Title: Kisspeptin-54 triggers egg maturation in women undergoing *in vitro* fertilisation

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Abstract

Background: Patients with inactivating mutations in kisspeptin signalling are infertile. Kisspeptin-54, the major circulating isoform of kisspeptin in humans, potently stimulates reproductive hormone secretion in humans. Animal studies suggest that kisspeptin is involved in the generation of the luteinising hormone surge which is required for ovulation. We hypothesised that kisspeptin-54 could be a novel method used to trigger egg maturation in women undergoing *in vitro* fertilisation therapy.

Methods: This was a proof of concept study. Following superovulation with follicle stimulating hormone, and gonadotrophin releasing hormone antagonist administration to prevent premature ovulation, 53 women were administered a single subcutaneous injection of kisspeptin-54 (1.6nmol/kg, n=2; 3.2nmol/kg, n=3; 6.4nmol/kg, n=24; 12.8nmol/kg, n=24) to induce a luteinising hormone surge and egg maturation. Eggs were retrieved transvaginally 36h after kisspeptin injection, assessed for maturation (primary outcome), and fertilised by intracytoplasmic sperm injection with subsequent transfer of one or two embryos.

Results: Overall, egg maturation was observed in 96% (51/53) of patients. Fertilisation of eggs and transfer of embryos to the uterus occurred in 92% (49/53) of patients. Biochemical and clinical pregnancy rates were 40% (21/53) and 23% (12/53), respectively.

Conclusion: This study demonstrates that a single injection of kisspeptin-54 can induce high rates of egg maturation in women with sub-fertility undergoing *in vitro* fertilisation therapy. Subsequent fertilisation of eggs matured following kisspeptin-54 administration and transfer of resulting embryos can lead to successful human pregnancy.

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Introduction

Kisspeptin is a group of recently identified, naturally occurring hypothalamic peptides (1-3) which are essential for normal human fertility. Patients with genetic inactivation of the kisspeptin signalling pathway fail to undergo puberty and are infertile (4-6). Conversely patients with activating mutations undergo precocious (early) puberty (7). Within the hypothalamus, kisspeptin is released from specialised neurones to stimulate the secretion of endogenous gonadotrophin releasing hormone (GnRH), which in turn stimulates the secretion of endogenous gonadotrophins (luteinising hormone, LH; follicle stimulating hormone, FSH) from the pituitary gland (8-9). Peripheral injection of kisspeptin has been shown to potently stimulate gonadotrophin secretion in many non-human mammalian species including rats (8, 10, 11), mice (12, 13), monkeys (14), sheep (15), and humans (16-23).

Recent evidence from animals suggests that kisspeptin may activate the LH surge required for egg maturation and ovulation. In rodents, the proestrus LH surge is abolished by a specific monoclonal antibody which blocks the actions of hypothalamic kisspeptin (24), and the LH surge has been observed to be absent in transgenic mice null for kisspeptin or its receptor (25). Conversely, peripheral administration of kisspeptin triggers egg maturation in adult female rats primed with gonadotrophins (26). In addition, exogenous administration of kisspeptin induces ovulation in the musk shrew (24), rats (26) and sheep (27). In keeping with this, human studies demonstrate that kisspeptin stimulates LH release most potently during the pre-ovulatory phase of the menstrual cycle (i.e. the time of the cycle just prior to ovulation) in females with regular menstrual cycles (17, 20, 28).

Based on this data we hypothesised that kisspeptin administration could be used as a novel method of triggering ovulation in humans. To test this hypothesis we performed a single-centre, proof of concept study to determine the effects of kisspeptin-54 administration on egg maturation (an objective marker of ovulation) in women undergoing *in vitro* fertilisation (IVF) therapy.

Results

Women with subfertility requiring IVF therapy underwent superovulation with recombinant FSH and GnRH antagonist administration to prevent premature ovulation. A single subcutaneous injection of kisspeptin-54 was used to trigger egg maturation (Figure 1). The number of patients assessed for eligibility, study enrolment, and dose allocation are shown in Figure 2.

Kisspeptin dose allocation and baseline characteristics: The first two patients were randomised to 1.6nmol/kg of kisspeptin-54; in these patients, egg collection was substantially more difficult when compared with a typical treatment cycle using human chorionic gonadotrophin (hCG) to induce egg maturation (Table 1). It was therefore decided to proceed to the second phase doses of 3.2, 6.4 and 12.8nmol/kg. Clinically difficult egg collection was reported in all patients at the kisspeptin-54 dose, 3.2nmol/kg. Subsequent patients were therefore randomised 1:1 between 6.4 and 12.8nmol/kg kisspeptin (n=24 per group). The dose allocation was approved by two clinical trialists who were independent of the study. No imbalances in baseline characteristics were observed between the dose groups (Table 2).

PRIMARY OUTCOME: Ability of kisspeptin to induce egg maturation in women undergoing IVF therapy

This study was designed to determine if egg maturation was achievable in women during IVF treatment following kisspeptin-54 administration. Egg maturation was observed following each dose of kisspeptin-54 (Table 3; Figure 3). Egg maturation with at least 1 mature egg was observed in 96% (51/53) of women studied irrespective of kisspeptin-54 dose.

SECONDARY OUTCOMES:

A descriptive analysis of secondary outcomes is presented.

(i) Circulating reproductive hormones following kisspeptin administration: Circulating reproductive hormones were measured immediately before, and 12h and 36h following

kisspeptin-54 injection in all patients, except for one patient who missed her 12h blood test. Serum LH, FSH and progesterone levels were observed to be elevated 12h following kisspeptin-54 injection (Table 4).

(ii) A surge in reproductive hormonal secretion is observed during the 12h following kisspeptin injection: Overnight blood sampling was performed to determine the time profile of hormone release in twenty of the subjects treated with the two highest doses of kisspeptin-54 (6.4 or 12.8nmol/kg; n=10/dose). Peak levels of plasma kisspeptin were observed approximately 1h after injection and then fell to pre-injection levels by 12h following injection (Figure 4). Serum LH levels peaked 4-6h following kisspeptin-54 injection, and decreased thereafter. Similar but less prominent patterns of elevation were observed in FSH and oestradiol secretion following kisspeptin-54 injection. In contrast, progesterone levels rose continually during the 12h following kisspeptin-54 injection (Figure 4).

