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The use of the Hormone Kisspeptin in *in vitro* fertilisation (IVF) treatment.

Statistical Analysis Plan, version 3.0

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Study: One versus 2 doses of kisspeptin (10hrs apart)

Participants will undergo controlled ovarian stimulation using a recombinant FSH/GnRH antagonist protocol and receive either one (Single) or two doses (Double) of kisspeptin-54 (9.6nmol/kg each dose) to trigger oocyte maturation.

On day 2 or 3 of the menstrual cycle, patients will receive daily SC recombinant FSH (recFSH) injections (Gonal F 112.5 IU, Merck Serono, Geneva, Switzerland, administered daily at midday); pelvic ultrasound scan will be performed five days after commencing recFSH, to determine ovarian follicular development. Daily subcutaneous GnRH antagonist injections (Cetrotide 0•25mg, Merck Serono, injected at 9pm daily) will be used to inhibit a premature LH surge, and will be commenced on day 5 of recombinant FSH injections. If serum LH is undetectable (<0•5 IU/L) on Day 7 of recombinant FSH injections, then the dose of cetrotide will be halved to 0•125mg daily. Further ultrasound scans will be performed according to follicular size and response to superovulation. When at least three ovarian follicles ≥18mm diameter are visible on ultrasound, all patients will receive a subcutaneous bolus injection of kisspeptin-54 administered by a study investigator to trigger oocyte maturation at 36hrs pior to egg collection (between 2030 and 2300h). The dose of kisspeptin-54 will be weight-adjusted (9.6nmol/kg). Patients will then be randomized to receive either a second dose of kisspeptin (9.6nmol/kg), or saline placebo injection at 10hrs following the first kisspeptin trigger injection.

The final dose of GnRH antagonist will be administered 24 hours prior to kisspeptin-54. Serum LH, FSH, oestradiol and progesterone will be measured immediately before, 12hrs and 36hrs after kisspeptin-54 injection, and at the time of egg retrieval in all women. Transvaginal ultrasound-directed oocyte retrieval (TVOR) will be carried out 36h following kisspeptin administration under intravenous anaesthesia (propofol). Intracytoplasmic sperm injection (ICSI) will be performed in all study cycles using sperm from the male partner to allow for assessment of oocyte maturation. All embryos will be graded at day 3 by an independent embryologist, blinded to doses of kisspeptin administered, using the British Fertility Society (BFS) and Association of Clinical Embryologist (ACE) embryo grading scheme for cleavage stage embryos, which describes embryos based on cell number, blastomere size and fragmentation.(30) If at day 3 at least 2 embryos have 6 or more cells, <20% difference in blastomere diameter and <50% fragmentation, they will be incubated until day 5 post oocyte retrieval in order that the strongest embryos can be identified for transfer. At Day 5, embryos will be graded for blastocyst expansion (1 to 6), inner cell mass (A to E) and trophectoderm (A to C).(30) At blastocyst stage, embryos which score at least 3 for blastocyst expansion and A or B for inner cell mass and trophectoderm will be classified as ‘high quality embryos’. One or two embryos of highest quality during morphological assessment will be transferred to the uterine cavity 3-5 days following oocyte retrieval.

Assessment of Ovarian Hyperstimulation Syndrome (OHSS)

All women recruited to the study will be regarded as being at high risk of OHSS. Therefore women will be routinely screened for the development of early OHSS (assessed at embryo transfer 3-5days following oocyte retrieval) and late OHSS (assessed 11days following embryo transfer). Women will be screened by symptoms (abdominal pain, abdominal bloating, diarrhoea, nausea, vomiting, subjective reduction in urine output), blood analysis (hemaglobin, hematocrit, white cell count, liver function, renal function, coagulation profile) and ultrasound parameters (ovarian size, presence of free

fluid in pouch of douglas, adnexae, abdomen or pleural cavity). OHSS will be graded according to the criteria of Golan et al. 1989 with updated categorization by Navot et al. 1992.(16,17) OHSS will ne independently graded by two experienced IVF clinicians external to the study team (S.L. and R.S.), who will ne provided with blinded data concerning patient symptom, blood analysis and ultrasound parameters. In the event of any discrepancy in categorization of OHSS, the more severe classification will be used.

Study outcomes

The primary outcome is proportion of patients achieving optimal oocyte yield (percentage of mature (metaphase 2; M2) oocytes collected from the number of follicles ≥14mm in size on final ultrasound scan prior to kisspeptin-54 trigger administration of at least 60%). All women will have an ultrasound on the morning of kisspeptin trigger administration. Oocytes will be independently classified as M2 by presence of the first polar body and round ooplasm, by an embryologist blinded to whether one or 2 doses of kisspeptin-54 were administered.

