Kisspeptin-10 stimulation of gonadotrophin secretion in women is modulated by sex steroid feedback

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STUDY QUESTION: Does sex-steroid feedback influence gonadotrophin responses to kisspeptin-10?

SUMMARY ANSWER: Gonadotrophin response to kisspeptin-10 is enhanced in sex-steroid deficient post-menopausal women and suppressed in women taking pharmacological doses of exogenous estrogen and progestogen.

WHAT IS KNOWN ALREADY: Kisspeptin, a novel hypothalamic neuropeptide, stimulates gonadotrophin secretion by stimulating GnRH secretion and has been shown in animal models to play a pivotal role in mediating sex steroid feedback. As estrogen feedback occurs at both the hypothalamus and the pituitary levels, we hypothesized that the stimulatory effect of kisspeptin-10 in women would be dependent on prevailing sex steroid milieu.

STUDY DESIGN, SIZE, DURATION: An experimental study of a novel neuropeptide in women—10 in the early follicular phase, 6 post-menopausal and 8 taking sex-steroid contraceptives (combined pill, n = 4; progestogen implants, n = 4) with suppressed LH secretion. Gonadotrophin secretion was followed for 60 min after kisspeptin administration.

METHODS AND PARTICIPANTS: The gonadotrophin response to intravenous kisspeptin-10 (0.3 μg/kg) in women in the early follicular phase was compared with that in the presence of low endogenous sex steroids/high gonadotrophin secretion (post-menopausal women) and in women taking sex-steroid contraceptives (combined pill, n = 4; progestogen implants, n = 4) with suppressed LH secretion. Area under the curve (AUC) of gonadotrophin secretion sampled at 15 min intervals over 60 min before and after kisspeptin-10 was analysed.

MAIN RESULTS AND ROLE OF CHANCE: Kisspeptin-10 stimulated LH secretion in follicular (ΔAUC 2.3 ± 0.8 IU/l h, P = 0.009), post-menopausal (5.3 ± 0.9 IU/l h P 0.002) and progestogen (2.6 ± 0.8 IU/l h P 0.05) groups but not in women taking combined pill (0.9 ± 0.4 IU/l h P 0.13). FSH secretion was significantly increased only in post-menopausal women (ΔAUC 2.6 ± 0.8 IU/l h P = 0.03) with changes of <0.5 IU/l h observed in the other three groups. Both LH and FSH responses in post-menopausal women were significantly larger than the other groups (one-way ANOVA analysis of ΔAUC; LH (P = 0.012) and FSH (P = 0.001)).

LIMITATIONS, REASONS FOR CAUTION: This study only assessed acute responses to an intravenous bolus of kisspeptin-10 administration, and the impact of continuous exposure to kisspeptin-10 on LH pulse frequency in women remains to be studied to fully understand the translational potential.

WIDER IMPLICATIONS OF THE FINDINGS: Gonadotrophin secretion in women is stimulated by kisspeptin-10. These results suggest that the pituitary gonadotrope is a functionally important locus of estrogen feedback in women and also inform potential translational applications of kisspeptin in reproductive endocrine disorders.

STUDY FUNDING: Medical Research Council (UK).

COMPETING INTERESTS: None.

Key words: LH secretion / estrogen feedback / kisspeptin / GnRH / menopause / progestogen
Introduction

Kisspeptin is a hypothalamic neuropeptide which has recently emerged as a key central regulator of gonadotrophin secretion (Oakley et al., 2009; Roseweir and Millar, 2009). Kisspeptin signalling is obligatory for normal puberty, as evidenced by a failure to progress through puberty in individuals with mutations in genes encoding kisspeptin and its receptor, GPR54 (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). There is now a wealth of evidence to suggest that kisspeptin acts directly on GnRH-containing neurones to stimulate GnRH secretion from the hypothalamus, that this is a key component of the neuroendocrine pathway regulating ovulation as well as puberty, and that the kisspeptin-GPR54 system contributes to the modulatory impact of metabolism on reproductive function (Oakley et al., 2009; Roseweir and Millar, 2009). Assesment of gonadotrophin response to the administration of exogenous kisspeptin thus provides a novel investigative tool to assess GnRH neuronal function in vivo in reproductive health and disease.

