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REC Ref. No. 16/LO/0391 Version 02 Date: 18 Mar 2019

Study Protocol: PHYSIOLOGICAL STUDIES OF HORMONES WHICH CONTROL METABOLISM AND REPRODUCTION

Background

Subfertility and weight disorders are very common problems, which can have significant and detrimental effects on the mental, physical and financial wellbeing of people affected by these conditions. Infertility affects 1 in 7 couples in the UK [1] and has a profound psychological and social impact on the couple involved. Furthermore, currently available treatments are only successful in a minority of cases. Obesity affects 1 in 4 adults in England [2] and obesity significantly increases the risk of diabetes (a metabolic disorder, which affects over 3 million adults in the UK), heart disease and cancer [3]. The currently licensed non-surgical obesity treatments have limited efficacy and are often poorly tolerated. Whilst surgical treatments for obesity are effective, they are not suitable for all patients and are associated with complications (some of which require life-long follow-up of patients who have had weight reduction surgery). Therefore there is a pressing need to develop better therapies for both infertility and obesity.

Fertility is dependent on the metabolic state of an individual but the physiological effects of interactions between the hormonal systems that govern reproduction and metabolism (including food intake, body weight, energy expenditure, and glucose regulation) are not well understood. People at either end of the body weight spectrum (i.e. underweight or obese) have low levels of reproductive hormones and therefore have reduced fertility. In addition, people with abnormal glucose regulation, which occurs in diabetes, also have low reproductive hormone levels and therefore reduced fertility. Fertility is regulated by the production of gonadotrophin releasing hormone (GnRH) from a small part of the brain, the hypothalamus, which stimulates the release of sex hormones (gonadotrophins) in a pulsatile manner into the bloodstream. However, we currently have a limited understanding of what factors control these hypothalamic cells to release gonadotrophins. Over the last ten years, the naturally-occurring hormones kisspeptin and neurokinin B (NKB) have been found to play critical roles in regulating GnRH release during puberty and adulthood in both men and women [4-9]. Animal studies have suggested that gut hormones (which regulate appetite and metabolism) also influence kisspeptin secretion [10, 11]. Additionally, animal studies have shown that kisspeptin and neurokinins affect the secretion of some gut hormones, appetite, energy expenditure and the regulation of glucose levels [10, 12-14].

Although reproduction and metabolism are closely linked, human studies exploring the important physiological effects of the hormonal regulators of reproduction and metabolism are lacking. Our (and other research groups') previous ethics-approved studies have demonstrated that administration of these gut hormones (i.e. glucagon, GLP-1, oxyntomodulin, peptide-YY (PYY) and substance P) and these reproductive hormones (i.e. kisspeptin and NKB) to humans is safe and well tolerated [15-30]. We therefore anticipate that by investigating the physiological effects of these gut hormones on LH pulsatility, and kisspeptin and NKB on metabolism, we will increase understanding of the human reproductive axis and control of metabolism, and identify potential targets for treatment of human infertility and/or weight disorders.

Objective

This study is designed to investigate the physiological effects of gut hormones and reproductive hormones on the human reproductive and metabolic systems.

Chief Investigator

The Chief Investigator is Professor Waljit S Dhillo, Professor of Endocrinology & Metabolism, Imperial College London, and Consultant Endocrinologist, Imperial College Healthcare NHS Trust.

Participants

Study participants will be healthy volunteers and people with conditions that predispose them to subfertility. Participants will be recruited by advertisements (print and online) and/or text (SMS) messages. The advertisements will include contact details for the researchers involved. The participants will initially be informed of the nature of the study by one of the researchers and will receive an explanatory information sheet to help decide whether they wish to take part in the study. Interested participants will be invited to attend a screening visit once they have been given at least 24hr to consider participation in the study with the relevant study information. General health will be determined at screening which will comprise a medical history, routine physical examination and basic investigations (full blood count, urea and electrolytes, liver function tests, thyroid function tests, plasma glucose, lipid profile and an electrocardiogram). Participants will be free to withdraw from the study at any time.

Inclusion criteria

- Aged 18 – 60 years
- Male or female
- Stable body weight for preceding 3 months
- 12-lead ECG with no clinically significant abnormalities as judged by the investigators.
- Full blood count, urea and electrolytes, thyroid function tests, liver function tests and glucose within 2 x the upper limit of normal.

