared to GnRH agonists, without a clear difference in ongoing pregnancy rate and live birth rate (22;24).

The accuracy of ORTs in ovarian response prediction in GnRH antagonist treatment regimens may differ from that in GnRH agonist protocols as there is a difference in the endocrine profile, early follicle recruitment and synchronisation of follicular development ultimately leading to a difference in number of oocytes retrieved (25). Hence, predictive models cannot be extrapolated from GnRH agonist to GnRH antagonist protocols.

Only a limited number of studies have addressed the value of ORTs for ovarian response prediction in GnRH antagonist cycles (66-69). In one study, both AMH and basal FSH were found to be predictive factors for high response, whereas AMH was the only significant factor for low response (66). Others also observed a high accuracy of AMH for the prediction of high and low response (67;69). However, among oocyte donors treated with a GnRH antagonist protocol, the predictive ability of AMH was only modest (68). The difference in the accuracy of AMH found among these studies may be caused by the use of different definitions for ovarian response.

The question therefore remains whether AMH is able to correctly predict ovarian response in GnRH antagonist cycles with similar accuracy as GnRH agonist cycles. The aim of this prospective cohort study was to determine the accuracy and clinical value of AMH in the prediction of ovarian response in IVF using GnRH antagonist co-treatment.

Materials and methods

Subject selection

For this prospective cohort study, we selected women from a cohort of 1031 patients who were treated at the IVF outpatient clinic of the Department of Reproductive Medicine and Gynecology of the University Medical Centre Utrecht. Informed consent was obtained from all patients for the banking and use of both serum and DNA samples for research purposes regarding assisted reproduction. This research protocol was approved by our Institutional Review Board and registered on www.clinicaltrials.gov (Protocol ID 13-109). Blood samples were collected, irrespective of the cycle day during routine screening for hepatitis B and C and HIV prior to IVF treatment, and stored at –20 °C. For the current study, we selected patients with a regular cycle who underwent their first IVF/ intracytoplasmic sperm injection (ICSI) cycle with GnRH antagonist treatment while receiving a starting dose of 150 or 225 IU recombinant FSH (recFSH). Patients who achieved a live birth after the previous IVF/ICSI treatment using 150/225 IU recFSH were also eligible candidates. Patients who fulfilled the inclusion criteria were treated between 2006 and 2011. The IVF treatment data were prospectively recorded in our electronic infertility patient data files. Serum samples were retrieved for the current study to determine serum AMH levels.

Controlled ovarian stimulation

Controlled ovarian stimulation was performed with recFSH (Gonal-f; Merck Serono, or Puregon; MSD, the Netherlands). A GnRH antagonist (Cetrotide; Merck Serono, or Orgalutran; MSD, the Netherlands) was used to prevent a premature LH surge. The patients were not pretreated with oral contraceptives. According to local protocol, recFSH (150-225 IU) was started on cycle day (CD) 2 or 3. The reasons to apply a starting dose of 225 IU recFSH/day instead of the standard dose of 150 IU recFSH in our clinic were female age > 41 years, a previous treatment leading to live birth with 225 IU recFSH/day or incipient ovarian failure (defined as a regular cycle, basal FSH > 10 IU/L and age < 40 years). GnRH antagonist treatment was commenced on stimulation day 5. Human chorionic gonadotropin (hCG, 10,000 IU, Pregnyl; MSD, the Netherlands or 6500 IU Ovitrelle; Merck Serono, the Netherlands) was administered to induce final oocyte maturation when at least three follicles of ≥ 17 mm in diameter were visualised by ultrasound. Oocyte retrieval was performed 36 hours after hCG administration. One or two embryos were transferred 3 or 4 days after oocyte retrieval. The luteal phase was supplemented with a daily dose of 600 mg vaginally administered micronized natural progesterone (Utrogestan; Besins Healthcare, Brussels, Belgium).

Anti-Müllerian hormone assay

After blood collection, plasma for assay of AMH was separated directly and frozen in aliquots within 3 – 4 hours. In December 2011 stored frozen samples were thawed overnight in the

refrigerator in order to determine AMH levels. All measurements were performed in a batch analysis using a single lot reagent by a DS2 ELISA analyser (AMH Gen II ELISA, A79765, Beckman Coulter; Inc., USA). In order to minimise possible interference from complement first a buffer was pipetted before the initial sample. Using this procedure the problem with complement interference was not detected. The lower limit of detection was 0,16 µg/L. Inter-assay variation was 10% at 0,27 µg/L and 4,7% at 3,9 µg/L (n = 18). The maximum time interval between serum sampling and the start of controlled ovarian stimulation was 7 months (range 1 day – 7 months).

Outcome measures

The primary outcome measure was ovarian response category. Since ovarian response definitions for GnRH antagonists are lacking, the most commonly used definitions for both high and low response in GnRH agonists were adapted for this study. A high response was arbitrarily defined as more than 15 retrieved oocytes or cancellation due to an anticipated risk of OHSS (62). A low response was defined as less than 4 retrieved oocytes or cancellation due to low ovarian response (less than 3 dominant follicles of >12 mm), or a switch to intra-uterine insemination (63). A normal response was therefore defined as 4-15 retrieved oocytes. Secondary outcomes included the duration of stimulation; total cumulative dose of recFSH consumed; number of oocytes retrieved; number of 2PN oocytes; number of suitable embryos for transfer and cryopreservation; ongoing implantation rate and ongoing pregnancy rate per started cycle.

Data analysis

All analyses for high and low response were performed for the whole group. Subgroup analyses were performed for the groups that received 150 and 225 IU recFSH. Data for continuous variables are presented as mean values and standard deviation. Between-group statistical comparisons of mean values were performed with ANOVA tests. Chi squared tests were used for categorical data. Differences were considered to be statistically significant if *P* value < 0.05. Receiving operating characteristic (ROC) curves were constructed to demonstrate the predictive accuracy of AMH and other patient characteristics as single predictors and in combination using univariate and multivariate logistic regression, for both high and low ovarian response. The corresponding area under the curve (AUC) was calculated for both response groups in order to express the overall accuracy. In order to illustrate the clinical usefulness of AMH for the prediction of both high and low response, the sensitivity, specificity, likelihood ratios, pre- and post-test probability and the percentage of women with an abnormal test result, were calculated for several cut-off values of AMH, which were derived from the ROC curve. To explore the association between the number of oocytes and ongoing pregnancy rate (OPR) per started cycle, for each oocyte number the mean and 95% confidence interval (CI) was calculated for ongoing pregnancy rate per started cycle. Finally, different cut-off values for the number of oocytes applied in the literature to define a high or low response, were used to compare our results with previously published GnRH antagonist studies.

Results

Patient demographics and clinical outcome

Supplemental Figure 1 depicts the number of patients at each stage of the selection process. A total of 487 patients were included in the study of which 389 were scheduled to undergo an IVF treatment, with ICSI to be performed in 98 patients. Two patients withdrew from treatment prior to ovum pick-up for personal reasons. The remaining patients were divided into three subgroups according to the ovarian response category; high (n = 58), normal (n = 326) and low (n = 101). The baseline characteristics of the total group are shown in Table 1. The subgroups differed significantly in age, bodyweight, BMI, AMH and type of subfertility.

Table 2 demonstrates the stimulation characteristics and clinical outcome per started cycle. The majority of patients used a dosage of 150 IU recFSH daily (n = 439), while in a small subset a dose of 225 IU (n = 48) was administered. Cycle cancellation due to a low response or switch to intra-uterine insemination occurred in 15 and 17 cases, respectively. Premature ovulation occurred once. Ten patients did not receive hCG due to a high response and thus a risk of OHSS. Twelve cases of mild OHSS and 1 case of severe OHSS, requiring hospitalisation, were observed. The recFSH dose was increased to 225 IU in 23 patients during stimulation. There was an expected, significant between-group difference for the number of oocytes retrieved, number of 2PN oocytes, number of suitable and transferred or cryopreserved embryos, and the percentage of single embryo transfer. The ongoing pregnancy rate per started cycle appeared not to differ significantly among the 3 ovarian response groups. However, logistic regression demonstrated that ongoing pregnancy rates rose with an increasing number of oocytes retrieved up to a number of 6 oocytes (OR 1.27, 95% CI 1.06 – 1,52, $p = 0.009$), whereas a non-significant trend towards lower pregnancy rates was observed beyond 15 oocytes (OR 0.84, 95% CI 0.62 – 1.14, $p = 0.3$). This is also illustrated by Figure 1 which depicts the number of retrieved oocytes in relation to the ongoing pregnancy rate per started cycle.

Table 1 Baseline characteristics per subgroup

	High response (N = 58)	Normal response (N=326)	Low response (N = 101)	*p* value
Demographics				
Age (years)	32.4 ± 4.7	34.7 ± 4.3	35.7 ± 3.5	**< 0.001**
Range	*(22.8 - 41.6)*	*(21.2 - 44.0)*	*(24.2 - 42.7)*	
Bodyweight (kg)	66.0 ± 11.0	69.1 ± 12.7	72.2 ± 14.5	**0.01**
Range	*(45.0 - 97.0)*	*(43.0 - 133.0)*	*(47.0 - 122.0)*	
BMI (kg/m²)	22.9 ± 3.1	23.8 ± 4.1	24.9 ± 4.9	**0.01**
Range	*(15.9 - 31.7)*	*(17.2 - 47.1)*	*(17.5 - 39.8)*	
Smoking, n (%)	11 (19.0)	46 (14.1)	19 (18.8)	0.5[a]
AMH prior to treatment (µg/L)	5.6 ± 3.4	2.5 ± 1.8	1.2 ± 1.2	**< 0.001**
Fertility characteristics				
Primary subfertility, n (%)	50 (86.2)	219 (67.2)	73 (72.3)	**0.01**[a]
Secondary subfertility, n (%)	8 (13.8)	107 (32.8)	28 (27.7)	
Duration of subfertility (years)	3.2 ± 2.0	3.3 ± 2.0	3.2 ± 2.4	1.0
Cause of subfertility, n (%)				
Male factor	28 (48.3)	139 (42.6)	41 (40.6)	0.4[a]
Unexplained	21 (36.2)	120 (36.8)	34 (33.7)	
Tubal factor	8 (13.8)	50 (15.3)	15 (14.9)	
Endometriosis	1 (1.7)	5 (1.5)	2 (2.0)	
Incipient ovarian failure	0 (0.0)	6 (1.8)	8 (7.9)	
Other	0 (0.0)	7 (2.1)	1 (1.0)	