Sex steroids can regulate Kiss1 gene expression both positively and negatively, depending on the physiological environment (Navarro et al., 2004; Smith et al., 2005; Rometo et al., 2007; Oakley et al., 2009). In contrast GnRH neurons do not express estrogen receptor alpha or androgen receptor (Shivers et al., 1983; Herbison et al., 1996; Herbison, 1998), thus kisspeptin-GPR54 signalling is now considered a major mechanism for steroid regulation of GnRH secretion in animal models (Oakley et al., 2009). However, the interaction between the kisspeptin system and sex-steroid feedback in humans has not been explored.

Administration of kisspeptin, as the longer kisspeptin fragment kisspeptin-54 (Kp54), stimulates gonadotrophin secretion in healthy women and in women with hypothalamic amenorrhoea (Dhillo et al., 2007; Jayasena et al., 2009, 2010). We have recently established dose responsiveness of kisspeptin-10, the smallest kisspeptin fragment with full intrinsic bioactivity, on LH secretion in men and have shown that intravenous infusion of kisspeptin-10 can stimulate LH pulse frequency in men (George et al., 2011). However, a recent study has suggested that kisspeptin-10 does not stimulate gonadotrophin secretion in women (Jayasena et al., 2011).

We report studies on kisspeptin-10 stimulation of gonadotrophin secretion in women, quantifying and establishing the time course of the stimulatory effect on LH and FSH secretion. We compared responses to intravenous kisspeptin-10 in women in the early follicular phase with a low sex steroid/high gonadotrophin condition (post-menopause) and with a high sex steroid/low gonadotrophin condition (combined contraceptive pill and progestogen contraceptive implants). To further characterize the potential translational therapeutic applications of kisspeptin-10, we also compared kisspeptin-induced gonadotrophin secretion with that following a maximally stimulatory dose of GnRH.

Materials and methods

Participants

Ten healthy women with regular menstrual cycles, 6 post-menopausal women and 8 women using progestogen implants (n = 4) or combined oral contraceptive pills (n = 4) for contraception were recruited to the study. All volunteers provided informed written consent and the study was approved by the local research ethics committee (Lothian REC Ref: 09/S1101/67). Post-menopausal women had experienced a natural menopause (median months since menopause: 90, range: 17–200) and had not taken hormone replacement therapy in the 6 months preceding their study visit. Women using progestogen implants (n = 4) had subcutaneous implants containing 68 mg etonogestrel (Implanon, MSD, Hertfordshire, UK) in the arm, inserted between 3 and 24 months previously. Combined oral contraceptive pills (COC) contained 20–30 μg ethinylestradiol and a synthetic progesterogen for 21 days of each menstrual cycle, and all women (n = 4) had been established on this for a minimum of 6 months prior to study. Premenopausal women not taking steroidal contraception were studied in the early follicular phase of the menstrual cycle (Days 2–7 inclusive) and women taking the combined contraceptive pill were studied 2–7 days after restarting therapy following a routine scheduled 7-day interruption.

Sample size calculations were made on the assumption that the effect sizes and variance of gonadotrophin responses to kisspeptin-10 would be comparable to previous studies of kisspeptin-54 in women (Dhillo et al., 2007; Jayasena et al., 2009, 2010). Baseline full blood count, renal function, liver function, electrolytes and glucose were within normal limits in all women.

Experimental protocol

Volunteers attended Edinburgh Clinical Research Facilities in a fasting state and were provided a standardized breakfast to minimize variation in metabolic feedback to the hypothalamic–pituitary–ovarian axis. Blood samples were obtained through intravenous cannulae at 15-min intervals for 3 h before and a further 3 h after kisspeptin-10 administration, as previous studies of kisspeptin-10 have demonstrated that LH returns to baseline within this duration. An intravenous bolus dose of 0.3 μg/kg (0.23 nmol/kg) of kisspeptin-10 was selected based on our recent dose–response study (George et al., 2011). At the end of this 6-h study period, a 100 μg bolus of GnRH was administered intravenously and four further samples obtained at 15-min intervals. This dose of GnRH and sampling regime is used routinely for clinical assessment of GnRH responsiveness (Zevenhuijzen et al., 2004). Importantly, evidence from studies involving repeated pulsatile administration of GnRH have not shown evidence of desensitization of GnRH response when subsequent pulses are at least 90 min apart (Crowley and Mcarthur, 1980, Seminara et al., 2000). Thus, the GnRH response is unlikely to be affected by the kisspeptin-10 bolus administered 3 h earlier. LH was measured at all time points and FSH at 30 min intervals.

