

**Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotrophin secretion in women with hypothalamic amenorrhea but chronic administration causes tachyphylaxis.**

**Short title:** Effects of kisspeptin in women with HA

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**Precis:** subcutaneous administration of kisspeptin-54 acutely and potently stimulates gonadotrophin release in women with hypothalamic amenorrhoea, but twice-daily administration over a 2 week period leads to desensitisation to its effects.

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1 **Abstract**

2

3 **BACKGROUND:** Kisspeptin is a critical regulator of normal reproductive function. A single  
4 injection of kisspeptin in healthy human volunteers potently stimulates gonadotrophin release.  
5 However, the effects of kisspeptin on gonadotrophin release in women with hypothalamic  
6 amenorrhea (HA), and effects of repeated administration of kisspeptin to humans are  
7 unknown.

8 **AIM:** To determine the effects of acute and chronic kisspeptin administration on  
9 gonadotrophin release in women with HA.

10 **METHODS:** We performed a prospective, randomised, double-blinded, parallel design study.  
11 Women with HA received twice-daily subcutaneous (sc) injections of kisspeptin (6.4nmol/kg)  
12 or 0.9% saline (n=5 per group) for 2 weeks. Changes in serum gonadotrophin and estradiol  
13 levels, luteinising hormone (LH) pulsatility and ultrasound measurements of reproductive  
14 activity were assessed.

15 **RESULTS:** On the 1<sup>st</sup> injection day, potent increases in serum LH and follicular stimulating  
16 hormone (FSH) were observed after sc kisspeptin injection in women with HA (mean  
17 maximal increment from baseline within 4 hours post-injection: LH, 24.0±3.5IU/l; FSH,  
18 9.1±2.5IU/l). These responses were significantly reduced on the 14<sup>th</sup> injection day (mean  
19 maximal increment from baseline within 4 hours post-injection: LH, 2.5±2.2IU/l, P<0.05;  
20 FSH, 0.5±0.5IU/l, P<0.05). Subjects remained responsive to GnRH post-kisspeptin treatment.  
21 No significant changes in LH pulsatility or ultrasound measurements of reproductive activity  
22 were observed.

23 **CONCLUSION:** Acute administration of kisspeptin to women with infertility due to HA  
24 potently stimulates gonadotrophin release, but chronic administration of kisspeptin results in  
25 desensitization to its effects on gonadotrophin release. This data has important implications  
26 for the development of kisspeptin as a novel therapy for reproductive disorders in humans.

1 **Introduction**

2 Hypothalamic amenorrhea (HA) is defined as the cessation of menstruation due to abnormal  
3 signalling between the hypothalamus and the pituitary gland (1), and accounts for  
4 approximately 30% of cases of amenorrhea in women of reproductive age (2). Functional HA  
5 is defined as HA occurring in the absence of a structural lesion, and often results from a  
6 relative energy deficit within the body (low body weight or weight loss) (3-7). Although  
7 current treatments for women with HA such as clomiphene, gonadotrophin injections and  
8 gonadotrophin releasing hormone (GnRH) pump therapy are efficacious, each has associated  
9 limitations (8;9).

10 The kisspeptins are a group of arginine-phenylalanine (RF) amide peptides encoded by the  
11 *KISS1* gene, which have been identified as potential novel agents for treating reproductive  
12 disorders. They act as endogenous ligands for the kisspeptin receptor (KISS1R, alternatively  
13 known as G protein coupled receptor 54) (10-12). *KISS1* and *KISS1R* are expressed in the  
14 hypothalamus, pituitary and placenta (10;12-14). Kisspeptin signalling exerts powerful effects  
15 on the mammalian reproductive system. Mice lacking kisspeptin or the kisspeptin receptor  
16 fail to undergo puberty and are infertile (15;16). In humans, inactivating mutations of *KISS1R*  
17 cause pubertal failure (16;17) and activating mutations lead to precocious puberty (18).  
18 Furthermore central or peripheral administration of kisspeptin induces gonadotrophin and sex  
19 steroid release in all mammalian species investigated, including rats (19-21), mice (22;23),  
20 monkeys (24) and sheep (23;25). We have previously demonstrated that intravenous infusion  
21 or subcutaneous bolus injection of kisspeptin-54 stimulates gonadotrophin secretion in  
22 healthy human male and female subjects, respectively (26;27). Kisspeptin may therefore be a  
23 potential novel therapy for treating reproductive disorders in humans. However the effects of  
24 kisspeptin administration in patients with infertility have not been previously investigated.

25

26 Although it has been consistently demonstrated that acute administration of kisspeptin  
27 stimulates gonadotrophin release (19-27), the effects of chronic administration of kisspeptin  
28 on reproductive function are less clear. Chronic administration of kisspeptin to non-human

1 mammals causes either sustained or non-sustained stimulation of reproductive function,  
2 depending on the mode of administration used. Intermittent administration of kisspeptin-10 to  
3 juvenile female rats (twice-daily injections) juvenile male monkeys (hourly injections) for 2  
4 days or to for 5 days, induces precocious reproductive maturation (28;29). In contrast,  
5 continuous peripheral infusion of kisspeptin-10 to monkeys or rats increases LH release only  
6 during the first 3 hours or first day of administration, respectively; LH concentrations  
7 subsequently return to levels observed before infusion of kisspeptin-10 (30;31). The long-  
8 term effects of administration of kisspeptin in humans have not been studied to date.