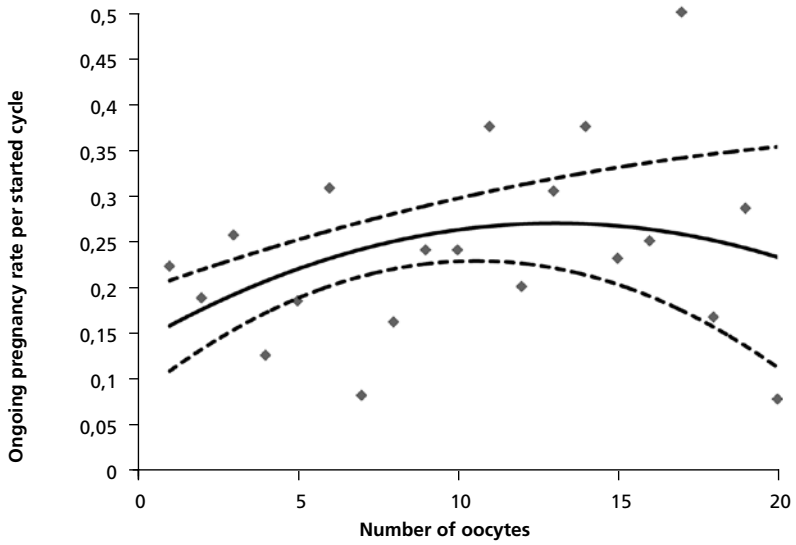
High response: > 15 oocytes or cancellation due to risk of OHSS. Normal response: 4-15 oocytes.
Low response: < 4 oocytes or cancellation due to poor response.
Data are presented as means ± standard deviation (SD). The ranges for age, bodyweight and BMI are depicted in italic font. P values are for between-group ANOVA tests unless otherwise stated.
P values in bold are statistically significant.
[a] *P value for between-group difference from Chi squared tests.*

Table 2 Stimulation characteristics and clinical outcome

	High response (N = 58)	Normal response (N=326)	Low response (N = 101)	*p* value
Stimulation characteristics				
Total dose of recFSH (IU)	1347 ± 298	1432 ± 392	1476 ± 502	0.2
Total duration of stimulation (days)	8.7 ± 1.4	9.0 ± 1.8	9.1 ± 2.8	0.4
Clinical outcome per started cycle				
Total number of oocytes	15.5 ± 7.8	8.4 ± 3.3	1.6 ± 1.3	**< 0.001**
Number of 2PN oocytes	8.0 ± 5.9	4.5 ± 2.7	1.0 ± 1.1	**< 0.001**
Suitable embryos for transfer or cryopreservation	6.7 ± 5.3	3.8 ± 2.5	0.9 ± 1.0	**< 0.001**
Number of embryos transferred	1.0 ± 0.7	1.2 ± 0.6	0.7 ± 0.7	**< 0.001**
Embryos cryopreserved	3.0 ± 4.0	1.2 ± 1.7	0.2 ± 0.5	**< 0.001**
Single embryo transfer (%)	33 (56.9)	197 (60.4)	41 (40.6)	**< 0.001**[a]
Ongoing implantation rate per embryo (%)	20 ± 37.5	20.7 ± 38.5	25.0 ± 42.1	0.7[a]
Ongoing pregnancy per started fresh cycle, n (%)	11 (19.0)	72 (22.1)	17 (16.8)	0.9[a]

High response: > 15 oocytes or cancellation due to risk of OHSS. Normal response: 4-15 oocytes.
Low response: < 4 oocytes or cancellation due to poor response.
Data are presented as means ± standard deviation (SD) and P values are for between-group difference from ANOVA tests unless otherwise stated. P values in bold are statistically significant.
[a] *P value for between-group difference from Chi squared tests.*

Figure 1 The number of retrieved oocytes in relation to ongoing pregnancy rate per started cycle



Prediction of high and low ovarian response

To examine the predictive accuracy of AMH and other possible predictors of ovarian response, the parameters listed in Table 1 were analysed by univariable en multivariable logistic regression. ROC curves were plotted for single and combined predictors (Figure 2). The levels of accuracy, as expressed by the AUCs, for ovarian response prediction are depicted in Table 3.

High response

For the prediction of high response, AMH had the highest accuracy (AUC 0.87) as compared to age, and BMI (Table 3). Although the AUC remained similar, multivariate logistic regression demonstrated that the addition of BMI slightly improved the predictive accuracy of AMH (OR 0.89, 95% CI 0.81 – 0.98, p = 0.01). The addition of age, smoking and type or duration of subfertility did not add prognostic value to the AMH model (p = 0.4, p = 0.8, p = 0.2, and p = 0.4, respectively). A separate analysis (data not shown) of the subgroups that used 150 IU or 225 IU recFSH demonstrated similar AUCs for age, AMH, BMI and the multivariate models.

Table 4 illustrates the clinical value of different AMH cut-offs for ovarian response prediction. When choosing a higher test cut-off level, the sensitivity and proportion of abnormal test results decreased, whereas the specificity, positive likelihood ratio and the post-test probability increased. At a specificity level of 90% and test cut-off of 4.5 µg/L, the test seemed to have the best performance level, indicating that in case of an abnormal test result, the chance of having an excessive response is 50%, while at the same time 60% of all true excessive responders will be identified.

Low response

AMH had an AUC of 0.79 for low response prediction, whereas age and BMI had absent accuracy (AUC 0.59 and 0.56, respectively, Table 3). In a multivariate logistic regression analysis again BMI added some predictive value to AMH (OR 1.08, 95% CI 1.02 – 1.15, p = 0.01). The factors age, smoking, type and duration of subfertility did not add prognostic value to AMH. Again, the subgroups that used 150 IU or 225 IU recFSH demonstrated similar AUCs for age, AMH, BMI and the multivariate models (data not shown). Table 4 demonstrates that the performance of AMH as a test for the prediction of low response was limited, as reflected by the low sensitivities corresponding with lower AMH thresholds. The optimal cut-off point, if any, seems to lie at a level of 0.80 µg/L, thus identifying 50% of all low responders and with a probability of 50% having a low response in case of an abnormal test.

Figure 2 Receiver operating characteristic curves for age, AMH and BMI for the prediction of high (panel A) and low response (panel B)

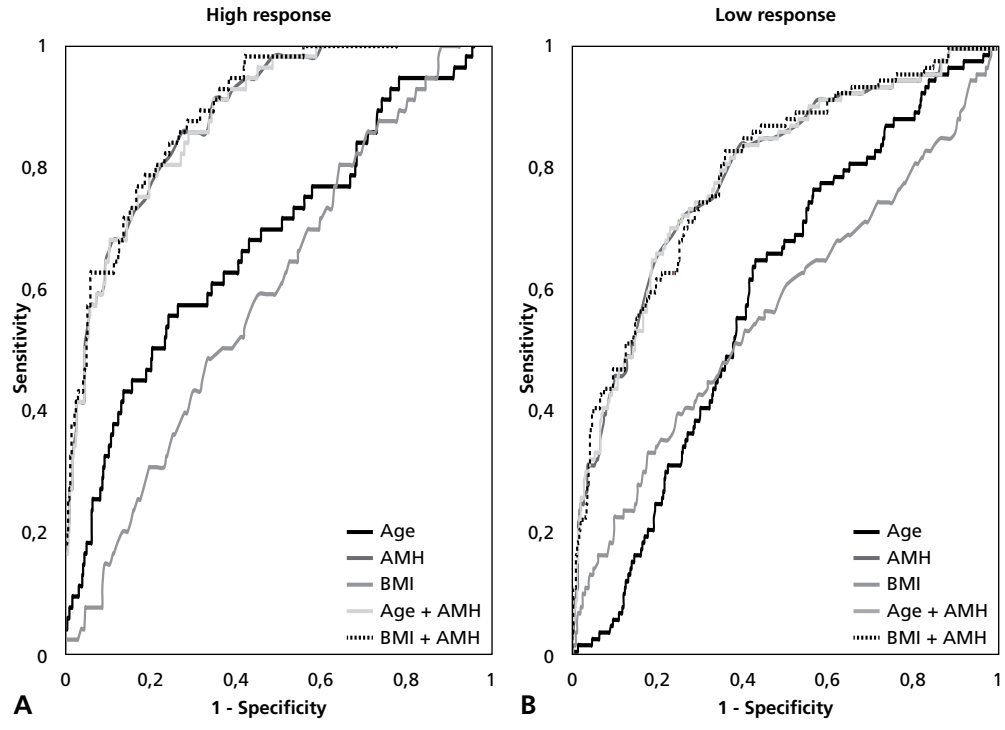


Table 3 The AUCs of prediction models of age, AMH and BMI for the prediction of high and low response

	High response		Low response	
	AUC	95% CI	AUC	95% CI
Univariable models				
Age (years)	0.66	0.57 - 0.74	0.59	0.53 - 0.65
AMH (µg/L)	0.87	0.82 - 0.91	0.79	0.74 - 0.84
BMI (kg/m^2)	0.58	0.50 - 0.65	0.56	0.49 - 0.63
Multivariable models				
Age + AMH	0.86	0.82 - 0.91	0.79	0.74 - 0.84
BMI + AMH	0.87	0.83 - 0.92	0.79	0.74 - 0.84
Age + AMH + BMI	0.87	0.82 - 0.92	0.79	0.74 - 0.84

High response: > 15 oocytes or cancellation due to risk of OHSS.
Low response: < 4 oocytes or cancellation due to poor response.
AUC: Area Under the Curve, CI: confidence interval

Table 4 Test characteristics for AMH as predictor of the outcome high and low response

	Cut-off value AMH (µg/L)	Proportion with abnormal test (%)	Sensitivity	Specificity	LR+	Pretest probability (%)	Posttest probability (%)
High response							
Optimal value	2.75	0.35	0.82	0.72	2.96	0.12	0.29
Possibly useful values	3.65	0.22	0.67	0.85	4.36	0.12	0.37
	4.45	0.15	0.57	0.90	5.93	0.12	0.45
	5.30	0.10	0.48	0.95	9.47	0.12	0.56
Low response							
Optimal value	1.45	0.37	0.74	0.73	2.70	0.21	0.42
Possibly useful values	0.94	0.23	0.53	0.85	3.50	0.21	0.48
	0.79	0.17	0.45	0.90	4.42	0.21	0.54
	0.52	0.11	0.34	0.95	6.88	0.21	0.64

LR+: likelihood ratio for positive test result.
High response: > 15 oocytes or cancellation due to risk of OHSS.
Low response: < 4 oocytes or cancellation due to poor response.

Discussion

This prospective cohort study demonstrates that AMH as a single test has substantial accuracy in the prediction of ovarian response using GnRH antagonist treatment for IVF. Furthermore, the accuracy curves indicate that AMH is a better predictor for high than for low ovarian response. The findings from the present study are in line with two systematic reviews on the predictive value of AMH for ovarian response using GnRH agonist treatment. These reviews have comprised a large number of studies, from which solid information has become available demonstrating an AUC of 0.81 for the prediction of high response (62) and 0.78 for the prediction of low response (63).

Conversely, the significance of AMH in a GnRH antagonist system has been addressed in only three other studies (66;67;69) which used a fixed start GnRH antagonist protocol with recFSH dosages of 150-225 IU. The reported accuracies of AMH for the prediction of high and low response differed among these studies which may be have been caused by differences in study population [maximum age 34 and 36 years, respectively in Arce *et al.* (2011) and Polyzos *et al.* (2013)] or by the use of different definitions for ovarian response making it difficult to compare results. Hence, we reanalysed our data using the different oocyte number cut-offs which were applied in the aforementioned GnRH antagonist studies. Arce *et al.* (2013) used similar definitions as in the present study and demonstrated a slightly lower AUC for predicting high response (0.81), but a higher accuracy for low response prediction (AUC 0.90). Andersen *et al.* (2011) demonstrated an AUC of 0.82 for high response (> 18 oocytes) and an AUC of 0.88 for low response (< 6 oocytes). The use of these definitions in our dataset improved the accuracy of AMH for high response, but not for low response (AUC 0.92 and 0.79, respectively). Finally, Polyzos *et al.* (2013) demonstrated an AUC of 0.80 for high response (> 20 oocytes) which cut-off substantially affected the accuracy of AMH in our dataset (AUC 0.94). In comparison, this study obtained a slightly poorer AUC (0.72) for low response (< 4 oocytes) despite using the same definition. Thus, the results of the present study seem to confirm most of the findings in previous studies.