(iii) Fertilisation of embryos and pregnancy: Although this study was primarily designed to investigate whether egg maturation could be achieved in women administered kisspeptin-54 during IVF treatment, we also recorded the rates of egg fertilisation, biochemical pregnancy (at 12 days following embryo transfer) and clinical pregnancy (6 weeks gestation). Results are shown in Table 5. Overall, fertilisation occurred in 92% (defined as at least 1 fertilised egg), rate of embryo transfer was 92%, high quality embryo transfer occurred in 58%, biochemical pregnancy rate was 40%, clinical pregnancy rate was 23% (12 of all 53 treated patients). The following outcomes were observed in the 12 women achieving a clinical pregnancy: seven women have each already given birth to healthy babies (five women had singleton pregnancies and two women had twins); a further three women have ongoing pregnancies (one in the 2nd trimester and two in the 3rd trimester); two women had miscarriages (one at 9 weeks and the other at 12 weeks gestation).

Adverse events:

Kisspeptin-54 administration was well tolerated in all 53 sub-fertile patients in this study (in keeping with the safety reported in previous studies which have administered kisspeptin in men and women (16-23). Five adverse events occurred which are established complications of *in vitro* fertilisation treatment and pregnancy (two patients experienced an ectopic pregnancy, one had a heterotopic pregnancy, and two had miscarriages).

Discussion

Kisspeptin signaling is obligatory for mammalian fertility (4-6), and exogenous kisspeptin potently stimulates endogenous reproductive hormones in all mammalian species studied to date (8, 10-23, 26). Kisspeptin therefore offers a potential novel method for treating patients with infertility. Animal studies suggest that kisspeptin may play a physiological role in generating the endogenous LH surge, which is required for ovulation. We therefore investigated whether a single injection of kisspeptin-54 could be used as a novel method to trigger egg maturation (an objective marker of ovulation) in patients during IVF therapy. Herein we demonstrate for the first time that a single injection of kisspeptin-54 triggers egg maturation sufficient to result in fertilisation, embryo implantation, and successful live birth in women with subfertility undergoing IVF therapy.

The major objective of this study was to investigate whether egg maturation was achievable in women administered kisspeptin-54 during IVF therapy. Egg maturation was assessed in two ways; firstly, the absolute number of mature eggs and secondly, the percentage of eggs collected that were mature. Mature eggs were observed in 96% of participants during the study, and the percentage of eggs collected that were mature, was between 75-85% following kisspeptin-54 at all doses tested. Circulating kisspeptin levels rose rapidly following kisspeptin-54 injection, and this led to a marked increase in LH secretion and smaller increase in FSH secretion (as expected from previous studies in healthy women). Kisspeptin-54 administration resulted in a rise in LH release sufficient to induce egg maturation in women undergoing IVF therapy; levels of serum LH peaked 4h post-injection, remained elevated 12h post-injection, and had returned to baseline 36h post-injection. Circulating progesterone levels subsequently increased following kisspeptin-54 injection, which is consistent with egg maturation. Our data therefore demonstrate that kisspeptin-54 can trigger egg maturation in women undergoing IVF therapy.

We prospectively tracked the progress of patients following treatment with kisspeptin-54. Embryogenesis was observed in almost all patients who ovulated following kisspeptin-54 at doses of 6.4 or 12.8nmol/kg. Furthermore, twelve clinical pregnancies were observed during

the study. To date, seven women have already delivered healthy babies and a further three women currently have ongoing second or third trimester pregnancies. The numbers of patients in each treatment group were very small, and numerous factors can affect pregnancy outcome following egg maturation; the observed pregnancy rate at each dose of kisspeptin-54 should therefore be interpreted with caution. However, our observations importantly demonstrate for the first time that successful human pregnancy can result from kisspeptin-54 treatment in women undergoing IVF therapy.

There were important ethical issues to consider in the design of this study of patients with subfertility undergoing IVF treatment. Firstly, it is known that omitting the trigger for egg maturation results in failed egg maturation (termed 'empty follicle syndrome') (29) therefore a placebo trigger was not considered. Secondly, kisspeptin had not previously been administered to treat patients with subfertility undergoing IVF treatment, so the effectiveness of kisspeptin to trigger egg maturation was not known. To minimise the number of patients exposed to clinically ineffective kisspeptin doses (i.e. doses not resulting in egg maturation) we chose a study design that allowed for rapid dose escalation. Therefore, although we have identified two doses of kisspeptin-54 which effectively induce egg maturation in subfertile patients undergoing IVF therapy, further research is required to ascertain the optimal dose of kisspeptin and verify that lower doses are insufficient to induce egg maturation.

It is interesting to consider the mechanism of action of kisspeptin-54 in relation to other hormones currently used to trigger egg maturation during IVF therapy. hCG is the most commonly used hormonal trigger for egg maturation in clinical practice (30), stimulating the ovaries directly by binding to the LH receptor. hCG has LH-like activity which is far longer in duration than the endogenous physiological LH surge (31), and its effects are not subject to any negative feedback. As such, hCG can be associated with excessive ovarian stimulation which can lead to ovarian hyperstimulation syndrome (OHSS) (32). GnRH agonists offer an alternative therapeutic approach to hCG for egg maturation (33). GnRH agonists stimulate the GnRH receptor on the pituitary gland to release LH. The use of GnRH agonists is associated with lower rates of OHSS but can also be associated with lower pregnancy rates (34)

compared to hCG. The latter has been hypothesised to be due to luteal phase insufficiency (35) which may be due to GnRH triggers causing excessive pharmacological stimulation and consequent desensitisation of the GnRH receptor. Kisspeptin-54 is implicated in generating the physiological endogenous LH surge by activating the hypothalamus to stimulate endogenous GnRH secretion which then stimulates endogenous LH release (8, 9, 13, 14). As such, the effects of kisspeptin administration depend upon an individual's endogenous GnRH reserve to stimulate LH release. The kisspeptin signalling pathway is therefore a novel mechanism which can be therapeutically exploited to trigger egg maturation in patients. Further research will be required to determine whether the novel therapeutic application of kisspeptin-54 to trigger egg maturation leads to better outcomes for patients undergoing IVF treatment than other hormones currently used for this purpose (such as hCG and GnRH agonists).

In summary, we provide proof of concept that a single injection of kisspeptin-54 induces high rates of egg maturation in women undergoing *in vitro* fertilisation therapy. Furthermore, eggs matured using kisspeptin-54 therapy can be fertilised *in vitro*, transferred to the uterus, and provide successful human pregnancy for couples with infertility. Further studies will determine the clinical utility of kisspeptin-54 during *in vitro* fertilisation therapy when compared with established pharmacological triggers of egg maturation.