9 In pre-pubertal female rats, caloric restriction leads to reduced gonadotrophin levels, delayed  
10 vaginal opening, and low hypothalamic kiss1 expression (32). Twice-daily administration of  
11 kisspeptin-10 to these animals restores vaginal opening and gonadotrophin secretion (32).  
12 Based on this data, we hypothesised that repetitive administration of kisspeptin would restore  
13 gonadotrophin secretion in human female subjects with HA.

14 A randomized double-blinded placebo-controlled parallel design study was conducted to  
15 determine if twice-daily administration of kisspeptin to human female subjects with HA  
16 would sustainably stimulate gonadotrophin release.

17

18 **Methods**

19 *Kisspeptin-54*

20 Kisspeptin-54 was synthesised by the Advanced Biotechnology Centre, Imperial College  
21 London and purified by reverse-phase high performance liquid chromatography (HPLC).  
22 Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide as  
23 previously described (26;27). The peptide was tested for bioactivity and toxicity as previously  
24 described (26). The *Limulus* ameocyte lysate assay (Associates of Cape Cod, Liverpool, UK)  
25 was negative for endotoxin, and the peptide was sterile on culture (Department of  
26 Microbiology, Hammersmith Hospital, London). Although kisspeptin-10, -13, -14, and -54

1 display similar potency *in vitro*, we used kisspeptin-54 due to its higher *in vivo* potency than  
2 the other kisspeptin fragments (31;33).

3

4 *Subjects*

5 Ethical approval was granted by the Hammersmith and Queen Charlotte's and Chelsea  
6 Hospitals Research Ethics Committee (registration number: 05/Q0406/142). Written informed  
7 consent was obtained from all subjects. This study was performed in accordance with the  
8 Declaration of Helsinki.

9 Subjects were recruited through advertisements placed in local newspapers. Responders to  
10 adverts were evaluated with a detailed menstrual history, clinical examination,  
11 electrocardiogram, and blood tests. Screening blood tests performed were as follows: full  
12 blood count; renal profile; liver profile; bone profile; glucose; thyroid profile; LH; FSH;  
13 estradiol; progesterone; androstenedione; dehydroepiandrostenone; testosterone; SHBG;  
14 prolactin; 17-hydroxyprogesterone and cortisol. Women were diagnosed with functional HA  
15 and included within the study if they fulfilled the following criteria: body mass index <  
16 25kg/m<sup>2</sup>; stable body weight over the previous 6 months; age between 18 and 40 years;  
17 secondary amenorrhea of at least 6 months duration; absence of oral contraceptive pill therapy  
18 for at least one year; absence of systemic disease co-morbidity; absence of active psychiatric  
19 illness; stable body weight; absence of therapeutic or recreational drug use; absence of  
20 clinical or biochemical hyperandrogenemia; structurally normal hypothalamo-pituitary region  
21 assessed by magnetic resonance imaging; structurally normal female reproductive tract  
22 visualised on ultrasound; absence of polycystic ovarian appearances on ultrasound; normal  
23 thyroid function tests; normal serum prolactin levels; serum LH:FSH ratio <1.5. Ten subjects  
24 with HA were recruited to the study. We have previously published baseline clinical and  
25 biochemical data for healthy women in the follicular phase of the menstrual cycle, and their  
26 responses to kisspeptin-54 administration (27).

27

1 *Protocol*

2 A randomized, double blinded, placebo-controlled, parallel design study was performed. Ten  
3 subjects with HA (see Table 1 for baseline characteristics) were randomised to either saline or  
4 kisspeptin treatment groups (five subjects per group). Prior to commencement of the 8 week  
5 study protocol, subjects were taught how to self administer subcutaneous injections of saline.

6 *Baseline period:* This initial 4 week control period (weeks 1-4) allowed the measurement of  
7 baseline values of reproductive hormones and ultrasound markers, and the acclimatisation of  
8 subjects to study conditions. During weeks 1-2 of the protocol, all women with HA self-  
9 administered twice-daily subcutaneous injections of saline (blinded to subjects only). No  
10 injections were administered during weeks 3-4 of the protocol.

11 *Treatment period:* During weeks 5-6 of the protocol, women with HA either self-administered  
12 twice-daily, double-blinded subcutaneous injections of saline (5 subjects) or kisspeptin-54 (5  
13 subjects), depending on the treatment group to which they had been assigned. The dose of  
14 kisspeptin administered was 6.4nmol per kg (equivalent to 37mcg per kg (26)). Twice-daily  
15 injections were self-administered at home by subjects during the treatment period, except on  
16 the last day (week 6, day 7) when just one injection was administered in the morning. This  
17 final injection was administered within the investigation unit as part of a 4 hour sampling  
18 study (see detailed protocol on page 8).

19 *Post-treatment period:* During weeks 7-8, subjects underwent a post-treatment observation  
20 period, in order to measure reproductive hormones and ultrasound markers. No injections  
21 were administered during this period.

22

23 *Kisspeptin injections:* All subjects were trained in self-administration of subcutaneous  
24 injections by an investigator at the start of the study protocol. At the beginning of each week  
25 when injections were to be performed, a box containing unlabelled vials of freeze-dried saline  
26 or kisspeptin-54, alcohol wipes, saline vials, needles and needle disposal bins was given to  
27 each subject. For injection, vial contents were reconstituted in 0.5ml of 0.5% saline. Then a

1 0.5ml insulin syringe was used to inject a weight-adjusted volume dissolved vial contents into  
2 the lower anterior abdominal region. Subjects were instructed to refrigerate vials stored at  
3 home.