The definitions commonly applied for ovarian response categories are mostly based on GnRH agonists. It can be debated whether the use of a higher number of oocytes to define a high response should be applied in GnRH antagonist regimens, as the use of GnRH antagonists is associated with a lower number of oocytes retrieved compared with GnRH agonists (25;26). The present study observed a non-significant trend towards lower pregnancy rates beyond 15 oocytes which is in line with Sunkara *et al.* (2011) who demonstrated that the optimal number of oocytes associated with the chance of achieving live birth was ~15, whereas a decline was shown with > 20 oocytes. The high estradiol levels associated with a high response, as well as the possible untimely changes in progesterone levels may explain the lower ongoing

pregnancy rate in this group of patients due to impaired endometrial receptivity and oocyte/embryo quality (2;18;39;46;49;50;52-54). Additionally, the presence of ≥ 18 follicles following GnRH antagonist treatment has previously been associated with an increased risk of developing OHSS (107). However, judging from the low incidence of ≥ 18 oocytes in the present study (n = 20, 4.1%), defining a high response as > 15 oocytes may be adequate in GnRH antagonists.

A low response following conventional ovarian stimulation is regarded as a sign of advanced ovarian aging (92;108), as this type of stimulation will induce maximal stimulation of the ovaries (25;70). Conversely, the retrieval of a low number of oocytes following mild stimulation is associated with much more favourable pregnancy chances and does not reflect a "poor" ovarian response (3;109). The present study demonstrated that pregnancy rates rose up to a number 6 oocytes retrieved, which is similar to what has been observed in GnRH agonist co-treated cycles (110). Hence, defining low response at a lower cut off than the accepted level of < 4 oocytes, may yield a subgroup with sufficiently poor prospects for pregnancy that predicting such group would be clinically relevant. Larger studies will be needed to support such a claim.

The current study demonstrated that the accuracy of AMH was slightly improved by BMI, whereas no improvement was observed after the addition of age, type or duration of subfertility and smoking. The biological availability of recFSH has been shown to be reduced in obese women. Moreover, BMI has been negatively associated with ovarian response (111-116). However, others have found no additional value of BMI to AMH in the prediction of high response (62). In the present study, the accuracy of BMI as a single predictor of ovarian response was very low which is in line with previous GnRH agonist (63), and GnRH antagonist (66) studies. Nevertheless, BMI could be used in GnRH antagonist protocols to improve the accuracy of AMH.

Ideally, a test for ovarian response prediction would identify all women with a high or low response and exclude all women with a normal response. Table 4, however, demonstrates that the performance of AMH for predicting high and low response is not optimal. Judging by the abnormal test rate corresponding with the optimal AMH cut-off for high response prediction, a considerable number of patients with a false positive test would be treated with a lower FSH dose and may therefore turn into a low responder. Furthermore, the performance of AMH as a test for the prediction of low response was rather limited, as reflected by the low sensitivities corresponding with lower AMH thresholds. It remains to be established whether increased stimulation dosages in expected poor responders will result in better pregnancy prospects, when using a GnRH antagonist protocol.

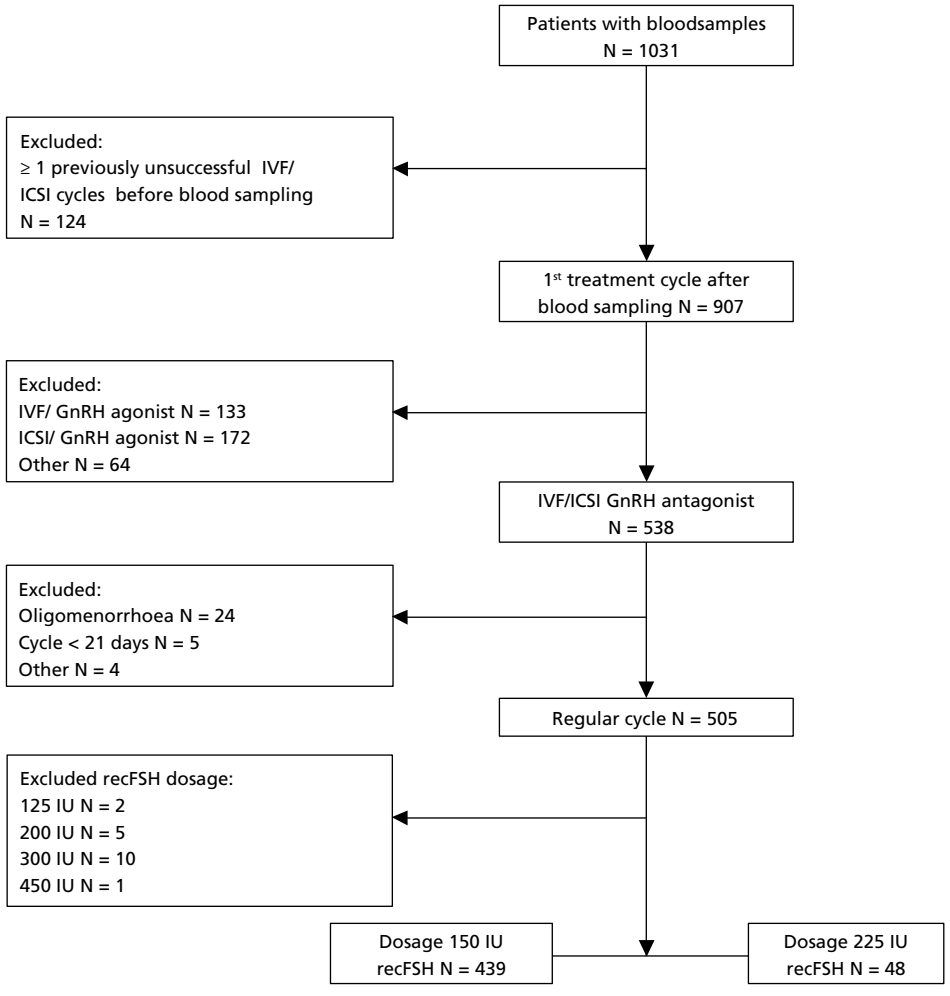
Strengths of this study include the absence of selection bias as all women starting their 1st IVF/ICSI in our hospital were asked to participate in this study and absence of verification bias as AMH values were not available at the start of and during IVF treatment. Furthermore, AMH was not measured during controlled ovarian stimulation which has been shown to decrease AMH

levels (117;118). A possible limitation is the time interval between serum sampling and initiation of controlled ovarian stimulation in the present study (7 months). However, this is not likely to have influenced the results as a time interval up to 12 months between serum sampling and initiation of stimulation has been shown not to affect the predictive ability of AMH (69).

Accurate prediction of response prior to IVF is important to individualise stimulation regimens by for example adjusting the starting dose of gonadotropins. Previous studies utilising AMH to tailor IVF treatment have shown a reduction in the incidence of high and low response as well as improved pregnancy rates compared with non-individualised treatment cycles (64;65). In the present study no large difference in pregnancy rates between the high, normal and low response groups was found. This may indicate that predicting a low response is clinically less relevant as opposed to predicting a high response, as here safety issues also play a role. One of the proposed preventive strategies for OHSS is the use of a GnRH agonist trigger to induce final oocyte maturation with or without cryopreservation of all embryos which has resulted in a decreased incidence of OHSS in high risk patients with no change in reproductive outcome (36). However, severe OHSS has recently been reported after GnRH agonist triggering with and without luteal-phase hCG supplementation, despite cryopreservation of all embryos (119;120). Therefore, it remains crucial to individualise IVF treatment in order to decrease the incidence of OHSS even though a direct benefit in terms of increased pregnancy rates remains to be established.

In conclusion, the current study demonstrates that AMH is an adequate predictor of both high and low response using GnRH antagonist treatment. The individualisation of GnRH antagonist protocols may be further improved by using an AMH-tailored approach. However, since AMH has a higher accuracy for the prediction of high response than for low response, and the significance of a low response in GnRH antagonists regarding pregnancy prospects has become doubtful, studies into individualisation may best focus on preventive strategies towards high response management.

Supplemental Figure 1 Flowchart depicting the number of patients throughout the selection process



IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; GnRH: gonadotropin releasing hormone; recFSH: recombinant FSH

Anti-Müllerian hormone: prediction of cumulative ongoing pregnancy rates in GnRH antagonist co-treated cycles

O. Hamdine, M.J.C. Eijkemans, E.W.G. Lentjes, H.L. Torrance, N.S. Macklon, B.C.J.M. Fauser and F.J. Broekmans

Submitted

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Abstract

Study question: What is the predictive ability of Anti-Müllerian hormone (AMH) for cumulative ongoing pregnancy within a one year treatment horizon in patients assigned to undergo ovarian stimulation for in vitro fertilisation (IVF) using a GnRH antagonist protocol?

Summary answer: Although AMH added some value in predicting ongoing pregnancy, its predictive accuracy was only modest.

What is known already: For several years, clinical research has focused on the prediction of ongoing pregnancy or live birth in assisted reproductive technology (ART) using GnRH agonist protocols. Little is known regarding the predictive ability and added value of AMH in GnRH antagonist co-treated cycles.

Study design, size, duration: Prospective cohort study at the University Medical Centre Utrecht, the Netherlands. A total of 487 patients scheduled for IVF/intracytoplasmic sperm injection (ICSI) between 2006 and 2011 were included in the study.

Participants/materials, setting, methods: Patients with a regular cycle who underwent their 1st IVF/ICSI cycle with GnRH antagonist co-treatment while receiving a starting dose of 150 or 225 IU recombinant FSH were included. Serum samples collected prior to the first IVF treatment were used to determine serum AMH levels. The IVF treatment data from the first cycle onward (with a maximum of one year) were prospectively recorded and used for this study.

Main results and the role of chance: The model for the prediction of ongoing pregnancy within one year in GnRH antagonist co-treated cycles included age at first IVF treatment, AMH level, type and duration of subfertility and the number of previous ART treatments. The c-statistic of this model was 0.60 (95% CI 0.57 – 0.64), indicating that this model discriminates between women who did or did not conceive with an accuracy of only 60%. AMH had intermediate added value (33.3%) in the prediction of ongoing pregnancy as assed by the continuous net reclassification improvement (NRI). A nomogram was developed by which a subgroup of patients could be identified with lower pregnancy prospects.

Limitations, reasons for caution: A proportion (19.3%) of the population received GnRH agonist downregulation in one of their subsequent cycles, reflecting common practice. However, a subanalysis including patients receiving GnRH antagonist co-treatment in all subsequent cycles demonstrated similar results.

Wider implications of the findings: Currently, factors that can accurately predict the probability of achieving an ongoing pregnancy and live birth are lacking, irrespective of the applied method of LH peak suppression. Although AMH is associated with pregnancy, its predictive accuracy is very modest even when assessing the cumulative pregnancy rate. AMH alone or in combination with female age is not very likely to alter clinical decisions based on the chance of ongoing pregnancy or live birth after ART and should preferably only be used for counseling or ovarian response prediction.