Study drugs

Kisspeptin-10 was custom synthesized under GMP standards (BachemGmbH, Weil am Rhein, Germany). Purity was assessed by high-performance liquid chromatography at 97% with a mass balance of 98.8% and stability assessed using in vitro receptor binding studies as previously described (George et al., 2011). For each participant, kisspeptin-10 was prepared within the hour prior to injection by diluting 1 mg of lyophilized kisspeptin-10 in 5 ml sterile normal saline. GnRH (Relefact) was sourced from Sanofi-Aventis, Frankfurt, Germany.

Assays

Blood samples were centrifuged at 4°C for 10 min at 1912 g and serum frozen at −20°C until analysis. LH and FSH were determined by ELISA as previously described (George et al., 2011). Inter-assay and intra-assay coefficient of variation for all hormonal assays was <5% at the concentrations measured. All samples from each study visit were analysed together in duplicate.
Data analysis

Primary analysis [area under the curve (AUC): 60-min AUC of gonadotrophin secretion before and after kisspeptin-10 was calculated by trapezoid integration and compared using paired t-tests as previously used to demonstrate dose responsiveness to kisspeptin-10 (George et al., 2011). By deducting 60-min mean baseline value from observed gonadotrophin concentrations, the inter-individual variability in baseline secretion was controlled for. Gonadotrophin responses (ΔAUC LH and ΔAUC FSH) in the four groups of women studies were compared using one-way ANOVA followed by Tukey’s test.

Secondary analysis (mean hormone concentrations): peak LH and FSH responses to kisspeptin and GnRH were identified as the highest concentration of each gonadotrophin measured within 60 min of kisspeptin or GnRH administration, respectively. Baseline (mean over 180 min before kisspeptin-10 administration) and peak gonadotrophin concentrations were compared within each of the study groups using paired Student’s t-tests.

Data are presented as the mean ± SEM. Data not normally distributed were log-transformed prior to statistical analysis. A two-sided P < 0.05 was regarded as statistically significant. The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used.

Results

Baseline age, weight, BMI, and serum LH, FSH and estradiol concentrations of the subjects are summarized in Table I. Post-menopausal women had significantly lower estradiol and higher gonadotrophin concentrations, and were older. The COCP group also had lower estradiol concentrations than in women in the follicular phase; LH concentrations tended to be lower in the groups taking steroid contraceptives, but these differences were not statistically significant. No adverse events were observed.

Kisspeptin-10 stimulates LH secretion in women

Acute intravenous administration of kisspeptin-10 stimulated LH secretion in follicular (ΔAUC LH 2.3 ± 0.8 IU/l h, P = 0.009), post-menopausal (5.3 ± 0.9 IU/l h, P = 0.002) and progestogen (2.6 ± 0.8 IU/l h, P = 0.05) groups but not in women taking combined pill (0.9 ± 0.4 IU/l h, P = 0.13). ΔAUC LH in post-menopausal women was higher than that in the other three study groups (P = 0.01). Analysis of 60-min ΔAUC of LH is summarized in Fig. 1.

Consistent with the 60-min ΔAUC analysis, peak analysis showed that LH rose in women in the follicular phase from 6.3 ± 1.2 at the baseline to a peak of 9.4 ± 1.3 IU/l (P = 0.006). Peak LH serum concentrations were achieved within 30 min, declining thereafter to reach pre-administration levels by 180 min. Post-menopausal women also responded to kisspeptin-10 administration with an increase in serum LH from 35.3 ± 2.8 IU/l at the baseline to a peak of 44.7 ± 3.4 IU/l (P = 0.005). Women with etonogestrel implants showed a significant rise in LH in response to kisspeptin-10 (4.6 ± 0.2 to 7.5 ± 0.9 IU/l, P = 0.02) but the COCP group showed a rise in LH of <1.5 IU/l, from 2.3 ± 0.9 at the baseline to 3.7 ± 1.4 IU/l (ns). Figure 2 and Table II illustrate the time course of LH responses.