Secondary outcomes are the occurrence of OHSS, fertilization rate (percentage of M2 oocytes which fertilize to form two pronuclear (2PN) zygotes following intracytoplasmic injection with sperm; ICSI), embryo formation and quality, biochemical pregnancy rate (serum βhCG >10mIU/mL eleven days after embryo transfer) and clinical pregnancy rate (intrauterine gestational sac with heartbeat on ultrasound at six weeks gestation).

Primary Outcome:

Proportion of patients achieving optimal oocyte yield (defined as proportion of mature eggs collected from follicles of at least 14mm in size on final ultrasound prior to egg collection being at least 60%) (Binary Outcome).

The main covariates to be considered are the number of follicles on final ultrasound scan prior to trigger (follicles ≥14mm on day of trigger), serum AMH, Antral Follicle Count, age, pretrigger estradiol level.

Absolute difference between two groups with CIs will be calculated.

Secondary outcomes:

-Change in reproductive hormones LH, FSH, Estradiol and progesterone from pretrigger values to 4hr, 10hr, 14hr and 20hr values.

Variables to be included in the model: weight, AMH, pretrigger reproductive hormone levels.

Pregnancy (binary) and Implantation rates (ordinal- can be 0, 50 or 100%)-

Baseline covariates which may need adjusting for include age, number of embryos transferred, number of high quality embryos transferred, cause of infertility and BMI.

-Rates of OHSS :

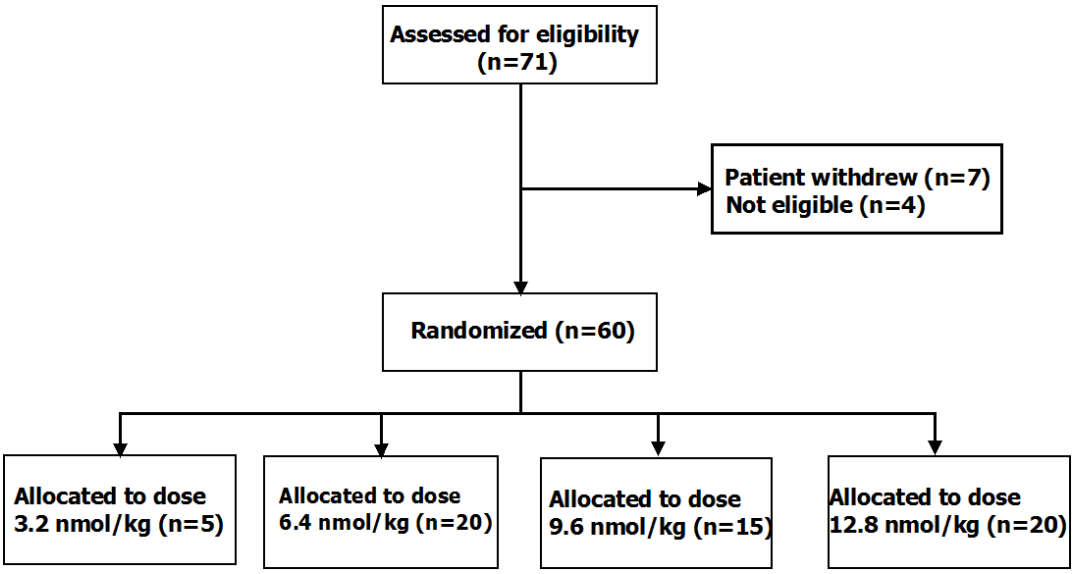
Covariates which may need to be adjusted for would include number of follicles >11mm on final ultrasound scan prior to trigger, serum AMH or antral follicle count, number of eggs collected, pretrigger oestradiol level.

List trial data as per CONSORT guidance:

Number of women screened, number who withdrew, number not eligible, number of women recruited and meeting inclusion criteria, but excluded prior to randomisation for complications requiring other IVF treatments prior to receiving kisspeptin eg hydrosalpinx.

We expect to recruit 62 women eligible for randomization (31 per group).

Example:



Tables to be generated following study completion:

Table 1:

Sort by column A N	SINGLE 31	DOIUBLE 31	BOTH 62
Age (y) C			
Weight (kg) I			
Body mass index (kg/m^2) J			
Antral Follicle Count (AFC) F			
Serum AMH* (pmol/L) E			
% with average cycle length ≥35 days D≥35			
Cause of infertility: G			
PCOS‡			
Tubal defect**			
Male Factor†			
Mixed			
Idiopathic (unexplained)			
Number of follicles¶ BF			
Number of Follicles ≥ 11mm¶ BK			
Number of Follicles ≥ 14mm¶ BN			

Table 1: Baseline characteristics of patients who received kisspeptin-54 trigger. Contains medians (lower quartile, upper quartile) for continuous variables and totals (percentages) for categorical variables.

* AMH = anti-Müllerian hormone

‡ Anovulation due to Polycystic Ovarian Syndrome.

** Blocked or removed Fallopian tubes.

† Infertility is due to a problem with male partner's fertility.