Trial registration number: www.clinicaltrials.gov, Protocol ID 13-109

Introduction

For several years, research in the field of reproductive medicine has focused on the prediction of clinical outcome in assisted reproduction. Several prognostic models using patient and/or treatment characteristics have been proposed for the prediction of the probability of an ongoing pregnancy or a live birth following in vitro fertilisation (IVF) with age being the most firmly established predictor (121;122). The purpose of all these efforts is to tailor treatment on an individual basis in order to optimise treatment outcome. Tailoring could imply adjustments in treatment schedule and dosage scheme, decisions on the source of the gametes, and even advice to refrain from treatment. Recently, Anti-Müllerian hormone (AMH) has been suggested as a predictor of both ovarian response and clinical outcome. AMH is strongly correlated to oocyte yield (123) and has been established as an accurate predictor of both excessive and poor ovarian response in IVF cycles using GnRH agonist (62;63) or GnRH antagonist co-treatment (66;67;69).

Conflicting results exist regarding the association of AMH with the outcome 'pregnancy' after assisted reproductive technology (ART) treatment. The value of AMH as a predictive factor for ongoing pregnancy or live birth, has mostly been studied in patients treated with a long GnRH agonist protocol and has been positively associated with the achievement of pregnancy or live birth in some studies (124-129) whereas others have observed limited or no predictive value of AMH for these outcomes (63;130;131). The ability of AMH to predict clinical pregnancy or live birth in studies using both GnRH agonists and antagonists was found to be modest (132-135). Predictive factors for GnRH antagonist co-treated cycles are still scarce (66). One study has demonstrated that AMH had poor accuracy for the prediction of ongoing pregnancy (67). Furthermore, most studies only analysed the treatment outcome of the first IVF cycle, either with or without frozen-thawed cycles (67;132;134;135). However, as ovarian stimulation in GnRH antagonist co-treated cycles is often milder than in the traditional GnRH agonist co-treated cycles (25), it is more realistic to assume that many patients need more than one IVF treatment to achieve a pregnancy. Hence, it would be useful for clinical practice to provide infertile couples scheduled to undergo an antagonist co-treated cycle with prognostic information regarding their cumulative probability of achieving an ongoing pregnancy within a certain time frame based on several fresh cycles and including frozen-thawed cycles.

The aim of this study was therefore to evaluate the relationship between possible predictors, including AMH, and cumulative ongoing pregnancy rate within a one year treatment horizon in patients assigned to undergo controlled ovarian stimulation for IVF using exogenous recombinant FSH (recFSH) and GnRH antagonist co-treatment.

Subjects and methods

Subject selection

To achieve the aim of this study, we selected women from a prospectively collected cohort of 1031 patients who were treated at the IVF outpatient clinic of the Department of Reproductive Medicine and Gynecology of the University Medical Centre Utrecht. Informed consent was obtained from all patients for banking and the use of both serum and DNA samples and clinical data for research purposes regarding assisted reproduction. This research protocol was approved by our Institutional Review Board and registered on www.clinicaltrials.gov (Protocol ID 13-109). Blood samples were collected, regardless of the cycle day as AMH has low inter– and intracycle variability (61;66), during routine screening for hepatitis B and C and HIV prior to IVF treatment, and stored at –20 °C. For the current study, we selected patients with a regular cycle who underwent their first IVF/ intracytoplasmic sperm injection (ICSI) cycle with GnRH antagonist co-treatment while receiving a starting dose of 150 or 225 IU recFSH. Women who achieved a live birth after a previous treatment episode and had a renewed child wish were also included in the study. These couples were thus intended to be treated with the GnRH antagonist system according to the local protocol in the first and subsequent cycles. Patients who fulfilled the inclusion criteria were treated between 2006 and 2011. The IVF treatment data from the first cycle onward (with a maximum of one year) were prospectively recorded in our electronic infertility patient data files and used for this study. Serum samples were retrieved for the current study to determine serum AMH levels.

IVF/ICSI procedure

Ovarian stimulation was performed with recFSH (Gonal-f; Merck Serono, or Puregon; MSD, the Netherlands). During the first treatment cycle a GnRH antagonist (Cetrotide; Merck Serono, or Orgalutran; MSD, the Netherlands) was used to prevent a premature LH surge. The patients were not pretreated with oral contraceptives. According to local protocol, recFSH (150-225 IU) was started on cycle day (CD) 2 or 3. The reasons to apply a starting dose of 225 IU recFSH/day instead of the standard dose of 150 IU recFSH in our clinic were female age > 41 years, a previous treatment leading to live birth with 225 IU recFSH/day or incipient ovarian failure (defined as a regular cycle, basal FSH > 10 IU/L and age < 40 years). GnRH antagonist co-treatment was commenced on stimulation day 5. Human chorionic gonadotropin (hCG, 10,000 IU, Pregnyl; MSD, the Netherlands or 6500 IU Ovitrelle; Merck Serono, the Netherlands) was administered to induce final oocyte maturation when at least three follicles of ≥ 17 mm in diameter were visualised by ultrasound. Oocyte retrieval was performed 36 hours after hCG administration. One or two embryos were transferred 3 or 4 days after oocyte retrieval. The luteal phase was supplemented with a daily dose of 600 mg vaginally administered micronized

natural progesterone (Utrogestan; Besins Healthcare, Brussels, Belgium). Supernumerary embryos of sufficient quality were cryopreserved on day 3 or 4 after oocyte retrieval. Patients who did not become pregnant after fresh transfer, could undergo frozen-thawed replacement cycles in either a natural or artificial cycle. Subsequent fresh IVF/ICSI cycles were also performed using the GnRH antagonist protocol. In some cases a switch was made to the long GnRH agonist protocol (Decapeptyl, Ferring, the Netherlands). The reasons to use GnRH agonist co-treatment in a subsequent cycle were as follows; premature ovulation or poor ovarian response in the previous GnRH antagonist cycle (< 4 oocytes), previously undiagnosed endometriosis, female age > 40 years or intercurrent indication for an ICSI treatment in case of previous total fertilisation failure with IVF.

Anti-Müllerian hormone assay

After blood collection, plasma for assay of AMH was separated directly and frozen in aliquots within 3 – 4 hours. In December 2011 stored frozen samples were thawed overnight in the refrigerator in order to determine AMH levels. All measurements were performed in a batch analysis using a single lot reagent by a DS2 ELISA analyser (AMH Gen II ELISA, A79765, Beckman Coulter; Inc., USA). In order to minimise possible interference from complement first a buffer was pipetted before the initial sample. Using this procedure the problem with the complement interference was not detected. The lower limit of detection was 0,16 µg/L. Inter-assay variation was 10% at 0,27 µg/L and 4,7% at 3,9 µg/L (n = 18). The maximum time interval between serum sampling and the start of ovarian stimulation was 7 months (range 1 day – 7 months).

Assessment of potential predictive factors

The following data were collected: age at first IVF/ICSI treatment, body mass index (BMI), cycle length, type and duration of subfertility, diagnosis, smoking status, obstetric history, previous ART treatment, scheduled for IVF or ICSI and serum AMH level.

Outcome measure

The main outcome was cumulative ongoing pregnancy rate within one year, defined as the presence of at least one fetus with heartbeat beyond 9 weeks of gestation.

Data analysis

The baseline characteristics were described for the entire group as well as for the pregnant and non-pregnant groups. Continuous data are presented as mean values and corresponding standard deviation. Categorical data are presented as frequencies and percentages. A Kaplan Meier curve was estimated for the cumulative chance of an ongoing pregnancy within one year. Time to event was defined as the time since start of treatment and the status at the end of follow-up was either ongoing pregnancy or no pregnancy. Women who stopped treatment before one year of follow-up without being pregnant were assumed not to have conceived during the remainder of the year (cumulative incidence approach) (136). We used a restricted cubic spline (3 df) to model the possible non-linear relationship of the continuous variables AMH, female age and BMI with the chance of ongoing pregnancy. Cox proportional hazards regression was applied to identify predictive factors for ongoing pregnancy. Backward elimination of variables using Akaike's Information Criterion (AIC) was used to select the best set of predictors, starting with 11 variables: AMH, age at first IVF treatment, BMI, smoking status, type and duration of subfertility, diagnosis, obstetric history (number of previous pregnancies and deliveries), previous ART treatments and scheduled for IVF or ICSI. The c-statistic was calculated to demonstrate the capacity of the model to distinguish between women who became pregnant earlier or later (or who did not become pregnant). Bootstrapping with 500 randomly replicated datasets obtained by drawing with replacement from the original data was used to estimate a) the shrinkage factor necessary to correct for overfitting and b) the correction for optimism of the c-statistic. In order to assess the added clinical value of AMH in predicting ongoing pregnancy, we compared a model with and without AMH, together with the selected variables, using the continuous version of the Net Reclassification Improvement (NRI). The NRI quantifies the improvement offered by a new marker by examining the extent to which the new marker reclassifies subjects with the event into a higher risk and subjects without the event into a lower risk (137). NRI values > 60% are considered strong, ~40% are intermediate and < 20% are weak (138). The maximum possible NRI is 200% as theoretically all women with and without an event could be reclassified in the correct direction (137).

Since part of the study population used a GnRH agonist protocol during subsequent cycles, a subanalysis was performed to assess the predictive value of AMH in cases strictly treated with a GnRH antagonist protocol. Finally, a simplified nomogram was created to show the probability of achieving an ongoing pregnancy within a year for combinations of age and AMH using all included cases. Data were analysed with SPSS version 20.0 (SPSS Inc, Chicago, Illinois,USA) and with R version 2.13 (http://www.r-project.org/).

Results

This study evaluated the IVF/ ICSI and subsequent frozen-thawed embryo treatment cycles performed in 1 year of 487 women. A 1st, 2nd, 3rd, 4th, 5th and 6th fresh IVF/ICSI treatment cycle was performed in 487, 296, 150, 35, 7 patients and 1 patient, respectively. Although the intention according to the local protocol was to use the GnRH antagonist system in all treatment cycles in these couples, 19.3% of patients were treated with a long GnRH agonist protocol in one of their subsequent cycles, for the reasons mentioned in the methods section. Sixty-six patients used GnRH agonist co-treatment in their 2nd cycle, 44 in their 3rd cycle, 15 in their 4th cycle, 2 in their 5th cycle and one during the 6th treatment cycle. The baseline characteristics are summarised in Table 1. The total group was divided into 2 subgroups: pregnant (N = 261) and not-pregnant (N = 226). These two groups differed significantly with regard to age at first treatment, AMH level, number of previous pregnancies, type and cause of subfertility.

Figure 1 demonstrates the estimated cumulative chance of an ongoing pregnancy within one year for the total group. After the one year period, 49.5% (95% CI 45.0 – 55.0) of the couples had achieved an ongoing pregnancy. Figure 2 represents the non-linear association of age and AMH with the chance of ongoing pregnancy, using restricted cubic splines. The chance to achieve an ongoing pregnancy declined beyond the age of 35. The chance to achieve an ongoing pregnancy increased with higher AMH levels up to approximately 3 µg/L, after which a plateau was reached.