Analysis of 60-min ΔAUC showed significant increase in FSH only in post-menopausal women (Fig. 3) with serum FSH rising from 2.4 ± 0.1 to 2.6 ± 0.1 IU/l h (P = 0.02). Peak analysis also showed a rise in serum FSH concentrations in post-menopausal women from 21.8 ± 4.9 at the baseline to a peak of 25.7 ± 4.4 IU/l (P = 0.04).

![Figure 1](https://example.com/figure1.png)

**Figure 1** Change in the AUC (IU/l h) of LH comparing the 60 min after kisspeptin-10 stimulation (0.3 μg/kg) with the 60 min before (basal) in 10 women in the follicular phase of the cycle (follicular) 6 post-menopausal women (post-menopausal) 4 women taking COCP and 4 women treated with progestogen implants (Progestogen Implant). Groups that do not share a letter are significantly different from each other (ANOVA followed by Fisher’s test): ΔAUC LH in post-menopausal women was higher than that in the follicular and COCP groups. *P < 0.05 **P < 0.01.

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<th>Table I Baseline characteristics of women undergoing intravenous kisspeptin-10 stimulation (0.3 μg/kg).</th>
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Data are shown as mean ± SEM. *Groups that do not share a letter are significantly different from each other (ANOVA followed by Fisher’s test). Post-menopausal women had significantly lower estradiol and higher gonadotrophin concentrations and were older.
white the other groups showed non-significant increases <0.5 IU/l; from 3.2 ± 0.4 to 3.4 ± 0.3 IU/l in the follicular phase, 2.4 ± 0.1 to 2.6 ± 0.1 IU/l in women with etonogestrel implants and from the 4.1 ± 1.8 to 4.7 ± 2.0 IU/l in the COCP group.

Comparison of gonadotrophin responses to kisspeptin and GnRH

Following 100-μg GnRH bolus, serum LH increased significantly in women in the follicular phase (6.3 ± 1.1 to 35.7 ± 7.2 IU/l, P < 0.001), post-menopausal women (35.3 ± 2.8 to 94.0 ± 10.3 IU/l, P < 0.001), as well as in women with etonogestrel implants (4.6 ± 0.2 to 26.5 ± 5.2 IU/l, P = 0.002). Women on the COCP showed a smaller response that did not reach statistical significance (2.3 ± 0.9 to 7.2 ± 2.1 IU/l, P = 0.17). Peak LH observed following kisspeptin-10 was 30.3 ± 4.3% of the GnRH-induced LH peak in women studied in the follicular phase, 49.3 ± 4.8% in post-menopausal women, 30.0 ± 4.1% in women with etonogestrel implants and 66.7 ± 26.0% in women on the combined contraceptive pill (Fig. 4A).

In response to 100 μg GnRH, serum FSH increased in women in the follicular phase (3.2 ± 0.4 to 4.4 ± 0.5 IU/l, P < 0.001), in post-menopausal women (21.8 ± 4.9 to 32.6 ± 4.6 IU/l, P = 0.008) and in women with etonogestrel implants (2.4 ± 0.1 to 3.6 ± 0.2 IU/l, P = 0.001). As with LH, women on the combined contraceptive pill showed no significant change in FSH in response to GnRH (4.1 ± 1.8 to 5.6 ± 1.9 IU/l, P = 0.22) (Fig. 4). Peak FSH observed following kisspeptin-10 was 73.9 ± 2.6% of the GnRH-induced LH peak in women studied in the follicular phase, 82.4 ± 11% in post-menopausal women, 72.4 ± 3.4% in women with etonogestrel implants and 75.0 ± 20.4% in women on the combined pill (Fig. 4B).