4  
5 *4 hour blood sampling post-injection of saline or kisspeptin:* All subjects underwent blood  
6 sampling in the 4 hour period immediately after the first (week 5, day 1) and final (week 6,  
7 day 7) injection of saline (5 subjects) or kisspeptin-54 (5 subjects) of treatment period. These  
8 studies were done in an investigation unit. An unused vial returned by each subject from  
9 home storage was used for their final injection of kisspeptin or saline. Saline or kisspeptin-54  
10 6.4nmol/kg was subcutaneously administered at 0 minutes by the investigator, and blood was  
11 sampled for serum LH, FSH, estradiol, and SHBG and plasma kisspeptin-IR at -30, 0, 15, 30,  
12 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes. In one subject, the study was extended to  
13 include blood sampling at 270, 300, 330, 360, 390, 420, 450 and 480 minutes post-injection.

14  
15 *Assessments of LH pulsatility:* Subjects underwent assessment of LH pulsatility on the first  
16 study day (week 1, day 1) and approximately 24 hours after the final injection of the treatment  
17 period (week 7, day 1). Blood was sampled sequentially every 10 minutes for serum LH over  
18 an 8 hour period. These studies were commenced between the hours of 0800h and 1200h.

19  
20 *Basal measurement of reproductive hormones:* Twice-weekly basal measurements of serum  
21 LH, FSH, estradiol, progesterone and SHBG and plasma kisspeptin-IR were taken from  
22 subjects throughout the 8 week study protocol between 0800 and 1800h. During weeks when  
23 injections were self-administered by volunteers (weeks 1, 2, 5 and 6), these blood tests were  
24 performed a mean of  $4.5 \pm 0.4$  hours after the previous injection, depending on the availability  
25 of volunteers. These twice-weekly basal measurements were used to calculate mean values  
26 for serum LH, FSH and estradiol, during the baseline period (weeks 1-4), treatment period  
27 (weeks 5-6) and post-treatment period (weeks 7-8) of the study protocol. Kisspeptin-IR was  
28 measured in order to confirm subject compliance to kisspeptin injections.

1

2 *Ultrasound scans.* Trans-abdominal ultrasound scans were performed once a week throughout  
3 the 8 week study period. During each scan the following parameters were measured:  
4 endometrial thickness in millimetres (mm); mean ovarian volume in cubic centimetres (cm<sup>3</sup>);  
5 mean follicles number; maximum diameter of largest follicle in each ovary in mm. Ovulation  
6 was confirmed by satisfaction of all of the following criteria: visualisation of a dominant  
7 follicle (diameter 11mm or greater); enlargement of dominant follicle into a pre-ovulatory  
8 follicle (diameter 18mm or greater); subsequent collapse of pre-ovulatory follicle or  
9 appearance of internal echoes on ultrasonography; a rise in serum progesterone to over  
10 10nmol/l.

11

12 *Other measurements:* Weight was measured on the first study day (week 1, day 1) and  
13 subsequently every 2 weeks during the 8 week protocol. During each study visit, urine was  
14 tested in order to exclude pregnancy (Clearview easy-HCG, Inverness Medical Innovations  
15 Inc., Waltham, MA).

16 Diastolic and systolic blood pressure, and heart rate were recorded every 30 minutes during  
17 the LH pulsatility studies, in order to compare mean values for each parameter prior to and  
18 after the treatment period (weeks 5-6). Blood pressure and heart rate were also recorded  
19 during 4 hour blood sampling studies performed post-injection of kisspeptin or saline.

20

21 *Response of HA subjects to GnRH before and after injections of kisspeptin:* A second group  
22 of 5 female subjects were recruited using identical inclusion criteria for HA described in this  
23 study to determine if subjects desensitised to the effects of kisspeptin retained sensitivity to  
24 the effects of GnRH. A baseline GnRH test was performed in all subjects in the investigation  
25 unit. In brief, subjects were cannulated and given a 100mcg iv bolus injection of GnRH  
26 (HRF<sup>®</sup>, Intrapharm Ltd, Kent, UK) at 0 minutes. Blood was sampled for measurement of  
27 serum LH, FSH and oestradiol at -30, 0, 15, 30, 45, 60, 90, 120 minutes. Seven days after the  
28 GnRH test, all 5 women self-administered twice-daily kisspeptin injections (6.4nmol/kg) for 2

1 weeks. The GnRH test was repeated in each subject 8-12 hours after their final kisspeptin  
2 injection.

3

4 *Collection and processing of blood samples*

5 Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Beckton  
6 Dickson, Franklin Lakes, NJ, USA). Samples were allowed to clot prior to centrifugation and  
7 separation of serum. Blood samples for plasma kisspeptin analysis were collected in lithium  
8 heparin tubes (Beckton Dickson, Franklin Lakes, NJ, USA) containing 5000 kallikrein  
9 inhibitor units of aprotinin (0.2ml Trasylo; Bayer, Newbury, UK). Samples were  
10 immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich  
11 International, Tuttlingen, Germany) for 10minutes at 3000rpm, and then separated. Serum and  
12 plasma samples were stored at -20°C until analysis.

13

14 *Analytical methods*

15 Serum LH, FSH, estradiol, and progesterone were measured using automated  
16 chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). SHBG was  
17 measured using a solid-phase automated enzyme immunoassay (Immulite; Siemens, Llanberis,  
18 UK). Reference ranges for females were as follows: LH (follicular), 2–10 IU/l; LH  
19 (midcycle), 20–60 IU/l; LH (luteal), 4–14 IU/l; FSH (follicular and luteal), 1.5–8 IU/l;  
20 estradiol (early follicular), less than 300 pmol/l; estradiol (midcycle), 400-1500 pmol/l;  
21 estradiol (luteal), 200-1000 pmol/l; and SHBG, 40–80 nmol/l. Interassay coefficients of  
22 variation were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; progesterone, 1.8%; and  
23 SHBG, 5.6%. Limits of detectability for each assay were as follows: estradiol 70pmol/l;  
24 FSH 0.05mIU/ml; LH 0.07mIU/ml; progesterone 0.1ng/ml; SHBG 0.1nmol/l.