Methods

Peptide

Kisspeptin-54 was synthesised, purified and tested to Good Manufacturing Practice standards by Bachem (Bachem Holding AG, Bubendorf. Switzerland). Vials of freeze-dried kisspeptin-54 were stored at -20°C and reconstituted in 0.5ml of 0.9% saline as described previously (16,17).

Subjects

Sixty patients requiring *in vitro* fertilisation treatment at Hammersmith Hospital, London, UK, were screened for participation between the start (August 2012) and end of patient recruitment (September 2013). Eligible patients received a single treatment cycle; all treatment costs for this study cycle were covered by study participation as the efficacy of kisspeptin to trigger egg maturation was unknown prior to starting the study. The inclusion criteria were: age 18-34years; early follicular phase level of serum FSH ≤12mIU/mL; serum anti-Müllerian hormone (AMH) 10-40pmol/L (1.4-5.6ng/mL); both ovaries intact, regular menstrual cycles of 24-35days duration; body mass index 18-29kg/m^2. Exclusion criteria were: moderate/severe endometriosis; poor response to, or more than one previous cycle of *in vitro* fertilisation treatment; clinical or biochemical hyperandrogenemia; polycystic ovarian syndrome. The number of patients assessed for eligibility, study enrolment, and dose allocation are shown in Figure 2.

Study outcomes

The primary outcome was egg maturation during a single treatment cycle, which was assessed in two ways: firstly, the absolute number of mature eggs; secondly, the percentage of eggs collected that were mature. Eggs were classified as mature by presence of the first polar body and round ooplasm (30).

Secondary outcomes were circulating levels of reproductive hormones following kisspeptin-54 injection; fertilisation rate, rate of embryo transfer, embryo quality, biochemical pregnancy

rate 12 days following transfer, and clinical pregnancy rate at 6 weeks gestation. Fertilisation rate was defined as the number of two pronuclear zygotes divided by the number of mature eggs injected (30). Biochemical pregnancy was defined by a plasma hCG concentration >10mIU/mL 12 days after embryo transfer. Clinical pregnancy was defined as an intrauterine gestational sac with heartbeat at 6 weeks gestation. Pregnancy outcomes and live births were documented in women with clinical pregnancy.

Dose allocation

Doses were to be allocated in two phases. During the first phase, the first nine women were to be randomised equally between the three lowest doses (0.4, 0.8 and 1.6nmol/kg) of kisspeptin. If fewer than 2 women in each group experienced clinically effective egg maturation, then during the second phase the next 9 women were to be randomised between the three higher doses (3.2, 6.4 and 12.8nmol/kg) of kisspeptin. The clinical ease of egg collection following kisspeptin-54 injection (compared with hCG which is the trigger most commonly used in clinical practice to trigger oocyte maturation in IVF treatment) was also noted. The rationale for this was that a clinically effective dose of kisspeptin would need to result in maturation of eggs which could be collected with similar ease to that observed with triggers of egg maturation currently used in clinical practice. Hence the clinical ease of collection was graded 1 to 4 by clinicians blinded to the dose allocation using the following as a guide: 1, not possible to collect any eggs; 2, substantially more difficult than during a typical IVF treatment cycle using hCG (excessive number of flushes were required in order to aspirate eggs from follicles and very scanty number of granulosa cells present in follicular aspirates); 3, slightly more difficult than using hCG (granulosa cells were present in the aspirates, although less abundant than following optimal triggering. Cumulus cells were more densely packed than following optimal triggering); 4, comparable level of difficulty when compared with hCG. Once a dose of kisspeptin had been shown to induce clinically effective egg maturation in at least 2 women (per cohort of 3) then further women would be recruited to increase the sample size at that dose to a minimum of 15 patients. The dose allocation was approved by two

clinical trialists who were independent of the study. Eggs were retrieved by clinicians blinded to the dose allocation of patients. Embryologists and patients were also blinded to the dose allocation. Within each phase of the study, patients were allocated to doses using simple randomisation.

Protocol

All of the women underwent superovulation using a recombinant FSH and GnRH antagonist protocol. Kisspeptin-54 was used to trigger egg maturation.

Superovulation protocol: On day 2 of the menstrual cycle, daily subcutaneous recombinant FSH injections (Gonal F 150IU, Merck Serono, Geneva, Switzerland, administered at 9am daily), were commenced in all patients; pelvic ultrasound scan was performed five days after commencing injections, to determine ovarian follicle development. Daily GnRH antagonist injections (Cetrotide 0.25mg, Merck Serono, injected at 9pm daily) were used to inhibit a premature LH surge, and were commenced when the lead follicle was >14mm diameter. Further ultrasound scans were performed according to follicular size and response to superovulation.

Kisspeptin-54 trigger of egg maturation: When at least three ovarian follicles ≥18mm diameter were visible on ultrasound, a subcutaneous bolus injection dose of kisspeptin-54 was administered by an investigator to trigger egg maturation. Kisspeptin-54 was administered 36h prior to egg retrieval (between 2030 and 2130h). Injections of FSH were stopped 12h prior to kisspeptin-54. In order to minimise any effect that the GnRH antagonist had on kisspeptin-54 response the last injection of GnRH antagonist was administered 24 hours prior to kisspeptin-54 (37, 38). Serum LH, FSH, oestradiol and progesterone were measured immediately before and 12h after kisspeptin-54 injection, and at the time of egg retrieval in all women.

Reproductive hormone secretion during the 12h following kisspeptin injection: A subgroup of women receiving the two highest doses of kisspeptin-54 (6.4 or 12.8nmol/kg, n=10/dose) volunteered to undergo overnight measurements of serum LH, FSH, oestradiol and

progesterone, and plasma kisspeptin immunoreactivity just prior to and after kisspeptin-54 injection (t=-30, -15, 0, 30, 60, 90, 120, 150, 180, 240, 360, 480, 600 and 705minutes post-injection). These hormonal measurements allowed us to determine the detailed time course of reproductive hormone release following kisspeptin-54 injection.