Discussion

The present study demonstrates that kisspeptin-10 stimulates LH and FSH secretion in women. Compared with the early follicular phase, gonadotrophin responses to kisspeptin-10 in women are enhanced in sex-steroid deficient post-menopausal women and suppressed in women taking pharmacological doses of exogenous estrogen and progestogen. As kisspeptin acts through GnRH secretion (Oakley et al., 2009; Roseweir and Millar, 2009), these observations support the notion that sex steroid feedback in women occurs both at the pituitary level and at the hypothalamic level (Crowley and McArthur, 1980; Hall et al., 1994; Pitteloud et al., 2008). In particular, the relatively smaller responses to kisspeptin-10 and GnRH in women taking combined estrogen/progestogen contraceptive are consistent with an inhibitory negative feedback effect of estrogen at the pituitary level (Shaw et al., 2010). Sex steroid feedback has been shown to regulate hypothalamic Kiss1 gene expression positively and negatively in animal models (Navarro et al., 2004; Smith et al., 2005; Rometo et al., 2007; Oakley et al., 2009). However, the present study involving the administration of exogenous kisspeptin-10 alone is not capable of differentiating the relative contributions played by pituitary gonadotropes and kisspeptin neurons in mediating sex steroid feedback. Clinical experiments involving kisspeptin antagonists currently in
development (Roseweir et al., 2009) may provide the necessary tools to elucidate this aspect.

As LH is used as a surrogate marker for GnRH release in this study, the parsimonious explanation of the present results is that sex steroids negatively regulate GnRH responsiveness to kisspeptin. However, without measuring GnRH concentrations directly in the portal circulation, other possibilities such as sex-steroid mediated alterations in gonadotrope sensitivity to GnRH (Shaw et al., 2010), in GnRH receptor signalling (Kottler et al., 2000) and in the differential early and late phase secretion of LH (Bremner and Paulsen, 1974) cannot be excluded. In particular, as continuous exposure to exogenous kisspeptin increases GnRH pulse frequency in the human (George et al., 2011; Young et al., 2012), it is plausible that such sustained stimulation by GnRH alters the pools of releasable LH, as has been shown in early studies employing varying exposure to GnRH (Bremner and Paulsen, 1974).

Our finding that kisspeptin-10 effects significant LH secretion in women appears to be at variance with the recently published report that kisspeptin-10 does not stimulate LH or FSH secretion in women studied in the follicular phase when administered as intravenous boluses of 1, 3 and 10 nmol/kg (Jayasena et al., 2011). The doses used by Jayasena et al. were 5–50 times higher than the 0.23 nmol/kg dose that we used in the present study and similar to the dose we have previously shown to result in a very limited increase in LH secretion compared with lower doses in healthy men (George et al., 2011). There are a number of potential explanations for the diminished LH response with higher doses of kisspeptin-10, including rapid desensitization and stimulation of inhibitory RF-amide receptors, as previously discussed (George et al., 2011). The present study also has a higher number of women studied in the follicular phase (n = 10 in the present study versus 5 in the study by Jayasena et al.) providing greater statistical power to detect small but significant changes. Moreover, the present study employed a 180 min baseline LH profile enabling comparisons of LH secretion before and after kisspeptin-10 administration in the same individual. This approach provides the opportunity to obtain a more accurate view of baseline secretion in gonadotrophin concentrations, thereby increasing the sensitivity to detect small changes. Finally, the present study involved the use of GMP grade kisspeptin-10 with the mass balance of 98.8% and demonstrated stability in solution and could have different potency from the preparation used by Jayasena et al. due to structure, storage or the vehicle used. However, it has to be noted that the increases in LH observed both in these studies and another recent study of kisspeptin-10 in women (Chan et al., 2012) are small (3 to 10 IU/l) and the physiological significance of such small increases in LH are as yet unclear.