25 Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established  
26 RIA (26;27). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and  
27 kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-  
28 releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43,

1 neuropeptide FF, and neuropeptide AF. The limit of detectability was 2pmol/l, and the intra-  
2 and interassay coefficients of variation were 8.3 and 10.2%, respectively.

3

#### 4 *Data analysis*

5 Data are presented as mean +/- standard error of mean (SEM). Hormone profiles during 4  
6 hour blood sampling studies and GnRH tests were analysed using repeated measures 2-way  
7 ANOVA with Bonferonni *post hoc* correction. Pairs of means were analysed using the  
8 unpaired two-tailed t-test. Multiple means were compared using one-way ANOVA with  
9 Bonferonni's Multiple Comparison Test. A previously described modified Santen and Bardin  
10 method was used to assess LH pulsatility (34;35). In all cases,  $P < 0.05$  was considered  
11 statistically significant.

12

## 1 **Results**

### 2 *Characteristics of subjects recruited to the study*

3 Baseline age, weight and BMI were not significantly different between kisspeptin and saline  
4 study groups (Table 1). Weight remained stable in both treatment groups during the study  
5 (mean weight change from beginning to end of study: saline, - 0.5kg; kisspeptin, -0.1kg;  
6  $P=0.09$ ). Subjects reported no increased incidence of nausea or other side effects following  
7 injection of kisspeptin or saline. Mean heart rate, systolic and diastolic blood pressure were  
8 similar prior to and after the treatment period (weeks 5-6) in all participants (data not shown).  
9 Furthermore no significant acute changes in heart rate, systolic and diastolic blood pressure  
10 were observed following kisspeptin administration when compared with saline control (data  
11 not shown).

12

### 13 *Kisspeptin immunoreactivity in plasma was raised following injection of kisspeptin*

14 Baseline plasma kisspeptin-IR was  $<2\text{pmol/l}$  and remained unchanged during the 4 hour  
15 period following injection of saline (Figure 1A, 1B). Kisspeptin injection resulted in a rise in  
16 plasma kisspeptin-IR, with peak mean kisspeptin-IR of approximately  $5000\text{pmol/l}$  at 45 min  
17 post injection (Figure 1A, 1B). Similar patterns of kisspeptin-IR were observed following  
18 injection of kisspeptin on the first and last injection days (Figure 1A, 1B).

19 In subjects randomised to receive saline injections, plasma kisspeptin-IR remained  $<2\text{pmol/l}$   
20 during basal measurements taken throughout the 8 week study protocol (Figure 1D). In  
21 subjects randomised to receive kisspeptin injections, plasma kisspeptin-IR was raised during  
22 the 2 week kisspeptin treatment period (weeks 5-6), but remained  $<2\text{pmol/l}$  for the remainder  
23 of the study protocol (Figure 1C). These twice weekly blood samples were taken at various  
24 times of the day (determined by subject availability) between the twice-daily injections.  
25 Accordingly the mean kisspeptin-IR values during the treatment period (week 5, mean plasma  
26 kisspeptin-IR  $416 \pm 217\text{pmol/l}$  and week 6, mean plasma kisspeptin-IR  $751 \pm 252\text{pmol/l}$ )  
27 were lower than the peak kisspeptin-IR observed 45 minutes after kisspeptin injection.

1

2 *Effects of first injection of saline or kisspeptin on serum reproductive hormones in women*

3 *with HA:* On the first injection day of the treatment period (week 5, day 1), saline injection

4 did not change serum LH, FSH or estradiol levels compared to baseline (Figure 2A-C).

5 Kisspeptin-54 injection acutely and potently increased serum LH levels in subjects with HA

6 in comparison to saline ( $P < 0.001$  at time-points 150 min to 240 min, Figure 2A). The mean

7 maximal increase in LH from baseline after kisspeptin injection was observed at 240 minutes,

8 and was  $24.0 \pm 3.5$  IU/l above baseline. Kisspeptin-54 injection also potently increased serum

9 FSH levels compared to saline ( $P < 0.001$  at time-points 180 min to 240 min, Figure 2B).

10 Following kisspeptin injection, the maximal FSH rise was observed at 240 minutes, and was

11  $9.1 \pm 2.5$  IU/l above baseline. Estradiol levels following kisspeptin injection were initially

12 similar to that following saline. However estradiol levels significant increased above baseline

13 between 180 and 240 min following kisspeptin administration ( $P < 0.05$ , Figure 2C).

14

15 *Effects of last injection of saline or kisspeptin on serum reproductive hormones in women*

16 *with HA:* On the last injection day of the treatment period (week 6, day 7), saline injection did

17 not change serum LH, FSH or estradiol levels compared to baseline (Figure 2D-F).

18 Kisspeptin administration resulted in a significant rise in LH only at 240 minutes post-

19 injection, and no significant rises in FSH or estradiol. Responses of LH, FSH and estradiol to

20 the kisspeptin administration were all significantly reduced following the last injection (week

21 6, day 7) when compared to the responses following the first kisspeptin injection (week 5, day

22 1), ( $P < 0.05$  for LH, FSH and estradiol responses).

23 Following the observation of significantly reduced gonadotrophin responses to kisspeptin on

24 the last injection day, we decided to further assess the duration of response to kisspeptin

25 injection in one volunteer in whom blood sampling was extended to 8 hours post-injection. In

26 this volunteer, kisspeptin IR was raised until 6 hours post-injection (data not shown).