Egg retrieval, fertilisation, and re-implantation of embryos: Transvaginal ultrasound-directed egg retrieval was carried out 36h following kisspeptin-54 injection, and intracytoplasmic sperm injection was performed in all study cycles using sperm from the male partner. One or two embryos of highest quality during morphological assessment were transferred to the uterine cavity 3-5days following egg collection. Progesterone (400mg twice daily suppository/pessary) and oestradiol valerate (2mg orally three times daily) were started after egg collection and continued until 12weeks gestation for luteal phase supplementation.

Hormonal assay methodology

Blood samples were collected as previously described (16, 17, 22, 23, 28). Serum LH, FSH, oestradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; oestradiol, 3.4%; progesterone, 1.8%. Limits of detectability for each assay were as follows: LH 0.07mIU/ml; FSH 0.05mIU/ml; oestradiol 70pmol/l (19pg/mL); progesterone 0.3nmol/L (0.1ng/mL).

AMH was measured using an enzyme linked immunosorbent assay (Beckman Coulter Inc, Brea, CA, USA). The reference range was 2.2-48.5pmol/L (0.3-6.8ng/mL). The lower limit of detection was 0.6pmol/l (0.08ng/mL). The interassay coefficient of variation was 4.6%; the intraassay coefficient of variation was 4.0%.

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established RIA (16, 17, 22, 23, 28). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related arginine-phenylalanine amide proteins, including prolactin-releasing peptide, arginine-phenylalanine amide-related peptide 1, arginine-phenylalanine amide-related peptide 2, arginine-

phenylalanine amide-related peptide 3, QRFP43, neuropeptide FF, and neuropeptide AF. The limit of detectability was 2pmol/l, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

Data analysis

The study data are summarised using standard descriptive methods. Data analysis was performed by AM, JW and DA, who had no involvement in patient management or data collection. Histograms and box-plots are used to assess the distributional assumptions, and check for possible outliers. Continuous variables following an approximately normal distribution are summarised using the mean and standard deviation. Skewed continuous variables are summarised using the median and inter-quartile range. Categorical variables (binary, ordered and multinomial) are presented in terms of frequencies and percentages. Where possible, outcomes are presented graphically, using scatter plots and box-plots.

Regulatory approval

The Hammersmith and Queen Charlotte's Research Ethics Committee, London, UK, approved this study (reference: 10/H0707/2). The study was carried out in the IVF Unit at Hammersmith Hospital under a license from the UK Human Fertilisation and Embryology Authority. Written informed consent was obtained from all subjects prior to inclusion in the study. Approval of this study as a Clinical Trial of an Investigative Medicinal Product was granted by the Medicines and Healthcare Products Regulatory Agency, UK. The study was registered on the National Institutes of Health Clinical Trials database (NCT01667406). The study was performed in accordance with the Declaration of Helsinki.

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Conflict of Interest

The authors declare that no conflict of interest exists.

Role of Funding Source

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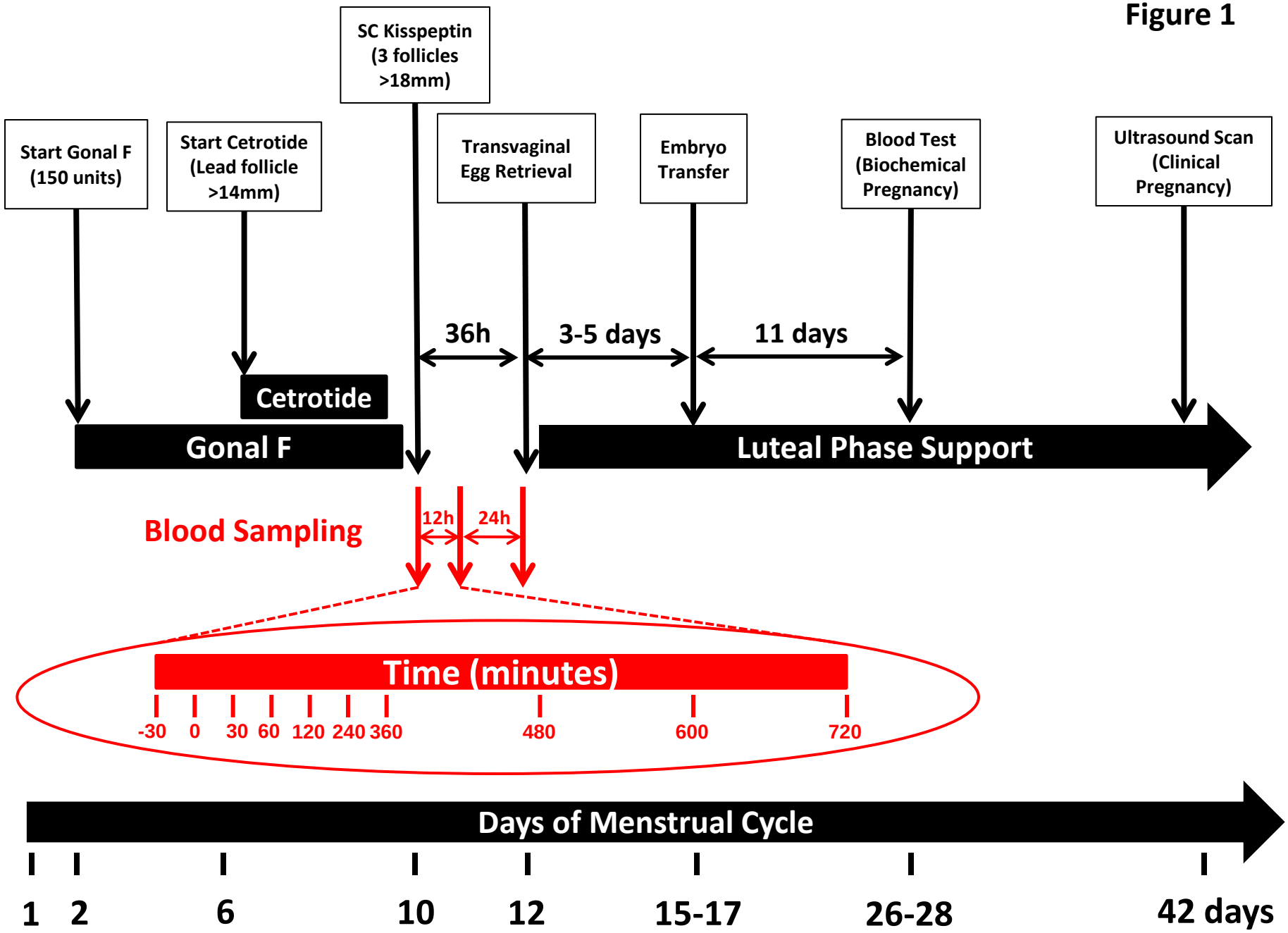
Figure 1
SC Kisspeptin (3 follicles >18mm)
Start Gonal F (150 units)
Start Cetrotide (Lead follicle >14mm)
Transvaginal Egg Retrieval
Embryo Transfer
Blood Test (Biochemical Pregnancy)
Ultrasound Scan (Clinical Pregnancy)
36h
3-5 days
11 days
Cetrotide
Gonal F
Luteal Phase Support
Blood Sampling
12h
24h
Time (minutes)
-30
0
30
60
120
240
360
480
600
720
Days of Menstrual Cycle
1
2
6
10
12
15-17
26-28
42 days