Table 1 Baseline characteristics for the total group

	Total group N = 487	**Pregnant N = 261**	**Not pregnant N = 226**	***p* value**
Demographics				
Age at 1st treatment (years)	34.6 ± 4.3	33.5 ± 4.2	35.9 ± 4.2	**<0.001**
BMI (kg/m²)	23.9 ± 4.2	24.0 ± 4.5	23.8 ± 3.8	0.5
Smoking, n (%)	77 (15.8)	48 (18.4)	29 (12.8)	0.2[a]
AMH prior to treatment (µg/L)	2.6 ± 2.3	3.0 ± 2.3	2.1 ± 2.2	**<0.001**
Fertility characteristics				
Primary subfertility, n (%)	343 (70.4)	197 (75.5)	146 (64.6)	**0.009[a]**
Secondary subfertility, n (%)	144 (29.6)	64 (24.5)	88 (35.4)	
Duration of subfertility (years)	3.2 ± 2.1	3.1 ± 2.0	3.4 ± 2.0	0.2
Previous ART treatments, n (%)	36 (7.4)	21 (8.0)	15 (6.6)	0.6[a]
IVF, n (%)	389 (79.9)	201 (77.0)	188 (83.2)	0.1[a]
ICSI, n (%)	98 (20.1)	60 (23.0)	38 (16.8)	
Obstetric history				
Number of pregnancies	0.6 ± 1.1	0.5 ± 0.9	0.8 ± 1.2	**0.01**
Number of deliveries	0.3 ± 0.6	0.3 ± 0.6	0.4 ± 0.6	0.2
Cause of subfertility, n (%)				
Male factor	207 (42.5)	126 (48.3)	81 (35.8)	**0.01[a]**
Unexplained	177 (36.3)	83 (31.8)	94 (41.6)	
Tubal factor	73 (15.0)	40 (15.3)	33 (14.6)	
Endometriosis	8 (1.6)	1 (0.4)	7 (3.1)	
Other	22 (4.5)	11 (4.2)	11 (4.9)	

Data are presented as means ± standard deviation (SD) and P values are for between-group difference from t-tests unless otherwise stated. P values in bold are statistically significant.

[a] *P value for between-group difference from Chi squared tests.*

Figure 1 The estimated cumulative chance of an ongoing pregnancy within one year

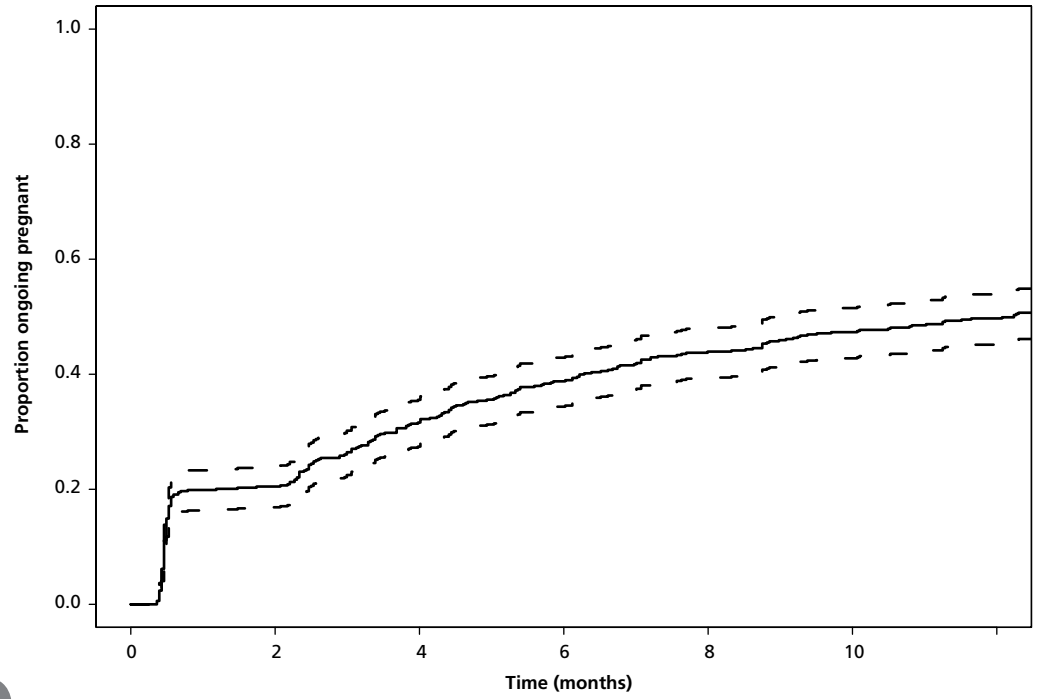
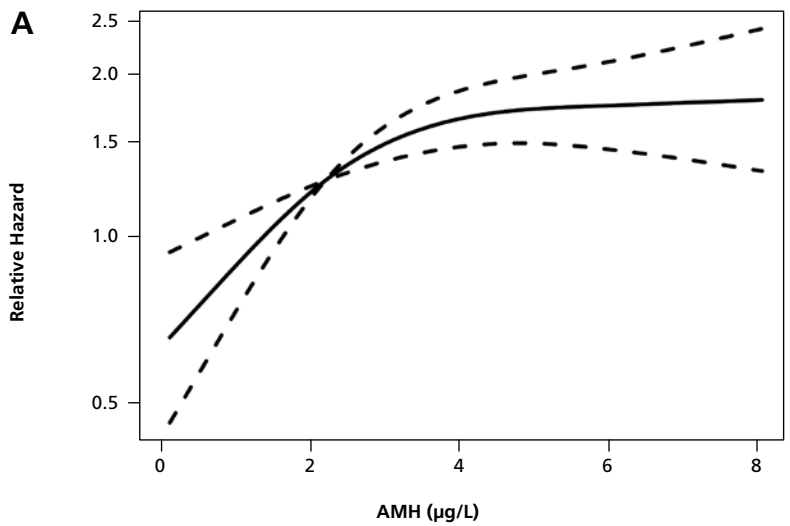
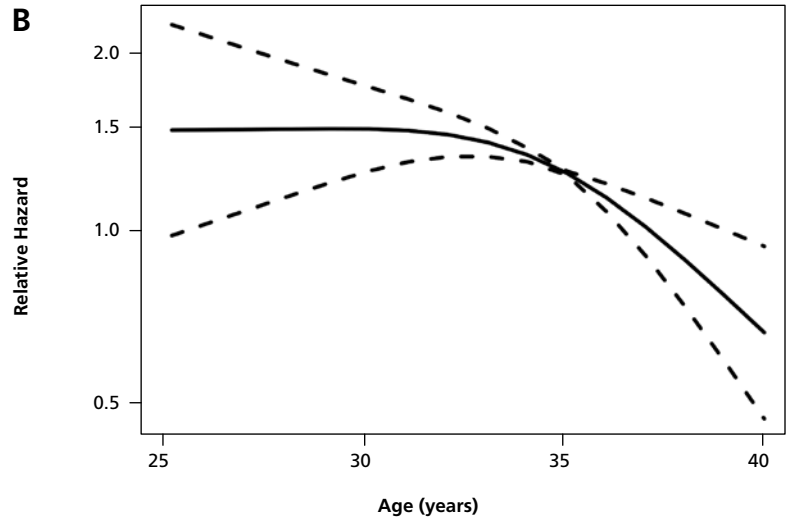


Figure 2 Restricted cubic splines representing the non-linear association of AMH (panel A) and age (panel B) with the chance of ongoing pregnancy





Backward elimination of possible predictors of ongoing pregnancy resulted in the selection of 5 predictors: age at first IVF treatment (HR 1.0, 95% CI 0.94 – 1.05), AMH level (HR 1.44, 95% CI 1.16 – 1.78), type and duration of subfertility (HR 0.73, 95% CI 0.53 – 1.02 and HR 0.94, 95% CI 0.88 – 1.01, respectively) and the number of previous ART treatments (HR 1.53, 95% CI 0.89 – 2.65). The c-statistic of the model including AMH was 0.60 (95% CI 0.57 – 0.64), which means that this model discriminates between women who did or did not conceive with an accuracy of 60%. Age and AMH as single predictors had similar accuracy for the prediction of an ongoing pregnancy (c-statistic: 0.60 [95% CI 0.57 – 0.64]). To assess the added value of AMH in predicting ongoing pregnancy, we used a model with and without AMH using the continuous NRI. The model with AMH correctly reclassified 17.0% of women who achieved an ongoing pregnancy into a higher probability category and correctly reclassified 16.3% of women who did not become pregnant into a lower probability category in comparison to a model without AMH. The total NRI therefore was 33.3%.

Table 2 shows a nomogram which depicts the predicted one-year probability of ongoing pregnancy from a simplified model containing only age and AMH, based on the data of all patients. The numbers in bold denote the number of patients within each combination of age and AMH and demonstrate that the extreme categories of young age and low AMH or high age and high AMH are quite rare.

Table 2 The predicted one-year probability (95% Confidence Interval) of ongoing pregnancy by a simplified model containing only age and AMH, based on the data of all patients

	AMH (µg/L)					
Age (years)	0-1	1-2	2-3	3-5	5-25	Total number of patients
0-30	0.45	0.57	0.60	0.74	0.74	
	(0.40 - 0.50)	(0.53 - 0.60)	(0.56 - 0.63)	(0.71 - 0.76)	(0.72 - 0.76)	
	3	***14***	***15***	***16***	***19***	***67***
30-35	0.41	0.52	0.55	0.69	0.69	
	(0.35 - 0.46)	(0.47 - 0.56)	(0.51 - 0. 59)	(0.66 - 0.71)	(0.66 - 0.72)	
	34	***43***	***48***	***37***	***20***	***182***
35-40	0.34	0.44	0.46	0.60	0.60	
	(0.28 - 0.39)	(0.38 - 0.49)	(0.41 - 0.51)	(0.56 - 0.63)	(0.57 - 0.64)	
	61	***61***	***30***	***22***	***18***	***192***
40-45	0.15	0.21	0.22	0.31	0.31	
	(0.07 - 0.22)	(0.13 - 0.27)	(0.15 - 0.29)	(0.24 - 0.37)	(0.24 - 0.37)	
	23	***9***	***7***	***5***	***2***	***46***
Total number of patients	***121***	***127***	***100***	***80***	***59***	

The numbers in (bold) italic represent the number of patients in each age/ AMH category.

We performed a subanalysis in 393 patients to assess the accuracy of AMH and other predictors in couples who strictly adhered to the GnRH antagonist protocol. A similar proportion of women achieved an ongoing pregnancy within one year 53.9% (95% CI 49.0 – 58.8). The prediction model included the same predictors of ongoing pregnancy, resulting in a similar c-statistic of the model including AMH [0.59 (95% CI 0.55 – 0.63)]. The accuracy of age and AMH as single tests in predicting pregnancy was also similar (0.60 [95% CI 0.56 – 0.64] and 0.59 [95% CI 0.55 – 0.63], respectively) to the primary analysis. The added value of AMH, as described by the NRI, was similar to the initial analysis. In comparison to a model without AMH, the model with AMH correctly reclassified a slightly higher percentage (17.9%) of women who achieved an ongoing pregnancy into a higher probability category and correctly reclassified a slightly lower percentage (12.7%) of women who did not become pregnant into a lower probability category (total NRI 30.6%).