1 Furthermore serum LH, FSH and oestradiol were still raised above baseline by the end of the  
2 8 hour sampling period (data not shown).

3 We also examined responsiveness to intravenous GnRH in 5 further subjects with HA, both  
4 before and after kisspeptin treatment. We observed LH responses to GnRH in all subjects  
5 prior to commencing kisspeptin treatment (mean peak LH increase during first 2 hours post-  
6 GnRH injection,  $14.4 \pm 4.6$  h.iU/l) (Figure 3). Furthermore these subjects remained  
7 responsive to GnRH injection 8-12 hours after their final kisspeptin injection ( $P = 0.23$  vs.  
8 baseline LH response, using 2-way ANOVA) (Figure 3).

9

10 *Reproductive hormones, LH pulsatility pattern, and radiological findings following saline or*  
11 *kisspeptin treatment*

12 Mean LH levels from twice-weekly basal blood tests during weeks 1 to 4 (the baseline period)  
13 were slightly lower in the kisspeptin group than the saline group (mean LH: saline  $3.7 \pm 0.6$   
14 vs. kisspeptin  $1.8 \pm 0.3$ ,  $P < 0.05$ ; Supplementary Table 1). Small rises in mean basal LH and  
15 FSH levels were detected during weeks 5 to 6 (the treatment period) in women randomised to  
16 kisspeptin *versus* saline (mean basal LH: saline  $-0.8 \pm 0.7$  vs. kisspeptin  $+1.2 \pm 0.7$ ,  $P = 0.09$ ;  
17 mean basal FSH: saline  $-1.8 \pm 0.4$  vs. kisspeptin  $+0.6 \pm 0.4$ ,  $P < 0.05$ ). Basal serum  
18 reproductive hormone levels were otherwise similar between kisspeptin and saline groups  
19 throughout the 8 week study period.

20 There was no significant change in mean LH, number of LH pulses or mean pulse amplitude  
21 observed in patients receiving saline or kisspeptin (Supplementary Table 2).

22 There was no significant change in mean values for endometrial thickness, ovarian volume,  
23 follicle number or maximum follicle diameter observed after kisspeptin versus saline  
24 treatment (Supplementary Table 3). One subject receiving kisspeptin developed radiological  
25 changes suggesting possible ovulation, with subsequent symptoms of pre-menstruation.  
26 Ultrasound scans of the subject revealed the rupture of a pre-ovulatory follicle (19mm

1 diameter) together with subsequent appearance of a corpus luteum. However this subject had  
2 no detectable rise in serum progesterone level, and reported no menstrual bleeding.

3

4 **Discussion**

5 We report the first study of kisspeptin administration in a human model of infertility, and the  
6 first investigation of the effects of chronic administration of kisspeptin in humans. Our results  
7 show that acute administration of kisspeptin-54 increased serum gonadotrophin levels in  
8 women with HA, but repeated injections lead to reduced effect.

9 Subjects with HA display a reproductive hormone profile which resembles the follicular  
10 phase of the menstrual cycle (low circulating gonadotrophin and estradiol levels) more closely  
11 than the other phases. Acute LH response to kisspeptin injection was approximately 4-fold  
12 greater in patients with HA than in previously studied healthy females in the follicular phase  
13 of the menstrual cycle given an identical weight-adjusted dose, (mean area under curve LH  
14 increase during first 4 hours post-kisspeptin injection in h.iU: women with HA in the current  
15 study, 40.2; healthy females in follicular phase, 9.8;  $P < 0.01$ ) (27). This is consistent with the  
16 observation that LH responses to kisspeptin-10 may be higher in under-nourished female rats  
17 when compared with those fed *ad libitum* (32). Further work in a single study comparing  
18 women with HA and women with normal menstrual cycles is required to confirm the  
19 observation made in this study. If confirmed, it would be interesting to determine if increased  
20 responsiveness of women with HA to kisspeptin is attributable to factors such as increased  
21 sensitivity to kisspeptin itself, or increased pituitary sensitivity to GnRH.

22 In juvenile female rats, twice-daily icv administration of kisspeptin-10 induces precocious  
23 vaginal opening in *ad libitum* fed animals (28), and restores vaginal opening under conditions  
24 of caloric restriction (32). Furthermore Plant *et al.* found that hourly intravenous kisspeptin-  
25 10 pulses were sufficient to induce a train of GnRH discharges characteristic of puberty in  
26 juvenile monkeys (29). We were therefore surprised to observe that LH, FSH and estradiol  
27 responses to the last kisspeptin injection were markedly lower than responses to the first

1 injection. In addition, reproductive ultrasound and basal reproductive hormone parameters  
2 were similar between the two treatment groups. The last kisspeptin injection, which was  
3 reconstituted using peptide returned from home storage by each volunteer, lead to similarly  
4 elevated plasma kisspeptin-IR to that observed after the first injection. This suggests that  
5 peptide degradation caused by home storage of kisspeptin did not account for the markedly  
6 reduced gonadotrophin responses to kisspeptin on the last injection day.