Figure 1: Superovulation protocol using subcutaneous kisspeptin-54 as a trigger to induce egg maturation

The timeline shows the day of menstrual cycle for a typical patient. On day 2 of the menstrual cycle, daily subcutaneous recombinant FSH (Gonal F 150 IU) were commenced. Daily GnRH antagonist injections (Cetrotide 0.25mg) were commenced when the lead follicle was >14mm diameter on ultrasound scan. When at least three ovarian follicles ≥18mm diameters were visible on ultrasound, a subcutaneous bolus injection dose of kisspeptin-54 (1.6, 3.2, 6.4 or 12.8 nmol/kg) was administered to trigger egg maturation (between 2030 and 2130h). Injections of GnRH antagonist and FSH were stopped 24h and 12h prior to kisspeptin-54, respectively. Transvaginal ultrasound-directed egg retrieval was carried out 36h following kisspeptin-54 injection, and intracytoplasmic sperm injection was performed using sperm from the male partner. One or two embryos were transferred to the uterine cavity 3-5 days following egg collection. Progesterone (400mg twice daily suppository/pessary) and oestradiol valerate (2mg orally three times daily) for luteal phase support were started after egg collection and continued until 12 weeks gestation. Biochemical pregnancy (βHCG >10iU/L) was assessed 11 days following embryo transfer and clinical pregnancy was assessed at 6 weeks gestation.

Reproductive hormone secretion during the 12h following kisspeptin injection (presented in red): A subgroup of women receiving the two highest doses of kisspeptin-54 (6.4 or 12.8nmol/kg, n=10/dose) underwent overnight measurements of serum LH, FSH, oestradiol and progesterone, and plasma kisspeptin immunoreactivity just prior to and after kisspeptin-54 injection.

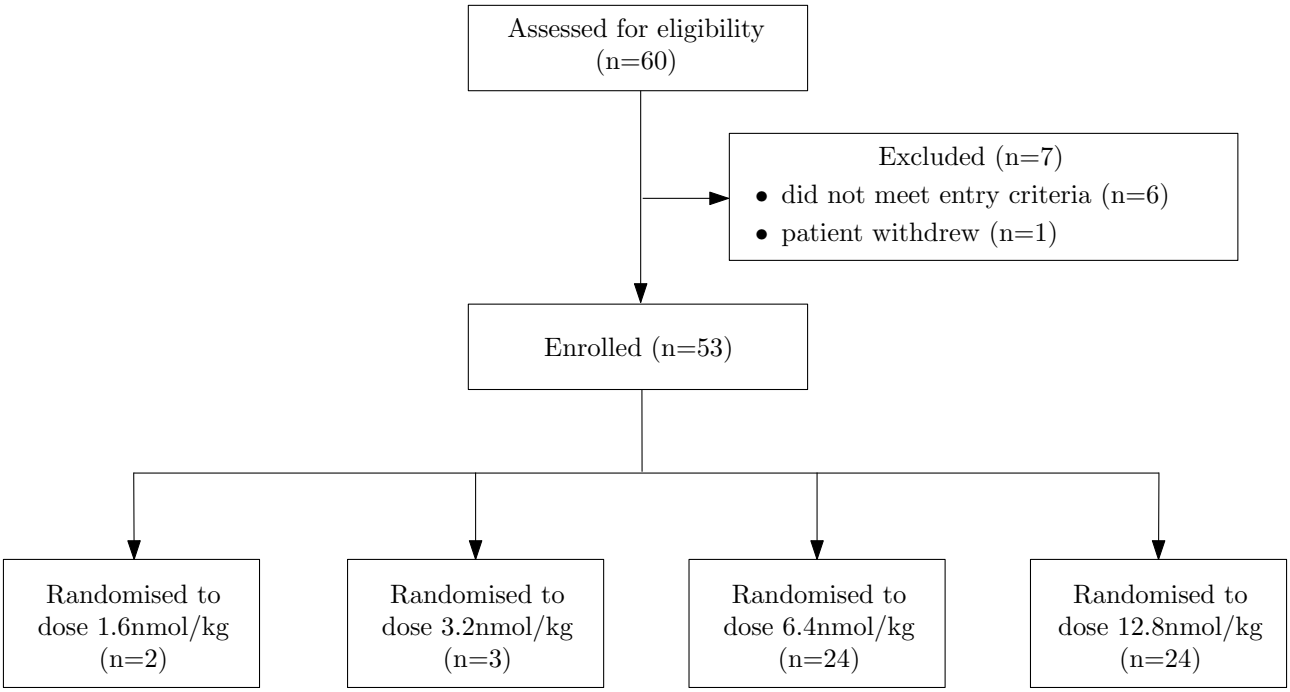

Figure 2: Enrolment of patients administered kisspeptin-54 to induce egg maturation during in vitro fertilisation therapy.

The first two patients were randomised to receive kisspeptin-54 at the dose of 1.6nmol/kg during in vitro fertilisation therapy. Clinically difficult egg collection was reported 36h following kisspeptin-54 injection in both patients. The subsequent nine patients were therefore randomised 1:1:1 between 3.2, 6.4 or 12.8nmol/kg. Clinically difficult egg collection was reported 36h following kisspeptin-54 injection in all patients in the 3.2nmol/kg cohort. Subsequent patients were therefore randomised 1:1 between 6.4 or 12.8nmol/kg kisspeptin-54 (n=24 per group). No patients were lost to follow up and no patients discontinued the intervention. The outcome data for all patients who were randomised was included in the final data analysis.