Discussion

This prospective study in GnRH antagonist co-treated cycles, has demonstrated an association between AMH and the chance of an ongoing pregnancy after ART occurring within a one year treatment horizon. However, the accuracy of AMH in forecasting outcome was very modest, thus preventing the use of such a marker for patient selection or treatment denial. Still, the ability of the AMH level to refine prognostic categories based on female age has become apparent from the current study, although the practical use of such refinement remains unclear. The association between AMH and clinical outcome has been mainly attributed to its primary relationship with oocytes yield (67;129), although others have suggested that AMH may also reflect oocyte quality (124). Conflicting results have been reported regarding the association of AMH with ongoing pregnancy or live birth. The present study has shown an association between AMH and ongoing pregnancy. Additionally, the NRI has demonstrated that AMH has intermediate added value in the prediction of pregnancy based on female age. However, it was also demonstrated that AMH is not the accurate predictor of cumulative ongoing pregnancy that the field has been striving to develop for years. Furthermore, AMH has a similar accuracy as female age, while adding only limited information to female age. These findings are in line with previously published research assessing the first fresh treatment cycle with subsequent cryo-thawed cycles. Arce *et al.* (2013) demonstrated that AMH was a poor predictor of pregnancy in GnRH antagonist co-treated cycles (area under the curve [AUC] = 0.48). Others using study populations co-treated with either a GnRH antagonist or agonist observed a higher accuracy of AMH for the prediction of cumulative live birth (AUC 0.62 – 0.64) (129;135).

AMH and female age have each provided certain prognostic information regarding the probability of achieving a pregnancy as demonstrated by Figure 2 and the nomogram in Table 2. Higher AMH levels resulted in an increased probability of an ongoing pregnancy, within age categories. However, pregnancy rates did not further increase when AMH levels were above 3 µg/L. Such findings are in line with Sunkara *et al.* (2011) who demonstrated a rise in live birth rates with an increasing number of oocytes up to 15 after which a plateau was reached. Ata *et al.* (2012) has demonstrated that the proportion of euploid embryos remained unchanged with increasing numbers of embryos available. Nonetheless, the proportion of women with at least one euploid embryo increased when more embryos were generated (139), explaining the way through which AMH, as a marker of quantity, is related to the chance of ongoing pregnancy. It should be noted that the aneuploidy rate among cleavage-stage embryos in young women (< 35 years) already exceeds 50%. The aneuploidy rate increases further with advanced female age, making it less likely to have at least one euploid embryo available that may be capable of leading to a live birth (139), which explains the decline in pregnancy chances beyond the age of 35 in the current study.

The presented nomogram demonstrates that the extreme categories of young age and low AMH or high age and high AMH are quite rare. Based on this nomogram it seems not feasible to select a patient category which should be advised not to start IVF treatment or which should be refrained from starting treatment as demonstrated by the relatively wide confidence intervals. However, the "low AMH/high age" category could be informed regarding their low pregnancy chances and perhaps discouraged from starting IVF, particularly when taking into account the low incidence of euploid embryos (6-17%) observed in women aged ≥ 40 years, as well as the high risk of not having any euploid embryos available for transfer (139). Additionally, in the current study expected poor responders (AMH 0-1 µg/L) younger than 40 years old appeared to have reasonable pregnancy chances (Table 2). This seems in contrast to a study using different age/AMH categories to predict live birth in GnRH agonist co-treated cycles in which poor prospects for cases with low AMH levels were demonstrated with only limited influence of female age category (126). Nevertheless, low oocyte numbers have been associated with a higher miscarriage rate, irrespective of age, which may be attributed to embryo aneuploidy as a consequence of oocyte aneuploidy and thus poor oocyte quality (140). Thus, the presented nomogram may be useful but only for counseling infertile couples, although validation in a different population is mandatory before any claims for general use can be laid down.

Cryopreservation and transfer of surplus embryos has become an integral part of modern ART programs and offers patients an extra chance to achieve a pregnancy. While most studies evaluated the clinical outcome of only one fresh cycle with or without frozen-thawed cycles, the current study assessed all fresh and frozen-thawed cycles performed within one year. This offers a realistic take on current clinical practice as patients can be offered prognostic information based on more than one IVF cycle. Another strength of this study was the absence of selection bias as all women starting their 1st IVF/ICSI in our hospital were asked to participate in this study. The AMH test results were not available at the start of and during the IVF treatments and could therefore not have altered clinical management. Furthermore, the nomogram is based on baseline characteristics, hence permitting it to be used by clinicians prior to commencing stimulation.

A possible limitation of this study is that a proportion of this patient population was down regulated with a GnRH agonist in subsequent cycles. However, the subanalysis included patients co-treated strictly with a GnRH antagonist protocol and has demonstrated similar results with regard to the modest accuracy of AMH in predicting ongoing pregnancy. Therefore, subsequent co-treatment with a GnRH agonist protocol has not influenced the results of this study. Furthermore, the heterogeneous population and the inclusion of couples that switched to GnRH agonist treatment based on clear definitions does reflect the common daily practice which allows generalizability of the findings.

The performance of assisted reproduction technology is far from optimal which results in an increased need to identify factors to improve IVF treatment, including the use of patient characteristics and ovarian reserve tests to predict pregnancy prognosis. So far, factors that can accurately predict the probability of achieving an ongoing pregnancy and live birth are lacking. Although AMH is associated with pregnancy, its accuracy for predicting pregnancy is very modest. A recent large individual patient data (IPD) analysis has demonstrated that in GnRH agonist co-treated cycles AMH, AFC and FSH did not add any value to the limited capacity of female age to predict ongoing pregnancy after IVF (63). Chances are that a similar IPD including GnRH antagonist co-treated cycles will offer similar results. Even when assessing cumulative pregnancy rate, which may be considered a better indicator of treatment outcome as opposed to the assessment of one stimulation cycle, the accuracy of the model utilising AMH was still only 60%. This is in line with previous research. External validation of the present prediction model and nomogram may still not provide accurate prognostic information. This may indicate that the probability of achieving a pregnancy is influenced by several other external factors such as embryo transfer policy and technique and variation in laboratory procedures. The question therefore remains whether predictive factors which can accurately forecast pregnancy will ever be found. Meanwhile, AMH alone or in combination with other possible predictors is unlikely to alter clinical decisions based on the chance of ongoing pregnancy or live birth after ART and is instead preferably used for ovarian response prediction.

In conclusion, the present study demonstrated that although AMH added some value in predicting ongoing pregnancy, its predictive accuracy was limited and may not yield much additional value on top of female age. Furthermore, a clear distinction between couples with a good or poor prognosis based on different age/AMH categories could not clearly be made. As such it would currently not be appropriate to withhold treatment purely based on the use of AMH. However, AMH may be used for treatment individualisation since its accuracy for ovarian response prediction has now been established. The true value of AMH may lie in the prediction of high response as here both efficacy and safety issues play a role. Nevertheless, its efficacy in terms of improved clinical outcome and cost-effectiveness still needs to be established in future trials.

General Discussion

For more than 30 years, the conventional long GnRH agonist protocol has been the most frequently used stimulation protocol for IVF treatment (2). The introduction of GnRH antagonists has allowed for the development of more patient-friendly protocols of which the fixed daily injection protocol starting on stimulation day 5-6 is currently the most frequently used regimen (28;29;34). However, there is a need to further optimise the GnRH antagonist system as the optimal application of GnRH antagonists has not yet been identified (25), and doubts still exist as to whether the GnRH antagonist protocol is equally effective compared to the long suppression agonist system (22). Additionally, individualisation of the treatment protocol both regarding the choice of the GnRH analogue and the FSH dosing level, has become increasingly important and knowledge of predictive factors, such as AMH, that can forecast ovarian response is desirable.

In this thesis we therefore evaluated the effect of modification of the current GnRH antagonist protocol on both the endocrine profile and live birth rate. Additionally, we evaluated the accuracy of AMH in the prediction of ovarian response and reproductive outcome in the GnRH antagonist regimen. The aims of the thesis were formulated as follows:

1. Can the GnRH antagonist co-treatment stimulation protocol for IVF be improved by a change in the timing of the co-treatment, with focus on:
   a. the stimulation phase endocrine profile
   b. the clinical outcome in terms of oocyte yield and pregnancy
2. Does AMH have a consistent role in ovarian response optimisation in GnRH antagonist co-treatment stimulation cycles, as expressed by:
   a. its accuracy in ovarian response prediction
   b. its accuracy in the prediction of treatment outcome

Current perspectives

The first aim reflects the optimisation of the commonly used fixed start GnRH antagonist protocol. The expected benefit of early initiation of GnRH antagonist co-treatment on clinical outcomes was based on three assumptions: moderation of the ovarian response related to early suppression of endogenous FSH by the GnRH antagonist and the mitigated subsequent exposure to estradiol (E_2) levels; tighter prevention of untimely LH surges, with reduction of premature ovulation rates; and a more consistent control on early and late follicular phase progesterone (P) levels, enabling improved conditions for endometrial receptivity.

Initially, slightly less follicles and oocytes were obtained following early initiation of GnRH antagonist co-treatment as compared to standard late initiation (chapter 2). This finding suggests that an early start might result in decreased follicular recruitment which may be beneficial when it comes to reducing the risk of OHSS in predicted high responders. However, this was not confirmed in chapter 3, leading to the conclusion that an early start may not aid in the mitigation of ovarian response.

In chapter 2 it was demonstrated that early initiation of GnRH antagonists resulted in lower and less variable LH and E_2 levels during the mid and late follicular phase, which were closer to normal cycle conditions. These findings indicate that an early start possibly improves the hormonal milieu in ovarian stimulation for IVF, where over-exposure to steroids becomes limited. This could be beneficial for both endometrial and oocyte quality and thus for the achievement of pregnancy.

The true impact of elevated serum P levels prior to the start of ovarian stimulation in a GnRH antagonist protocol on clinical outcome remains a subject of debate. Chapter 4 revealed that elevated P levels are indeed associated with reduced chances of pregnancy. However, we observed no difference between early or late GnRH antagonist initiation on the effect of elevated or normal P on ongoing pregnancy rate.

Contrary to expectations, in chapter 3 a trend towards higher ongoing pregnancy and live birth rates in favour of the standard midfollicular phase fixed start regimen was observed. This may mean that a more stable endocrine profile does not necessarily result in improved endometrial receptivity and hence better pregnancy prospects. The clinical importance of this small non-significant difference in live birth rates is probably negligible. Additionally, lengthening the number of GnRH antagonist injections by four days may increase both treatment burden and costs and imposes the risk of losing some of the benefits of a GnRH antagonist protocol.

The second aim reflects the role of AMH in the optimisation of GnRH antagonist co-treated cycles. The value of AMH as a predictor for ovarian response has already been established in long GnRH agonist co-treated cycles (62;63;123;141), whereas the significance of AMH measurements in GnRH antagonist regimens has been addressed in only three other studies

(66;67;69) which show varying results possibly due to heterogeneity in definitions for ovarian response. Chapter 5 therefore addresses the role of AMH for response prediction in GnRH antagonist cycles. AMH as a single test had substantial accuracy in the prediction of ovarian response in GnRH antagonist co-treated cycles for IVF. Furthermore, AMH had a higher accuracy for the prediction of high response than for low response. Although AMH has clinical value, its test performance is far from optimal, especially with regard to low response prediction which is reflected by the low detection rate corresponding with lower AMH thresholds. Judging by the abnormal test rates corresponding with the optimal AMH cut-off for high response prediction, a considerable number of patients with a false positive test would be treated with a lower dose and may therefore turn into a low responder. In reality, tests like AMH may never be absolutely accurate in the prediction of ovarian response.