7 Kisspeptin-10 was used during the animal studies of repetitive kisspeptin administration  
8 (28;29), whereas the 54 amino acid form of kisspeptin was administered during this study.  
9 Our results reveal that plasma kisspeptin-IR is raised for up to 6 hours following each  
10 subcutaneous injection of kisspeptin-54. Sustained exposure of monkeys and rodents to  
11 kisspeptin also leads to desensitisation to its effects. Seminara *et al.* demonstrated that  
12 continuous intravenous kisspeptin-10 administration to male rhesus monkeys for 98 hours  
13 lead to increased LH release lasting only 3 hours followed by a return of gonadotrophin  
14 concentrations to levels similar to those observed prior to the kisspeptin-10 infusion (30).  
15 Similarly, Thompson *et al.* observed increased LH levels only during the first day of a  
16 continuous three day sc kisspeptin-54 infusion to adult male rats (31). A recent publication by  
17 Keen *et al.* demonstrates the pattern of kisspeptin release within the monkey hypothalamic  
18 median eminence to be pulsatile (36). Animal data suggests that a protocol using intermittent  
19 administration of kisspeptin (28-29;32) may be less likely to result in desensitisation than a  
20 protocol using continuous administration (30-31). However, given the prolonged action of  
21 subcutaneous kisspeptin-54 injection on plasma kisspeptin-IR and reproductive hormone  
22 levels, our protocol of twice-daily kisspeptin-54 injections may have resulted in  
23 desensitisation through prolonged and non-pulsatile kisspeptin exposure. An intermittent,  
24 intravenous method of kisspeptin administration might minimise or prevent the  
25 desensitisation of gonadotrophin responses observed in this study.

26 We observed GnRH administration to stimulate LH secretion in HA subjects even after 2  
27 weeks of kisspeptin treatment. A study by Ramaswamy *et al.* demonstrated that  
28 responsiveness GnRH bolus, was maintained in adult male rhesus monkeys during an infusion

1 of kisspeptin-10 delivered at 200mcg/h, but reduced at a higher infusion rate of 400mcg/h  
2 (37). In our study, a lower LH response to GnRH administration was observed after kisspeptin  
3 treatment when compared with the baseline response; however this difference was not  
4 significant. Our results therefore suggest that the protocol of kisspeptin administration used  
5 during this study lead to desensitisation upstream of the pituitary gland. It is possible that our  
6 observations are explained by KISS1R down-regulation, which has been previously  
7 demonstrated *in vitro* (38).

8 Kisspeptin antagonism has been shown to inhibit pulsatile GnRH release in pubertal female  
9 rhesus monkeys and pulsatile LH release in adult female sheep (39). Furthermore  
10 Ramaswamy et al. observed that LH pulse amplitude and frequency was reduced by infusion  
11 of kisspeptin-10 at 400mcg/h (but not 200mcg/h) in adult male monkeys (37). In the current  
12 study, neither LH pulse amplitude nor LH pulse frequency were significantly altered after  
13 kisspeptin treatment in women with HA. Our results might be explained by rapid recovery  
14 from kisspeptin exposure during the 24 hour period between the final kisspeptin injection and  
15 the second assessment of LH pulsatility study. It is also plausible that a protocol using higher  
16 or more frequent doses of kisspeptin injections would have significantly altered LH pulsatility.

17  
18 This study demonstrates that acute subcutaneous administration of kisspeptin-54 potently  
19 stimulates pituitary-gonadal function in human females with HA. However, significantly  
20 reduced gonadotrophin responses to kisspeptin-54 administration were observed after two  
21 weeks of twice-daily kisspeptin-54 injections, suggesting desensitisation. These results have  
22 important implications for the therapeutic potential of kisspeptin to treat patients with  
23 reproductive disorders.

24

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1 **Table 1: Comparison of baseline characteristics of women with hypothalamic**  
 2 **amenorrhea randomised to saline versus kisspeptin-54.** Values are provided for subjects  
 3 randomised to receive saline (n=5) or kisspeptin-54 (n=5). Data is shown as mean +/- SEM.

4  
 5  
 6

Characteristic	Study Group		P value
	Saline	kisspeptin-54	
Age (years)	24.8 ± 0.5	26.8 ± 2.4	0.28
Weight (kg)	51.8 ± 3.3	54.5 ± 1.1	0.46
Body mass index (kg/m <sup>2</sup> )	19.0 ± 0.7	19.9 ± 0.4	0.25
Duration of amenorrhea (months)	22.4 ± 9.9	23.2 ± 12.7	0.96
Serum LH (iU/l)	4.5 ± 1.6	2.6 ± 0.9	0.32
Serum FSH (iU/l)	6.6 ± 0.7	6.1 ± 1.0	0.69
Serum estradiol (pmol/l)	105 ± 13.9	78 ± 4.9	0.10

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1 **Figure Legends**

2

3 **Figure 1.** Effect of injections of saline or kisspeptin-54 on plasma kisspeptin-IR in women  
4 with HA. **A-B:** Mean  $\pm$  SEM plasma kisspeptin-IR after bolus sc injection of saline or  
5 kisspeptin 6.4 nmol/kg (n=5 per group) on the first day (**A**) or last (14<sup>th</sup>) day (**B**). Injections  
6 were administered at 0 min. **C-D:** Mean  $\pm$  SEM basal plasma kisspeptin-IR during each week  
7 of the 8 week study protocol in subjects randomized to receive kisspeptin (**C**) or saline (**D**)  
8 injections during weeks 5-6. Data is shown as mean  $\pm$  SEM. \*, P < 0.05; \*\*, P < 0.01; \*\*\*,  
9 P < 0.001.

10

11 **Figure 2.** Effects of the first and last injections of saline or kisspeptin-54 on serum  
12 reproductive hormones, in women with HA. **A-C:** changes in serum LH (**A**), FSH (**B**) and  
13 estradiol (**C**) after bolus sc injection of saline (n=5) or 6.4 nmol/kg kisspeptin-54 (KP54, n=5)  
14 on first day (week 5, day 1) of treatment period are shown. **D-F:** changes in serum LH (**D**),  
15 FSH (**E**) and estradiol (**F**), after bolus sc injection of saline or kisspeptin-54 on the last day  
16 (week 6, day 7) of the treatment period are shown. Injections were administered at 0 min.  
17 Data is shown as mean  $\pm$  SEM. \*, P < 0.05; \*\*\*, P < 0.001.