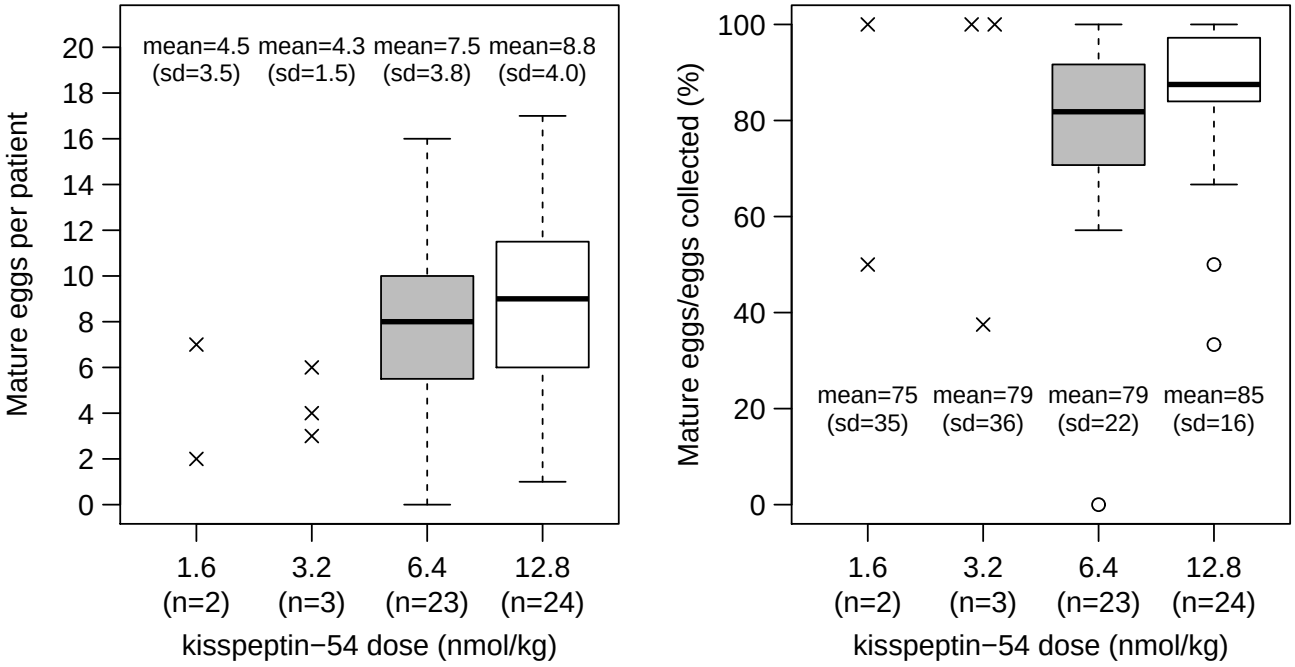

Figure 3: Egg maturation in patients following administration of kisspeptin-54.

Individual data are shown by crosses for the 1.6 and 3.2 nmol/kg doses. Boxplots are shown for the 6.4 and 12.8 nmol/kg doses: line = median, box = interquartile range, whiskers extend to the extremes of the data (1.5 × the interquartile range), open circles = very extreme outliers. The difference in absolute number of mature eggs between the 12.8 and 6.4nmol/kg doses was 1.2 (95% confidence interval -1.0 to 3.5). For the percentage of eggs collected that were mature, this difference was 6% (95% confidence interval -5% to 17%). SD, standard deviation.

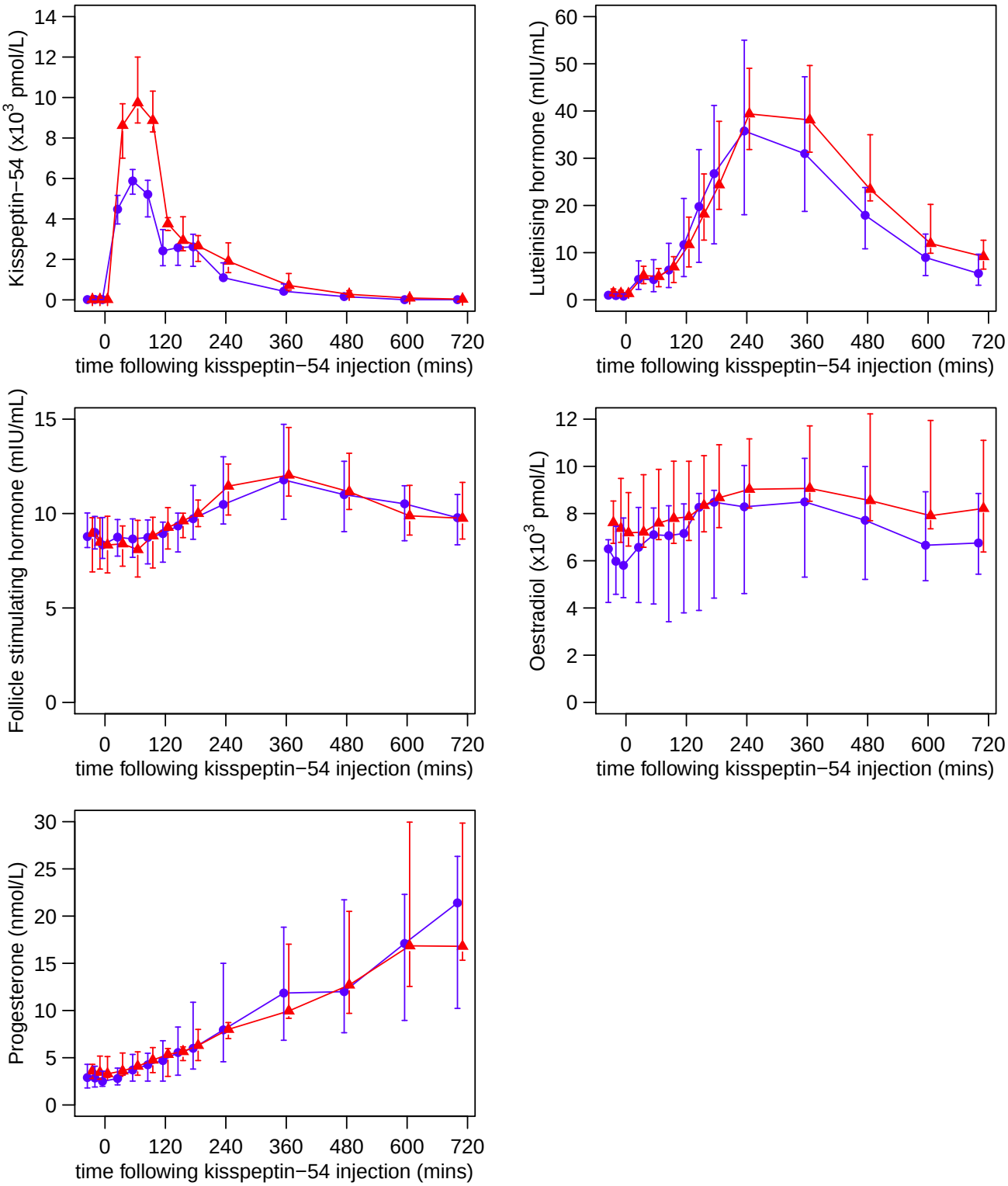
Kisspeptin−54 (x10^3 pmol/L)
Luteinising hormone (mIU/mL)
Follicle stimulating hormone (mIU/mL)
Oestradiol (x10^3 pmol/L)
Progesterone (nmol/L)
time following kisspeptin−54 injection (mins)
0
120
240
360
480
600
720