Exclusion criteria

- History of any medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study or potentially cause harm to the volunteer.
- Pregnancy or breastfeeding.
- History of hypersensitivity to any of the components of the infusions.
- Treatment with an investigational drug within the preceding 2 months.
- Volunteers who have or intend to donate blood or blood products within three months before or following study completion.
- A history of alcoholism or substance abuse within the preceding 5 years.
- A history of major haematological, renal, thyroid or hepatic abnormalities or significant cardiovascular disease.
- A history of cancer.
- Volunteers with a medical or psychological condition that would impair their ability to participate reliably in the study or give informed consent.

Hormones

The naturally-occurring hormones to be used in this study are glucagon, GLP-1, oxyntomodulin, PYY, substance P, kisspeptin and NKB. Over the last 25 years our group has infused synthetic human gut hormone peptides to a large number of volunteers (over 1000 in total) with no untoward side effects. Natural human sequence glucagon has been administered by our laboratory and by others without adverse effect [31-33]. Furthermore, glucagon and insulin are licensed in the UK for administration by subcutaneous, intramuscular and intravenous routes as a treatment for hypoglycaemia and for diagnostic purposes. Natural human sequence GLP-1, oxyntomodulin, PYY and substance P have also been

administered by our laboratory and by others without adverse effect [15-18, 34]. Similarly, kisspeptin (which increases over 1000-fold in pregnancy) and NKB are naturally occurring hormones, and have been administered safely by our research group and other researchers [19-22, 24, 26, 30]. Insulin is naturally produced by the pancreas in response to a rise in blood glucose

Following the MHRA algorithm, "Is it a clinical trial?", and previous discussions with the MHRA, this proposed study is not a clinical trial but rather an investigation of the physiological roles of gut and reproductive hormones in the regulation of reproduction and metabolism. All the hormones to be used in this study will be prepared using our standard operating procedure for preparation of peptides for human volunteer studies, which has been previously described and has received Ethics approval (see Appendix 1) or obtained from a licensed pharmacy.

Energy Expenditure

A key component of metabolism is energy expenditure. Activation of brown adipose tissue depots located in the neck increases energy expenditure and we have previously shown that this can be safely and reliably detected with thermal imaging [29]. Therefore we will use this non-invasive technique during some of the visits in Group B to assess the effect of these natural hormones on brown adipose tissue activation and hence energy expenditure.

Protocol

Study design: This study is a single blinded physiological study of naturally occurring hormones. This study will be carried out in 2 groups (Group A and Group B, further details below). It is a physiological study of natural hormones given by infusion to overcome their very short half-lives. Doses of hormones used will be those that have been used in previous studies that have been demonstrated to safely produce a biological effect without untoward side effects. At least two clinically trained investigators will be present throughout each study visit. Participants will have their blood pressure, pulse and blood glucose (BM, via rapid bedside testing) monitored and recorded on arrival and at regular intervals throughout each visit.
There will be a maximum of 9 study visits per participant, each lasting up to 11 hours and participants will either be allocated to Group A or Group B (further details below). Participants will be assigned a number generated by a random number generator. Participants assigned an odd number will be allocated to Group A and participants assigned an even number will be allocated to Group B.

Pre study visits: Participants will be asked to avoid alcohol and strenuous exercise for 24 hours prior to each study visit. Other instructions specific to Group A and Group B are outlined in the relevant sections below.

Study visits: Study visits will be scheduled at least 3 days apart. Study visits will also be scheduled in such a way that the maximum blood taken from each participant will not exceed 500ml per month. For comparison, the volume of blood taken during a standard blood donation session is 470ml [35]. Female participants will have their urine tested for ß-hCG on each occasion to ensure that they are not pregnant. After arrival, 2 intravenous cannulae will be inserted (1 in each arm), 1 cannula will be used to administer the infusions and 1 cannula will be used for blood sampling.

- **GROUP A**

Primary outcome: To determine whether there is a difference in luteinising hormone (LH) secretion when participants receive a gut hormone compared with vehicle in healthy men and women, and in men and women who have conditions that predispose them to subfertility.

The effect of kisspeptin on LH secretion in men and women has been well characterised and as such it is not necessary to infuse kisspeptin and measure LH in this part of the study. Therefore in this part of the study only vehicle and gut hormones will be infused and their effects on the reproductive system and metabolism determined by measuring kisspeptin, LH, and FSH levels, as well as gut hormones, glucose and insulin levels.