In keeping with previous studies, kisspeptin-10 preferentially stimulates LH over FSH (Dhillo et al., 2005, 2007; Jayasena et al., 2009, 2010, 2011; Chan et al., 2011; George et al., 2011). FSH secretion has a constitutive component, which is less dependent on GnRH pulsatility than that of LH (Padmanabhan et al., 1997); therefore the magnitude and/or duration of GnRH release in response to a brief kisspeptin stimulus will be expected to stimulate LH more than FSH.
This observation is consistent with earlier studies using a low dose (25 μg) of GnRH which elicited isolated LH secretion (Franchimont et al., 1974). Another plausible explanation for preferential secretion of LH is kisspeptin-mediated increase in GnRH pulse frequency. High pulse frequency preferentially increases gonadotrope α and LH-β gene expression whilst lower pulse frequency increases FSH-β gene transcription (Dalkin et al., 1989; Haisenleder et al., 1991; Kaiser et al., 1997). We have previously demonstrated that GnRH pulse frequency is increased by exogenous kisspeptin-10 administration (George et al., 2011). A decrease in GnRH pulse frequency has been reported in animal models administered kisspeptin antagonists (Li et al., 2009). GnRH neurons from mice of both sexes have also been shown to be sensitive to kisspeptin in vitro, with sustained activation in explanted GnRH neurons observed immediately after kisspeptin exposure (Dumalska et al., 2008). It is therefore reasonable to speculate that exogenous kisspeptin-10, even when administered as an intravenous bolus, rapidly enhances GnRH pulse frequency as a result of which LH synthesis and secretion is prefered over FSH.

In the present study, we have also demonstrated that kisspeptin-10-induced LH secretion is less than that observed with a maximally stimulatory dose of GnRH, irrespective of the steroidal milieu. We compared the magnitude of secretion of LH following the administration of a dose of kisspeptin-10 previously shown in men to be maximally stimulatory (George et al., 2011) with that observed following the administration of a maximally stimulatory dose of GnRH (100 μg intravenous bolus). LH secretory response to GnRH was higher than the response to kisspeptin-10 in all groups, consistent with studies in rodent models (Tovar et al., 2006). This finding easily explained by the amount of GnRH available to stimulate secretion of LH, as it has been shown that the GnRH doses required to mimic a kisspeptin-induced LH surge are much smaller than the pharmacological dose used in the present study (Chan et al., 2011). Kisspeptin independent GnRH secretion has been demonstrated in Kiss1 and GPR54 knock-out rodents models (Chan et al., 2009) and kisspeptin antagonists, unlike GnRH antagonists, do not lower LH to sub-basal concentrations (Roseweir et al., 2009; Millar et al., 2010). Our observations are consistent with these studies and the notion that while the principal mode of action of kisspeptin is GnRH mediated (Oakley et al., 2009), there is a kisspeptin-independent component of GnRH secretion. This suggests potential therapeutic application of kisspeptin and its agonist analogs will primarily be in areas where modest rises in serum concentration of gonadotrophins are desirable. However, intravenous infusions of kisspeptin-10 in men have been shown to effect significantly more gonadotrophin secretion than its administration as boluses (George et al., 2011). Experiments involving continuous exposure to kisspeptin-10 are therefore needed to characterize fully the translational therapeutic potential of kisspeptin and its analogues.

The preferential stimulation of LH secretion following kisspeptin administration observed in the present study is consistent with studies in animal models (Roa et al., 2008). Consistent with these, ED90 value for the FSH-releasing effects of kisspeptin in vitro has been shown to be ~100-fold higher than that of LH (Navarro et al., 2005). However, the presence of a distinct inhibitory sex-steroid input to the kisspeptin neurons in the antero-ventral periventricular nucleus in rodents (Oakley et al., 2009) complicates extensive comparison between studies in rodents and present human data.

We did not measure circulating, administered kisspeptin for a number of reasons. First, to obtain an accurate picture of kisspeptin, the sampling regime would need to be much more frequent, given the short half-life of kisspeptin-10 (Mikkelsen et al., 2009). Consistent with this, mass spectroscopic assays of kisspeptin-10 in men administered kisspeptin-10 infusions showed the concentrations of kisspeptin-10 and its metabolites below detection limits (George et al., 2011). Second, kisspeptin antibodies currently available are not specific enough to differentially detect kisspeptin fragments with intrinsic bioactivity (Oakley et al., 2009).

In conclusion, prevailing sex steroid concentrations modulates the stimulatory effect of kisspeptin-10 on LH in women. We have also demonstrated that kisspeptin-induced LH secretion in women is only a proportion of that observed with a maximally stimulatory dose of GnRH.

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Authors’ roles

J.T.G., R.A.A. and R.P.M. contributed to the conception and design of this study. J.T.G. led the data acquisition and analysis of data. J.T.G. drafted the manuscript which was revised by R.A.A. and R.P.M. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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