¶ On final ultrasound scan during controlled ovarian stimulation prior to kisspeptin-54 trigger administration.

Table 2:

	SINGLE (n = 31)	DOUBLE (n = 31)	BOTH (n = 62)
Number of Oocytes BZ			
Number of Mature (M2) Oocytes CA			
Oocyte Maturation Rate (%) * CD			
Oocyte Yield (%) † CG			
Proportion achieving ≥60% oocyte yield (%) CH			
Number of 2 pronuclei (2PN) zygotes CE			
Proportion with at Least 4 eggs collected (%) CB			
Fertilization rate (%) ‡ CF			
Number of patients with embryo transfer CN≥1			
Number of Cleaved embryos at day 3 post ICSI CI			
Number of Embryos at day 3 graded as ≥623 CJ			
Number of Patients with day 5 transfer % with CM=5			
Number of Embryos at day 5 CK			
Number of High quality blastocysts (≥3A/B) CL			
Number of High quality blastocysts (>3A/B) transferred CO			
Number of Embryos suitable for freezing CV			
Biochemical Pregnancy rate per protocol (%) CP including those who did not have transfer as 0			
Biochemical Pregnancy rate per transfer (%) CP not including those who did not have transfer			
Clinical Pregnancy rate per protocol (%) CQ			

including those who did not have transfer as 0	
Clinical Pregnancy rate per transfer (%) CQ **not including those who did not have transfer**	
Implantation rate (%)Ψ CU	
Live Birth rate per protocol (%)	

Table 2: IVF outcome measures following kisspeptin-54 trigger. Contains means (standard deviation) for continuous variables and totals (percentages of N) for categorical variables

* Oocyte Maturation Rate is the percentage of oocytes collected which were mature.

† Oocyte Yield is the percentage of mature oocytes collected from the number of follicles

≥14mm in diameter on the final ultrasound scan prior to kisspeptin-54 trigger administration.

‡ Fertilization rate is the percentage of mature oocytes which fertilize following intracytoplasmic injection with sperm (ICSI).

Ψ Implantation rate is defined as the percentage of embryos transferred which implant on assessment by ultrasound at 6 weeks of gestation.

Table 3:

Kisspeptin-54 Dose	SINGLE	DOUBLE	BOTH
N	31	31	62
At least 1 oocyte retrieved BZ ≥1			
At least 1 mature oocyte retrieved CA ≥1			
At least 1 2PN zygote formed ‡ CE ≥1			
Segmentation Ψ			
Number of patients with embryo transfer CN≥1			
Number of patients with day 5 transfer* % with CM=5			
High quality embryo transfer† CO≥1			
Biochemical pregnancy at 11 days CP			
Clinical pregnancy at 6 weeks CQ			
Miscarriage / Still Birth (PENDING)			
Live Birth (PENDING)			

Table 3: Summary of patient response following kisspeptin-54 triggering.

The number of patients completing each criterion is shown (% in brackets).

‡ 2PN is two pronuclear zygote assessed on day following oocyte retrieval

Ψ Segmentation refers to cryopreservation of all embryos and embryo transfer in a subsequent frozen cycle due to high risk of OHSS

* Embryo transfer on day 5

† Transfer of at least one high quality embryo. High quality embryos were blastocyst embryos graded on day 5 as being at least 3A or B in quality (Gardner et al. 2000).

Table 4:

	Early OHSS	Late OHSS
	By single vs double	By single by double
OHSS Symptomatology		
Number of patients screened by OHSS symptoms		
At least 1 symptom potentially consistent with OHSS		
Number of patients requiring medical intervention or hospitalization for OHSS		
Sonographical Screening		
Number of patients screened with pelvic ultrasound		
Mean ovarian volume (mls)		
Increase in ovarian volume as proportion of baseline scan		
Max ovarian diameter (mm)		
Number of patients with maximum ovarian diameter >5cm		
Number of patients with maximum ovarian diameter >8cm		
Number of patients with pleural effusion		
Number of patients with free fluid in abdomen		
Number of patients with fluid in POD / adnexa		
Blood parameters		
Number of patients screened with Blood analysis		
Number of patients with Hematocrit >45%		
Number of patients with White Cell Count >15 $x10^9/L$		
Number of patients with ALT or AST >2xULN		
Number of patients with total protein >80 g/L		
Number of patient with creatinine >110 µmol/L		

Number of patients with OHSS ¥	
Normal	
Mild	
Moderate	
Severe	
Critical	

Table 4: OHSS in high risk women following kisspeptin-54 trigger

¥ Diagnosis of OHSS was performed by two experienced IVF physicians independent of the study team provided with blinded data according to the criteria of Golan et al. 1989 with updated categorization of severe and critical OHSS by Navot et al. 1992.(16,17)

POD, Pouch of douglas; ALT, Alanine transaminase; AST, Aspartate transaminase; ULN, Upper Limit of Normal.