Prior to each study visit, participants will consume a standardised dinner of their own choice at 8 pm, i.e. if they consumed a specific meal before the first study visit, they will instructed to consistently consume the same meal on the evening before each study visit. Then, the volunteers will eat a standardised snack at 10pm, and will be asked not to have anything further to eat or drink (apart from plain water) until the next morning. On the morning of each study visit, they will consume a standardised breakfast at 6am, and asked not to eat or drink anything else until they arrive for the study visit at the Clinical Research Facility.

Participants will attend the following visits in a random order.

Visit 1: Intravenous infusion of vehicle

Visit 2: Intravenous infusion of glucagon (up to 14pmol/kg/min) [25]

Visit 3: Intravenous infusion of GLP-1 (up to 0.8pmol/kg/min) [25]

Visit 4: Intravenous infusion of oxyntomodulin (up to 3pmol/kg/min) [34]

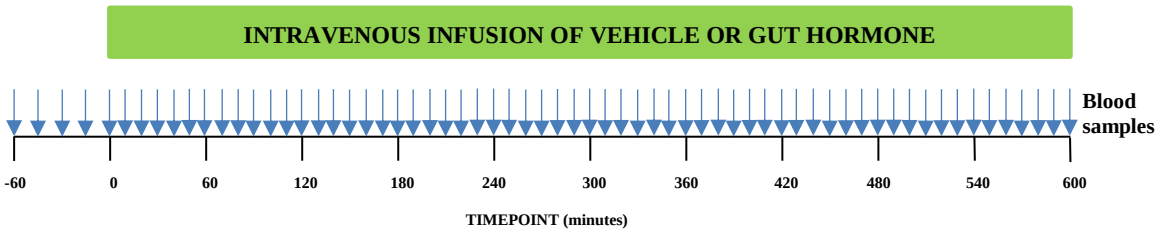
Visit 5: Intravenous infusion of PYY (up to 0.8pmol/kg/min) [36]

Visit 6: Intravenous infusion of substance P (up to 1.5pmol/kg/min) [16]

These doses have been selected based on biological effect and safe tolerability [16, 25, 34, 36].

Small amounts of blood will be taken via the cannula (not more frequently than every 10 minutes) for up to 11 hours in total (see figure 1). Blood samples will be analysed for measurement of sex hormones e.g. kisspeptin, LH, FSH, testosterone (in men), oestradiol (in women), as well as gut hormones, glucose and insulin. A maximum of 250ml of blood (about three quarters of the volume in a can of soft drink) will be taken in total during each study visit.

Figure 1 – Group A Study Visits



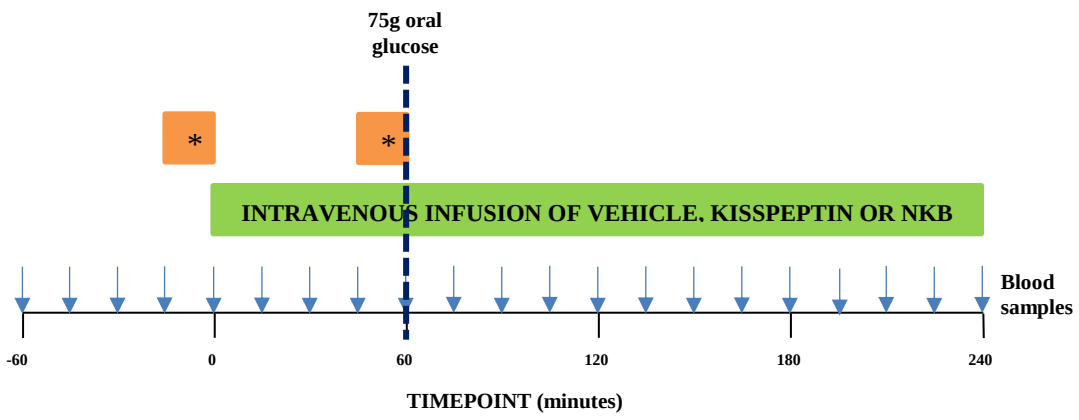
- **GROUP B**

Primary outcome: To determine if there is a difference in metabolic parameters (glucose, insulin, energy expenditure, appetite) when participants are administered kisspeptin or NKB compared with vehicle in healthy men and women, and in men and women who have conditions that predispose them to subfertility.