Furthermore, although AMH was associated with pregnancy, its accuracy with regard to the prediction of cumulative ongoing pregnancy was rather modest (chapter 6), thus preventing the use of such a marker for patient selection or rejection from a treatment program.

Future perspectives

Based on the findings in this thesis we can conclude that further improvement of the GnRH antagonist protocol in terms of clinical outcome cannot be achieved by adjusting the timing of GnRH antagonist administration. Therefore, the current late fixed start regimen starting on stimulation day 5 or 6 still offers the best clinical outcome profile and remains the best protocol at present.

One of the problems encountered in GnRH antagonist regimens is the occurrence of premature LH surges. The incidence varies and a wide range from 1.4 – 35% has been reported (29;35;78). Although early initiation of GnRH antagonist co-treatment resulted in a more stable hormonal milieu during the follicular phase (chapter 2), it was not able to completely prevent premature ovulation as an incidence of 1.3% was still observed compared with 0.3% in the late start arm (non-significant difference, chapter 3). It is possible that in the 24 hours between the GnRH antagonist injections, the pituitary is not continuously protected against the feedback effects of estradiol, resulting in activation of intracellular mechanisms that enhance gonadotropin secretion (79). This emphasises the fact that GnRH antagonists act through competitive receptor binding, and any strong signal from the hypothalamic region, elicited by fast rising estradiol levels, may win the competition and provoke an LH burst form the pituitary. In case of premature ovulation, the time interval between hCG administration and the oocyte pick-up could be shortened in a subsequent cycle (i.e. 34 hours). A more likely approach would be the use of a long GnRH agonist protocol in the subsequent cycle as GnRH agonists cause profound

pituitary receptor desensitisation and LH surges are less likely to occur (25).

Another problem encountered in GnRH antagonist co-treated cycles is the possibility of elevated early follicular progesterone levels at initiation of ovarian stimulation. The reported incidence of this phenomenon is rather low, 4.9 – 13.1% (48;51;98;142). Chapter 4 has confirmed existing literature and demonstrated that elevated baseline P levels are indeed associated with reduced chances of pregnancy.

It is unknown how women with elevated baseline P levels should be managed in order to optimise or normalise their prospects for successful IVF outcome. So far 3 treatment options have been proposed to normalise baseline P levels: delaying initiation of ovarian stimulation by 1 or 2 days (51), administering a GnRH antagonist during 3 consecutive days prior to initiation of ovarian stimulation (71) and early commencement of GnRH antagonist co-treatment (chapter 4). Nevertheless, none of these approaches has resulted in a clear improvement of pregnancy rates. In view of the relatively low incidence of this condition, the absence of a proven effective treatment strategy and the increased treatment costs, routine screening for P is not recommended. Large randomised controlled trials (RCT) are needed to develop an effective treatment strategy. This may involve GnRH agonist co-treatment as this is associated with basal levels of steroid hormones at initiation of stimulation and therefore consistently normal P levels (95). However, it can be debated whether the estimated costs of such a large RCT are justified, especially in an era where cost reduction has become an important issue.

Elevated P levels at the end of the follicular phase have been reported in up to 38% of GnRH antagonist cycles and are associated with decreased pregnancy rates (49;50;52;53;55;56). A recent systematic review and meta-analysis in > 60,000 cycles using both GnRH antagonists and agonists confirmed the association between elevated P levels on the day of hCG and a decreased probability of pregnancy after fresh embryo transfer (143).

Solid evidence regarding the most effective way to manage women with elevated P levels on the day of hCG administration is still lacking and further research is warranted. The development of prediction models to distinguish patients at risk for elevated P levels on the day of hCG may aid in the prognostic counseling of patients. However, the efficacy of such models remains debatable in the absence of an effective treatment strategy. Some have proposed that the transfer of cryopreserved embryos in subsequent frozen-thawed cycles may be a way to bypass impaired endometrial receptivity (144;145). However, recently it was demonstrated that elevated P levels 2 or more days prior to the LH surge negatively affected pregnancy outcomes in frozen-thawed natural cycles (146), indicating that this condition may be ovarian stimulation independent. Possible strategies to avoid these effects may be the use of artificial replacement cycles (146) or the transfer of blastocysts instead of cleavage-stage embryos as clinical outcome was not affected in cycles with elevated P levels and blastocyst transfers (55). Larger studies are needed to confirm the true value of such strategies.

In the efforts to improve GnRH antagonist protocols, the focus may also be put on individualised treatment regimens based on ovarian reserve tests such as AMH. The use of an AMH tailored approach has previously resulted in a reduced incidence of high and low response as well as improved pregnancy rates compared with non-individualised treatment cycles (64;65). However, these studies did not meet basic criteria for evidence based medicine, as they were not randomised controlled trials.

In this thesis we have established that AMH is an accurate predictor of ovarian response in GnRH antagonist co-treated cycles with similar accuracy as reported in GnRH agonist co-treated cycles. We have also demonstrated that AMH had a higher accuracy for the prediction of high response than for low response. A low response following conventional ovarian stimulation is regarded as a sign of advanced ovarian ageing and poor oocyte quality (14;64;65;108) as this type of stimulation will induce maximal stimulation of the ovaries (25;70). Conversely, the retrieval of a low number of oocytes following mild stimulation is associated with much more favourable pregnancy chances and does not reflect a poor ovarian response (3;109). It has been suggested that the oocytes retrieved following mild stimulation represent a more homogeneous group of good quality oocytes, possibly due to subtle interference with natural selection or the minimised exposure of growing follicles to the negative effects of ovarian stimulation (11).

Therefore, the relevance of predicting a low response in a GnRH antagonist system can be debated. The low responders described in chapter 5 and 6 had reasonable pregnancy prospects compared to normal responders. Unexpected low responders treated with a standard dose of 150 IU FSH may benefit from dose adjustment as they have sufficient ovarian reserve and may therefore have better pregnancy prospects (147). It is also possible that these women will become normal responders when treated with a long GnRH agonist protocol as a result of more synchronised follicular development. Conversely, a low response in women already receiving maximum stimulation, is more likely to be the result of a depletion of the ovarian pool of FSH-sensitive antral follicles. It is unclear whether the latter patient category will benefit from a long GnRH agonist protocol. It is well known that the proportion of women with at least one euploid embryo increases when more embryos are generated (139). Additionally, low oocyte numbers have been associated with a higher miscarriage rate, irrespective of age, which may be attributed to embryo aneuploidy as a consequence of oocyte aneuploidy and thus poor oocyte quality (140). As a result expected low responders will be less likely to have at least one euploid embryo available for transfer that may be capable of leading to a live birth (139). Thus, predicting a low response in a GnRH antagonist system may have little clinical value as opposed to low response prediction in a conventional protocol.

On the other hand, predicting a high response may be more useful as here safety issues play a role. Expected high responders can be treated with a GnRH antagonist protocol which in itself will result in a more mitigated response compared with a long GnRH agonist protocol.

In cases where patients are still at risk of OHSS, a GnRH agonist trigger can be administered with or without cryopreservation of all embryos, depending on patient discomfort. It has been demonstrated that this is an effective strategy for the reduction or prevention of the risk of OHSS (148). With the advent of GnRH agonist triggering, the relevance of prior prediction of a high response in a GnRH antagonist system can be debated. By treating all patients with a GnRH antagonist protocol and triggering with a GnRH agonist if necessary, the OHSS free clinic was supposed to become reality. However, despite this treatment regimen severe OHSS still has been reported, which is probably due to the use of too high FSH dosages in predicted high responders (119;120). So, caution is needed when using this approach and the value of prior identification of high responders and FSH dose adjustments, even in GnRH antagonist systems really need to be studied.

In this thesis we have also demonstrated that the accuracy of AMH for the prediction of cumulative ongoing pregnancy is limited (chapter 6), which is in line with previous studies using GnRH antagonist co-treatment (67;132-134). This may indicate that the association between AMH and clinical outcome reflects oocyte quantity and not quality. Our findings also indicate that the probability of achieving a pregnancy is influenced by several other external factors such as embryo transfer policy and technique and variation in laboratory procedures. It is questionable whether factors which are able to accurately predict pregnancy will ever be found.

The nomogram as described in chapter 6 demonstrated the ability of AMH to refine prognostic categories based on female age. Others have also proposed a nomogram for the prediction of live birth based on AMH and age (126). However, the practical use of such refinement remains unclear as it is still impossible to select a patient category which should be advised not to start or be excluded from IVF treatment. However, the nomogram may be used as for counseling. For example, older women with low AMH levels could be informed regarding their low pregnancy chances and could perhaps be discouraged from starting IVF, especially when taking into account the low incidence of euploid embryos observed in women aged ≥ 40 years (139). Nevertheless, validation in a different population is mandatory before any claims for general use can be made.

Summarising conclusion

This thesis has demonstrated that the late start GnRH antagonist protocol remains the best currently available protocol. Further adjustments in the timing of GnRH antagonist co-treatment are thought to be useless. In view of the low incidence of elevated early follicular P levels and the absence of an effective treatment strategy, routine screening for elevated early follicular P levels is not recommended.

It may be more valuable to focus on the individualisation of GnRH antagonist co-treated cycles by using ovarian reserve tests as this may be useful when it comes to safety issues. However, a direct benefit of treatment individualisation in terms of increased pregnancy rates and cost-effectiveness remains to be established. Future studies focussing on treatment individualisation and cost-effectiveness for the prevention of high and low response, such as the Optimist trial (106), may shed some light on this issue. Meanwhile, AMH alone or in combination with other possible predictors, such as age, is unlikely to alter clinical decisions based on the chance of ongoing pregnancy or live birth after IVF treatment. AMH should therefore preferably only be used for ovarian response prediction.

Summary

8

This thesis focuses on the optimisation of controlled ovarian stimulation for IVF using exogenous FSH and GnRH antagonist co-treatment, by studying the timing of the initiation of GnRH antagonist co-medication and the role of ovarian reserve markers in optimising ovarian response and reproductive outcome.

The introduction **(Chapter 1)** addresses the concept of ovarian stimulation and its complications. It also describes the development and optimisation of GnRH antagonist protocols over the past 15 years into what is now considered the standard protocol. Finally, this chapter addresses the current evidence on optimisation and individualisation of IVF treatment with regard to ovarian response prediction.

Chapter 2 describes the results of a nested study, including 160 IVF/ICSI patients, within a multicentre randomised controlled trial (RCT) which studied the effect of early [cycle day (CD) 2] versus late (CD 6) GnRH antagonist initiation on the stimulation phase endocrine profile. This study has demonstrated that early initiation of GnRH antagonist co-treatment resulted in a more stable endocrine profile, with more physiological levels of estradiol and LH during the follicular phase. It was hypothesised that a more stable hormonal milieu might result in improved clinical outcomes.