Figure 4

Figure 4: Detailed overnight blood sampling following kisspeptin-54 injection reveals a surge in reproductive hormonal secretion in patients undergoing in vitro fertilisation therapy.

A subgroup of women receiving the two highest doses of kisspeptin-54 (6.4 or 12.8nmol/kg; n=10 per group) at t= 0min, underwent overnight measurements of circulating kisspeptin-54, LH, FSH, oestradiol and progesterone at t= -30, -15, 0, 30, 60, 90, 120, 150, 180, 240, 360, 480, 600 and 705minutes. Blue triangles show median for 6.4nmol/kg kisspeptin-54; red circles for 12.8nmol/kg kisspeptin-54. Vertical lines indicate the interquartile ranges. Conversion factor for serum oestradiol: 1pmol/L=0.27pg/mL; conversion factor for serum progesterone: 1nmol/L=0.31ng/mL.

Clinical ease of collection	dose=1.6		dose=3.2		dose=6.4		dose=12.8	
1	0	(0%)	0	(0%)	2	(8%)	1	(4%)
2	2	(100%)	3	(100%)	5	(21%)	3	(12%)
3	0	(0%)	0	(0%)	3	(12%)	2	(8%)
4	0	(0%)	0	(0%)	14	(58%)	18	(75%)
Total	2	(100%)	3	(100%)	24	(100%)	24	(100%)

Table 1: Clinical ease of egg collection 36h following kisspeptin-54 injection in patients undergoing *in vitro fertilisation* therapy. Clinical ease of collection was graded as follows: 1, not possible to collect any eggs; 2, substantially more difficult than during a typical *in vitro fertilisation* treatment cycle using human chorionic gonadotrophin; 3, slightly more difficult than using human chorionic gonadotrophin; 4, comparable to human chorionic gonadotrophin. Doses of kisspeptin-54 injection are in nmol/kg.

	Kisspeptin-54 dose (nmol/kg)							
	1.6		3.2		6.4		12.8	
N	2		3		24		24	
Age (y)	30	(28,32)	31	(31,32)	32	(29,34)	30	(29,33)
Weight (kg)	66	(65,67)	59	(58,61)	62	(55,67)	64	(59,69)
Body mass index (kg/m^2)	23	(22,23)	22	(22,23)	22	(21,24)	23	(23,25)
Antral Follicle Count (AFC)	30	(28,31)	24	(20,28)	20	(16,26)	20	(17,26)
AMH*	21	(18,23)	13	(12,19)	18	(14,26)	21	(12,25)
Length of menstrual cycle (d)	28	(28,29)	29	(28,30)	28	(28,30)	28	(28,30)
Cause of infertility:								
Male factor†	1	(50%)	0	(0%)	9	(38%)	7	(29%)
Tubal defect‡	0	(0%)	0	(0%)	5	(21%)	5	(21%)
Other known cause§	0	(0%)	1	(33%)	2	(8%)	5	(21%)
Idiopathic	1	(50%)	2	(67%)	8	(33%)	7	(29%)
Number of follicles¶	16	(16,16)	20	(18,30)	20	(16,24)	19	(15,24)
Follicles >14mm¶	8	(8, 9)	11	(11,14)	11	(8,12)	9	(8,12)

Table 2: Baseline characteristics of patients administered kisspeptin-54 during *in vitro* fertilisation treatment. Contains medians (lower quartile,upper quartile) for continuous variables and totals (percentages) for categorical variables.

* serum levels of anti-Mullerian hormone in pmol/L

† infertility due to a problem with the partner's fertility

‡ blocked or removed Fallopian tubes

§ includes endometriosis and endometriotic cyst

¶ immediately prior to the kisspeptin-54 trigger

	Kisspeptin-54 dose (nmol/kg): mean (sd)			
	1.6 (n=2)	3.2 (n=3)	6.4 (n=23/24*)	12.8 (n=24)
M2†	4.5 (3.5)	4.3 (1.5)	7.5 (3.8)	8.8 (4.0)
%M2‡	75 (35)	79 (36)	79 (22)	85 (16)

Table 3: Summary of egg maturation following administration of kisspeptin-54.