The effect of gut hormones on glucose and insulin is well known therefore it is not necessary to infuse gut hormones and measure these parameters in this part of the study. Consequently, in this part of the study only vehicle and kisspeptin or NKB will be infused (low dose on one visit and high dose on a separate visit, up to a maximum of 0.72nmol/kg/min for kisspeptin [24] and up to a maximum of 5.12nmol/kg/min for NKB [30]), and their effects on metabolism will be determined by measuring glucose, insulin, C-peptide and glucagon levels (as well as reproductive hormone levels) during standardised oral glucose tolerance tests (OGTT), intravenous glucose tolerance tests (IVGTT) and appetite assessments. A low dose as well as a high dose of reproductive hormones will be investigated as previous data in animals suggest that there may be different effects at different doses [37]. In keeping with standard preparation for these tests, participants will be asked to eat their evening meal at 8pm on the night preceding each study visit, and refrain from eating and drinking anything (apart from plain water) until they attend for the study visit at the Clinical Research Facility. These tests are routinely used in clinical practice and research to assess insulin secretion, insulin sensitivity and diagnose abnormalities in glucose regulation such as diabetes. Participants will attend the following visits in a random order.

Oral glucose tolerance test (OGTT) Visits 1-3: After an acclimatisation period, a 10 minute baseline thermal imaging recording will be taken. The infusion of vehicle, kisspeptin or NKB will be started at timepoint 0 minutes and when the blood levels are at steady state (i.e. up to 60 minutes after the start of the infusion) a 2nd 10 minute thermal imaging recording will be taken. 75g of glucose will then be administered orally. Small amounts of blood will be taken via one cannula (not more frequently than every 10 minutes) for up to 5 hours in total (see Figure 2). Blood samples will be analysed for measurement of glucose, insulin, C-peptide, glucagon, kisspeptin, LH, FSH, testosterone/oestradiol. A maximum of 190ml of blood (about half the volume in a can of soft drink) will be taken in total during each study visit.