Chapter 3 describes the results of an open-label multicentre randomised controlled trial including 617 patients. This study assessed the impact of early initiation of GnRH antagonist co-treatment on CD 2 for IVF on live birth rate per started cycle and on the cumulative live birth rate compared to standard late initiation on CD 6. There were no significant differences in clinical outcomes between the two groups. However, a non-significant trend towards a higher live birth rate per started cycle and cumulative live birth rate was observed in the late start group compared with the early start group. The study was terminated prematurely because no significant difference was observed in clinical outcomes after 617 inclusions. Thus, despite a more stable endocrine profile early initiation of GnRH antagonist does not appear to improve clinical outcomes of IVF compared with midfollicular initiation.

In **Chapter 4**, the impact of elevated early follicular progesterone levels in GnRH antagonist co-treated cycles on ongoing pregnancy rate was assessed using prospective data in combination with a systematic review and meta-analysis. The prospective data, including 158 patients, were derived from the nested study of the main RCT described in Chapter 3. The systematic search identified 2 studies analysing elevated early follicular progesterone levels in GnRH antagonists. The prospective data demonstrated a non-significant difference in ongoing pregnancy rate in favour of the group with normal baseline progesterone levels. No differential impact of

early or late GnRH antagonist initiation on the effect of elevated or normal progesterone levels on ongoing pregnancy rate was observed. The meta-analysis demonstrated that in GnRH antagonist co-treated cycles, elevated early follicular progesterone levels significantly decreased the ongoing pregnancy rate.

Chapter 5 assesses the clinical value of AMH and other patient characteristics for the prediction of high or low ovarian response in controlled ovarian stimulation using GnRH antagonist co-treatment. This prospective cohort study included 487 IVF/ICSI patients. It was demonstrated that AMH had a better accuracy for the prediction of both high and low response as compared to age and BMI. Furthermore, AMH had a better accuracy for the prediction of high response than for low response and showed similar accuracy as reported in previous studies on GnRH agonists. In a multivariate model, including the factors age, AMH, BMI, smoking, type and duration of subfertility, only BMI added some predictive value to AMH for both high and low response prediction.

In **Chapter 6**, the predictive ability of AMH for cumulative ongoing pregnancy within a one year treatment horizon was assessed in 487 IVF/ICSI patients using a GnRH antagonist protocol. A prediction model was proposed including age at first IVF treatment, AMH level, type and duration of subfertility and the number of previous ART treatments. This model discriminated between women who did or did not conceive with only modest accuracy. Although AMH was associated with pregnancy, its predictive accuracy was very modest even when assessing the cumulative pregnancy rate. Finally, a nomogram was developed by which a subgroup of patients could be identified with lower pregnancy prospects. However, based on this nomogram it was not possible to detect a patient category which should be excluded from treatment.

Finally, **Chapter 7** discusses the conclusions that can be drawn from this thesis. This thesis has demonstrated that although early initiation of GnRH antagonists resulted in a more stable hormonal milieu, it did not result in improved clinical outcomes. Therefore the current late start GnRH antagonist protocol remains the best protocol at present and further adjustments in the timing of GnRH antagonist co-treatment are deemed useless. It was also demonstrated that elevated early follicular progesterone levels have a negative impact on the chance of achieving a pregnancy. In view of the low incidence of elevated early follicular progesterone levels and the absence of an effective strategy, routine screening is not recommended.
It may be more useful to focus on the individualisation of GnRH antagonist co-treated cycles by using ovarian reserve tests such as AMH. Indeed, AMH was shown to be an accurate predictor of both low and in particular high response. Predicting a high response may be more relevant as here safety issues play a role and individualisation of treatment may be more feasible (by

adjusting the gonadotropin dose or using a GnRH agonist trigger). However, a direct benefit of this approach in terms of increased pregnancy rates and cost-effectiveness remains to be established. Although AMH is a good predictor of ovarian response, its accuracy in predicting ongoing pregnancy was very modest. Thus, AMH alone or in combination with age, is unlikely to alter clinical decisions based on the chance of ongoing pregnancy or live birth after IVF treatment and should therefore preferably only be used for ovarian response prediction.

9

Dit proefschrift concentreert zich op het optimaliseren van gecontroleerde ovariële stimulatie voor IVF middels exogeen FSH en GnRH antagonist downregulatie, door het tijdstip van de start van de GnRH antagonist en de rol van markers voor ovariële reserve voor het optimaliseren van de ovariële respons en reproductieve uitkomst te bestuderen.

In de introductie (**Hoofdstuk 1**) wordt ovariële stimulatie ten behoeve van IVF alsmede de bijbehorende risico's en complicaties besproken. Tevens wordt de historische ontwikkeling van het GnRH antagonist protocol beschreven. Tot slot bespreken we in dit hoofdstuk ook de huidige kennis ten aanzien GnRH antagonisten en het belang van het optimaliseren en individualiseren van IVF behandelingen door het voorspellen van ovariële respons.

In **Hoofdstuk 2** worden de resultaten beschreven van een substudie die deel uitmaakt van de multicenter gerandomiseerde studie die beschreven wordt in Hoofdstuk 3. Het effect op het hormonale profiel van het vroeg-folliculair versus het standaard midfolliculair starten van de GnRH antagonist werd onderzocht in 160 IVF/ICSI patiënten. Deze studie liet zien dat een vroege start een stabieler endocrien profiel geeft met lagere LH en oestradiol waarden tijdens de folliculaire fase van de stimulatie. Dit gunstige hormonale milieu zou de zwangerschapskansen positief kunnen beïnvloeden.

Hoofdstuk 3 beschrijft de resultaten van het multicenter gerandomiseerde onderzoek waaraan 617 IVF/ICSI patiënten hebben deelgenomen. Het doel van deze studie was om het effect te bestuderen van een vroege start van de GnRH antagonist in vergelijking tot een standaard late start op de kans op een levend geboren kind. Deze studie toonde geen significant verschil tussen beiden groepen, echter, er was wel een trend zichtbaar in het voordeel van de late start. Aangezien er geen verschil werd gezien in kans op zwangerschap na 617 inclusies, werd deze studie voortijdig beëindigd. Een vroege start leidde dus niet tot een toename van de kans op zwangerschap en dus op een levend geboren kind, ondanks een stabieler endocrien profiel.

In **Hoofdstuk 4** wordt het effect van verhoogde vroeg-folliculaire progesteronwaarden binnen een GnRH antagonistensysteem bestudeerd. Voor deze studie zijn de prospectieve gegevens van 158 IVF/ICSI patiënten uit het multicenter gerandomiseerde onderzoek gebruikt en gecombineerd met de resultaten uit een systematische review en meta-analyse. De prospectieve data lieten een niet significant verschil zien in kans op zwangerschap in het voordeel van de groep met normale vroeg-folliculaire progesteronwaarden. De invloed van progesteron op de kans op zwangerschap werd niet beïnvloed door het vroeg of laat starten van de GnRH antagonist. De meta-analyse liet zien dat de kans op zwangerschap significant verlaagd werd indien sprake was van verhoogde vroeg-folliculaire progesteronwaarden.

In **Hoofdstuk 5** wordt de waarde van AMH en andere patiëntkarakteristieken voor het voorspellen van een hoge of lage ovariële respons in GnRH antagonist cycli beoordeeld. Deze prospectieve cohort studie bevatte 487 IVF/ICSI patiënten. AMH had een hogere accuratesse voor het aantonen van een hoge/lage respons in vergelijking tot leeftijd en BMI. Tevens bleek AMH een betere voorspeller te zijn van een hoge respons dan voor een lage respons. In een multivariate analyse bleek alleen BMI de voorspellende waarde van AMH iets te verbeteren.

In **Hoofdstuk 6** wordt de waarde van AMH met betrekking tot het voorspellen van de cumulatieve kans op zwangerschap binnen het GnRH antagonistensysteem bestudeerd. De cumulatieve data van de patiënten die beschreven worden in **Hoofdstuk 5** zijn hiervoor gebruikt. Er werd een predictiemodel ontwikkeld met de volgende factoren: leeftijd ten tijde van de 1e IVF behandeling, AMH, type en duur van de subfertiliteit en het aantal voorafgaande behandelingen. Met behulp van dit model kon geen goed onderscheid gemaakt worden tussen vrouwen die wel/niet zwanger werden. AMH was wel geassocieerd met zwangerschap maar bleek een slechte voorspeller te zijn van cumulatieve zwangerschap. Tot slot werd er ook een nomogram opgesteld voor leeftijd en AMH waarbij er een groep geïdentificeerd kon worden met lagere zwangerschapskansen. Op basis van dit nomogram kan er echter geen patiëntencategorie benoemd worden bij wie behandeling weerhouden zou moeten worden.

Hoofdstuk 7 bespreekt de conclusies die uit dit proefschrift getrokken kunnen worden. We hebben aangetoond dat een vroege start van de GnRH antagonist weliswaar een stabieler hormonaal profiel geeft, maar dat dit niet leidt tot een verhoogde kans op zwangerschap. Vooralsnog blijft de standaard late start het beste GnRH antagonisten protocol. Nieuwe wijzigingen met betrekking tot de timing van de GnRH antagonist lijken derhalve niet zinvol. Daarnaast hebben wij laten zien dat verhoogde vroeg-folliculaire progesteronwaarden de kans op zwangerschap negatief beïnvloeden. Aangezien de incidentie van verhoogde vroeg-folliculaire progesteronwaarden laag is en er nog geen effectieve methode is om dit probleem te behandelen wordt het routinematig bepalen van vroeg-folliculair progesteron afgeraden.
Voorts is gebleken dat AMH een goede voorspeller is van zowel een lage als in het bijzonder een hoge ovariële respons binnen een GnRH antagonistensysteem. Het voorspellen van een hoge respons lijkt klinisch relevanter omdat de veiligheid van de patiënt hier in het geding zou kunnen komen en de behandeling eventueel aangepast zou kunnen worden om de risico's te verlagen (bijvoorbeeld verlaging van de dosering gonadotrofinen of trigger met een GnRH agonist). Of dit vervolgens zal leiden tot verbeterde zwangerschapskansen en kosteneffectiviteit zal moeten blijken uit nieuwe gerandomiseerde studies. Tot slot hebben we aangetoond dat AMH geen goede voorspeller is van zwangerschap. Het lijkt dan ook onwaarschijnlijk dat klinische beslissingen ten aanzien van de kans op zwangerschap voor een

bepaald individu beïnvloed zullen worden door AMH, hetzij alleen, hetzij in combinatie met andere factoren zoals leeftijd. Het is dan ook aan te bevelen om AMH alleen te gebruiken voor responspredictie.

9

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Ouijdane Hamdine was born in Rhenen, the Netherlands on September 12th 1981. After graduating from "the Rembrandt College" in Veenendaal in 2000 she started studying medicine at the University of Utrecht. She discovered a strong interest in the field of Gynecology during her first internship at the Meander Medical Centre in Amersfoort and subsequently completed a second internship at this hospital in her last year of medical training. After graduating as a medical doctor in October 2006, she decided to broaden her horizon by working as a junior doctor in the Emergency department and Surgery department at the Beatrix Hospital, Gorinchem. In September 2007 she obtained a position as a junior doctor at the Gynecology department of the University Medical Centre Utrecht which she extended to a position as a fertility physician and PhD student a year later. To gain experience in the field of Obstetrics she started working at the Tweesteden Hospital in Tilburg in January 2013 where she discovered that her main interests were Fertility and Gynecology. Therefore, she started working as a fertility physician at the Radboud University Medical Centre in Nijmegen from January 2014. During this period she completed her PhD.