18

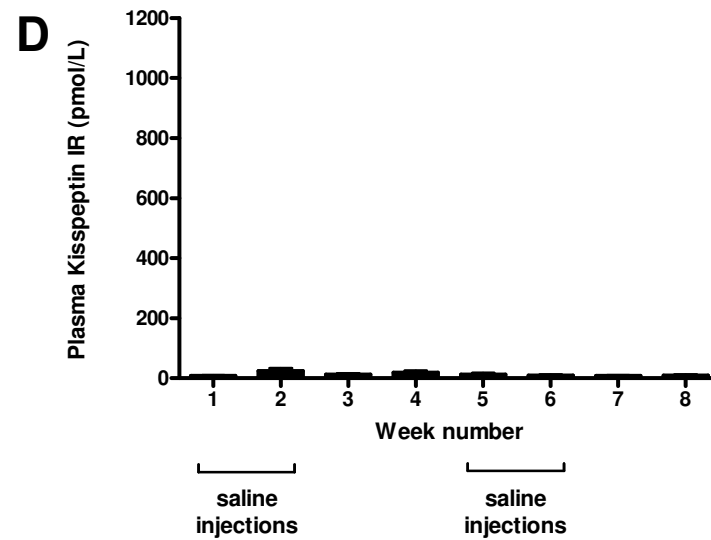
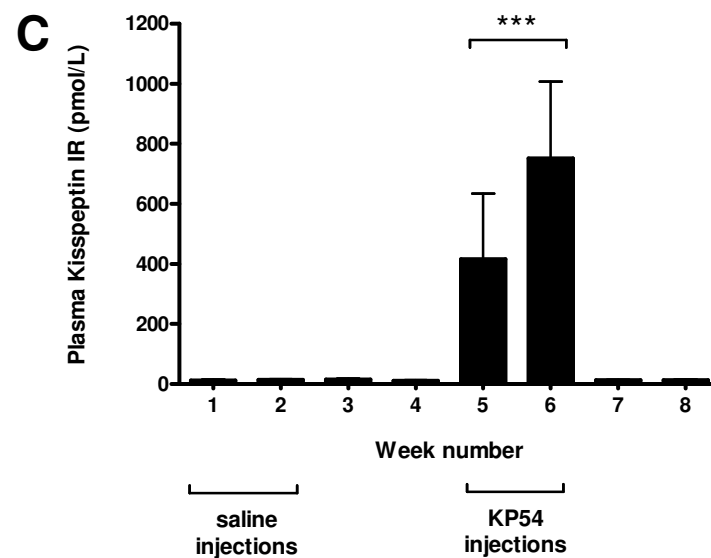
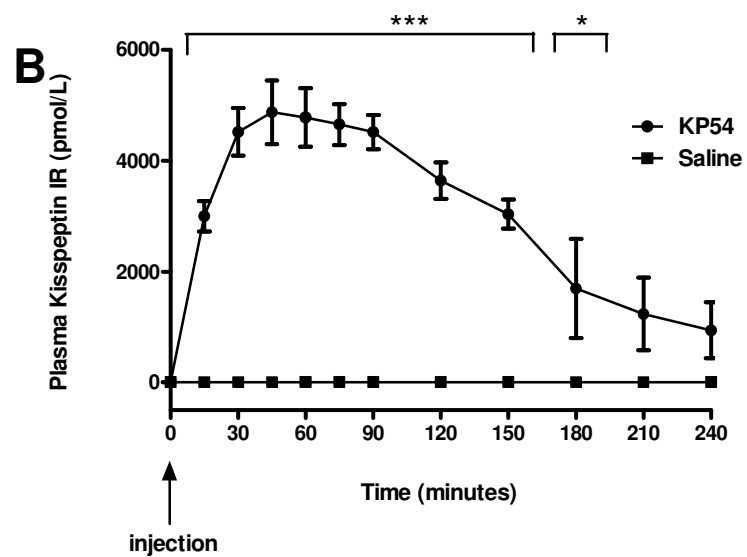
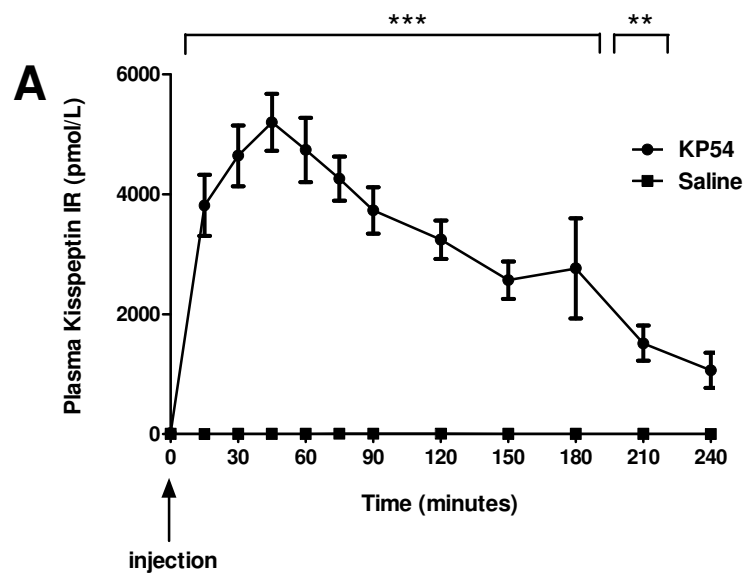
19 **Figure 3.** Comparison of LH responses to GnRH administration both before and after  
20 kisspeptin-54 injections, in women with HA. Intravenous GnRH (100mcg) was administered  
21 7 days prior to commencement of a 14 day, twice-daily regime of subcutaneous kisspeptin  
22 injections (6.4nmol/kg) (*baseline response to GnRH*) (n=5). The GnRH test was repeated 8-12  
23 hours after the last injection of kisspeptin (*post-treatment response to GnRH*). When  
24 comparing baseline and post-treatment LH responses to GnRH injection, overall responses  
25 were similar (P = 0.23), as were LH changes at each time-point after GnRH injection.  
26 Injections were administered at 0 min. Data is shown as mean  $\pm$  SEM.