$\star$ n=24 for M2 and n=23 for %M2

$\dagger$ absolute number of mature eggs per patient

$\ddagger$ percentage of collected eggs that were mature

	N	mean (sd)		range	median (lq,uq)		gm	N	mean (sd)		range	median (lq,uq)		gm
	dose=1.6 nmol/kg							dose=3.2 nmol/kg						
LH (mIU/mL)														
0 hours⋆	2	0.8	(0.1)	(0.7,0.8)	0.8	(0.7,0.8)	0.7	3	1.4	(1.3)	(0.4,2.9)	0.8	(0.6,1.8)	1.0
12 hours⋆	2	6.6	(4.7)	(3.3,9.9)	6.6	(4.9,8.2)	5.7	2	3.8	(3.3)	(1.5,6.2)	3.8	(2.7,5.0)	3.0
36 hours⋆	2	2.2	(0.2)	(2.1,2.4)	2.2	(2.2,2.3)	2.2	3	1.1	(0.6)	(0.4,1.5)	1.3	(0.8,1.4)	0.9
FSH (mIU/mL)														
0 hours⋆	2	6.2	(1.8)	(4.9,7.4)	6.2	(5.5,6.8)	6.0	3	8.3	(3.4)	(6.1,12.2)	6.7	(6.4,9.4)	7.9
12 hours⋆	2	6.9	(0.7)	(6.4,7.4)	6.9	(6.7,7.2)	6.9	2	9.7	(3.3)	(7.3,12.0)	9.7	(8.5,10.8)	9.4
36 hours⋆	2	4.9	(0.7)	(4.4,5.4)	4.9	(4.7,5.2)	4.9	3	5.2	(1.4)	(3.7,6.5)	5.3	(4.5,5.9)	5.0
Oestradiol ($\times 10^3$ pmol/L)														
0 hours⋆	2	4.0	(1.7)	(2.8,5.2)	4.0	(3.4,4.6)	3.8	3	13.3	(2.9)	(10.5,16.2)	13.2	(11.8,14.7)	13.1
12 hours⋆	2	5.9	(0.8)	(5.3,6.5)	5.9	(5.6,6.2)	5.9	2	16.6	(2.0)	(15.2,18.0)	16.6	(15.9,17.3)	16.5
36 hours⋆	2	2.2	(0.1)	(2.1,2.2)	2.2	(2.2,2.2)	2.2	3	7.4	(1.5)	(5.9,8.9)	7.4	(6.7,8.1)	7.3
Progesterone (nmol/L)														
0 hours⋆	2	1.0	(0.0)	(1.0,1.0)	1.0	(1.0,1.0)	1.0	3	3.7	(3.1)	(1.0,7.0)	3.0	(2.0,5.0)	2.8
12 hours⋆	2	7.5	(0.7)	(7.0,8.0)	7.5	(7.2,7.8)	7.5	2	22.0	(5.7)	(18.0,26.0)	22.0	(20.0,24.0)	21.6
36 hours⋆	2	5.5	(2.1)	(4.0,7.0)	5.5	(4.8,6.2)	5.3	3	8.0	(2.0)	(6.0,10.0)	8.0	(7.0,9.0)	7.8
	dose=6.4 nmol/kg							dose=12.8 nmol/kg						
LH (mIU/mL)														
0 hours⋆	24	1.3	(1.1)	(0.4,4.2)	0.8	(0.4,1.8)	0.9	24	1.3	(1.4)	(0.4,5.7)	1.0	(0.5,1.4)	1.0
12 hours⋆	24	7.9	(8.0)	(0.7,39.2)	5.5	(2.8,11.2)	5.4	24	8.8	(5.3)	(1.0,25.4)	8.0	(5.0,12.3)	7.3
36 hours⋆	24	1.4	(2.1)	(0.4,10.6)	0.7	(0.6,1.2)	0.9	24	1.2	(0.9)	(0.4,3.9)	0.9	(0.6,1.4)	0.9
FSH (mIU/mL)														
0 hours⋆	24	9.1	(2.6)	(5.3,16.0)	8.7	(7.3,10.5)	8.8	24	8.6	(2.2)	(5.0,13.2)	8.9	(6.8,10.1)	8.3
12 hours⋆	24	10.3	(2.3)	(6.1,14.8)	10.2	(8.5,12.3)	10.1	24	10.8	(2.5)	(6.1,17.9)	10.3	(9.1,12.7)	10.6
36 hours⋆	24	6.2	(1.4)	(4.1,9.7)	6.3	(5.3,6.8)	6.1	24	6.1	(1.3)	(3.8,8.6)	6.0	(5.2,6.9)	5.9
Oestradiol ($\times 10^3$ pmol/L)														
0 hours⋆	23	7.5	(3.8)	(1.1,16.6)	6.7	(5.8,9.1)	6.4	24	7.5	(3.8)	(2.2,17.5)	7.3	(5.3,9.5)	6.6
12 hours⋆	24	8.9	(3.3)	(2.6,16.4)	9.3	(6.8,10.3)	8.3	24	8.4	(3.7)	(2.1,15.8)	7.6	(5.9,11.2)	7.5
36 hours⋆	24	4.1	(2.5)	(1.3,12.0)	3.2	(2.8,4.4)	3.5	24	3.6	(2.6)	(1.0,13.1)	3.0	(2.2,4.3)	2.9
Progesterone (nmol/L)														
0 hours⋆	24	2.7	(1.9)	(0.5,9.0)	2.0	(1.0,3.2)	2.1	24	3.5	(2.9)	(1.0,15.0)	3.0	(2.0,4.2)	2.7
12 hours⋆	24	16.8	(8.5)	(3.0,33.0)	17.0	(9.8,20.5)	14.5	24	20.2	(10.1)	(8.0,45.0)	15.8	(12.0,28.5)	18.1
36 hours⋆	24	10.2	(5.9)	(2.0,21.0)	9.0	(5.8,14.2)	8.3	24	14.5	(7.0)	(4.0,30.0)	12.5	(9.8,19.0)	12.9

Table 4: Circulating reproductive hormones following kisspeptin-54 administration. Serum luteinising hormone (LH), follicle stimulating hormone (FSH), oestradiol and progesterone levels were measured immediately before injection of kisspeptin-54 (0 hours). Repeat measurements were made 12 hours following injection of kisspeptin-54, and at the time of egg collection (36 hours following injection). lq = lower quartile; uq = upper quartile; gm = geometric mean. $\star$ time following kisspeptin-54 injection.

	dose=1.6			dose=3.2			dose=6.4			dose=12.8		
	success (%)		N	success (%)		N	success (%)		N	success (%)		N
Patients treated			2			3			24			24
At least 1 egg collected	2	(100)	2	3	(100)	3	23	(96)	24	24	(100)	24
At least 1 M2 egg	2	(100)	2	3	(100)	3	22	(96)	23	24	(100)	24
At least 1 fertilised egg	1	(50)	2	3	(100)	3	22	(100)	22	23	(96)	24
Embryo transfer	1	(100)	1	3	(100)	3	22	(100)	22	23	(100)	23
High quality embryo transfer	0	(0)	1	1	(33)	3	15	(68)	22	15	(65)	23
Biochemical pregnancy at 12 days	1	(-)	0	1	(100)	1	11	(73)	15	8	(53)	15
Clinical pregnancy (6 weeks)	1	(100)	1	0	(0)	1	7	(64)	11	4	(50)	8

Table 5: Summary of response to treatment and progression of pregnancy following *in vitro* fertilisation treatment. Shows number of successes (percentage of N). Doses of kisspeptin-54 injection are in nmol/kg.