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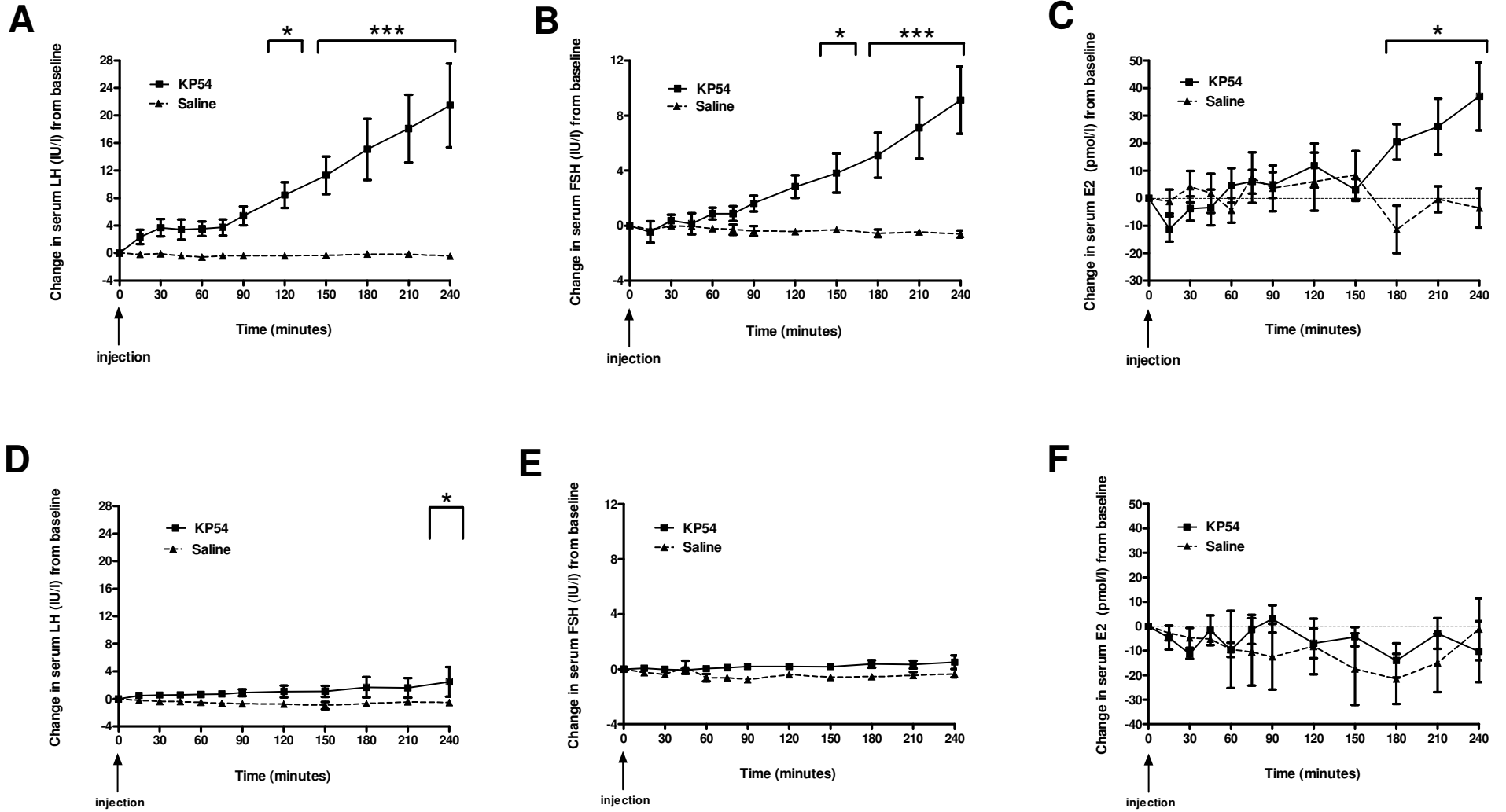
1 **Supplementary Figure 1.** Summary of 8 week study protocol. During weeks 1-2 all subjects  
2 were given saline injections. Each patient was randomised to receive twice-daily double-  
3 blinded saline or kisspeptin-54 (KP54) 6.4nmol/kg injections during weeks 5-6 of the study.  
4 During weeks 3-4 and 7-8 patients received no injections. Four hour blood sampling was  
5 performed immediately following the first and last saline or kisspeptin injection of the  
6 treatment period. LH pulsatility was assessed before and after the treatment period. Once-  
7 weekly ultrasound scans and twice-weekly blood sampling for measurement of LH, FSH, E2  
8 and kisspeptin-IR, were also performed.

9

**Figure 1**



# Figure 2



**Figure 3**