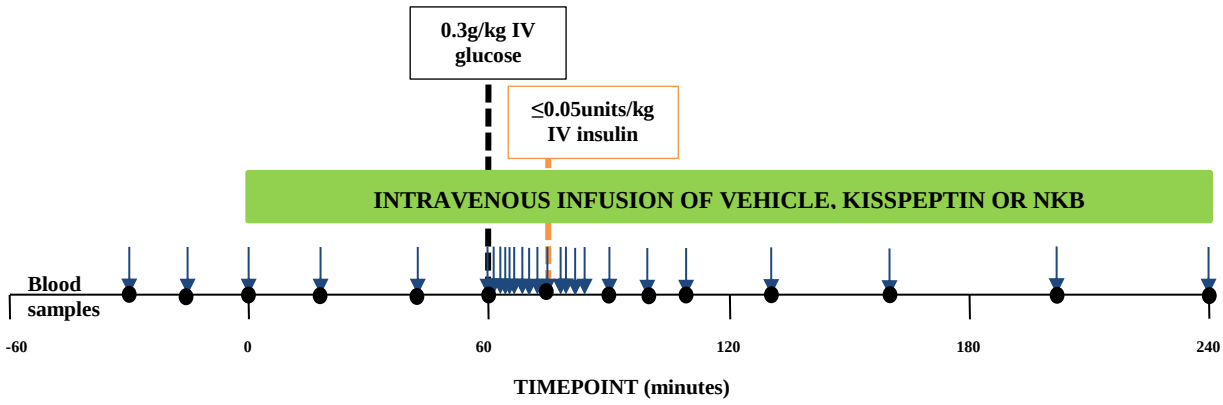
Figure 2 – Group B OGTT Study Visits



*Thermal imaging

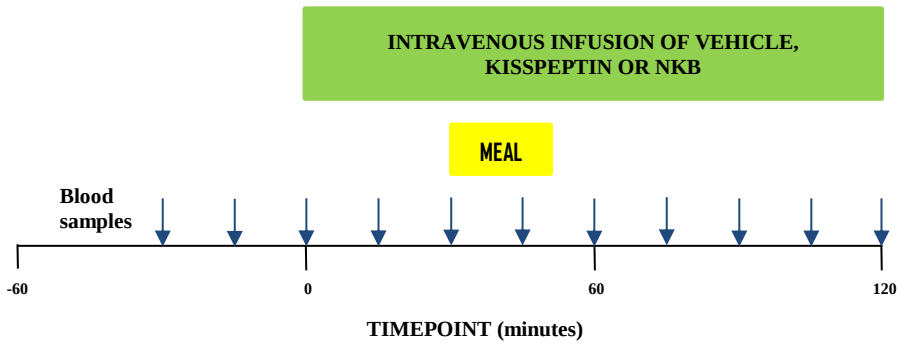
Intravenous glucose tolerance test (IVGTT) Visits 4-6: After a baseline period, vehicle, kisspeptin or NKB infusion will be started at timepoint 0 minutes. To evaluate the acute insulin response to glucose (AIRg) and insulin sensitivity index (S_I), a standard frequently sampled iv glucose tolerance test (FSIVGTT) will be performed with an iv glucose bolus of 0.3 g/kg administered manually over 2 minutes at up to +60 minutes. A dose of insulin (up to 0.05units/kg) may be given intravenously about 30 minutes after the dose of intravenous glucose [38-40]. This will enable calculation of the insulin sensitivity index using the minimal model in people with abnormal glucose homeostasis (whose insulin secretion is impaired) and ensure that glucose levels do not remain elevated for a prolonged length of time after the dose of intravenous glucose. Blood samples will be taken for glucose, insulin and C-peptide at −30, -15, 0, 20, 40, 60, 62, 63, 64, 65, 66, 68, 70, 72, 74, 78, 80, 82, 85, 90, 100, 110, 130, 160, 200, and 240 minutes (blue arrows in Figure 3). This is a well-established protocol used in metabolic studies [27, 38-40]. Blood samples for kisspeptin, LH, FSH, testosterone/oestradiol and glucagon will be taken approximately every 15 minutes (black circles in Figure 3). About 190ml of blood (about half the volume in a can of soft drink) will be taken in total during each study visit.

Figure 3 – Group B IVGTT Study Visits



Appetite Visits 7-9: The IV infusion of vehicle, kisspeptin or NKB will be started at t= 0 minutes and will last up to 120 minutes (Figure 4). Blood samples for the above mentioned measurements will be taken at time points (-30, -15, 0, +15, +30, +45, +60, +75, +90, +105, +120 min). Simultaneously volunteers will be asked to fill in visual analogue scales (VAS) to record appetite and nausea levels at t= -30, 0, +30, +90 and +120 minutes. An ad libitum meal, provided to excess, will be served at t= +30 minutes. Volunteers will be allowed 20 minutes to eat until t= +50 minutes. The maximum amount of blood taken will be less than 190 ml. Thermal imaging and non-invasive measurement of energy expenditure may be performed before the infusion starts and during the infusion.

Figure 4 – Group B Appetite Study Visits



Safety and protection of volunteers

Human natural sequence glucagon is a licensed medication, dispensed to patients with diabetes so that their friends or relatives may provide treatment for hypoglycaemia at home, without medical assistance, at a dose up to ten times higher than used in this study. Glucagon and insulin are also used routinely in the investigation of endocrine diseases. Glucose levels will be measured at regular intervals throughout the study visits using bedside capillary tests. Glucagon, insulin, PYY, GLP-1, oxyntomodulin, substance P, kisspeptin and NKB have been administered without adverse effects in numerous studies worldwide over many years [15-27, 29, 30, 34, 38-40] As with all physiological satiety signals, increasing levels of gut hormones cause gradual reductions in appetite, with nausea occurring at the highest levels as the most significant manifestation of loss of appetite.

Participants will remain in the investigation unit for a short period of observation after termination of the infusion. During the infusion, up to two experienced physicians will monitor the volunteers at all times with regular observations (pulse and blood pressure). Participants will be encouraged to report any unusual or unpleasant sensation to the investigator immediately. Any significant adverse effects will lead to withdrawal of the individual and any serious adverse effects will terminate the whole study.

Throughout the study there will be at least one physician available on 24 hour call via a direct line, with a second physician on back up, and a secondary direct line to Professor Dhillo. Although we do not anticipate any serious adverse effects, participants will be provided with contact numbers and clear instructions that, if they feel unwell, they should call us. However, since the peptides are very rapidly cleared, and circulating concentrations reduce to normal within minutes, participants will be under direct observation by the investigators whilst they have elevated hormone levels, and would not be anticipated to experience any delayed effects after clearance of the peptides.

It will be made clear to participants that they will be free to withdraw from the study at any time without providing any reason. Any possible adverse event will be reviewed with the senior clinicians (principally Professor Dhillo). Any significant adverse effects would lead to withdrawal of the individual and any serious adverse effect which is related to the study intervention may result in termination of that intervention from the clinical study. Any serious adverse event suspected to be related to the study procedures would be

reported to the ethics committee and the sponsor (Imperial College London), as well as to the data monitoring committee. The data monitoring committee will be convened prior to initiation, and an internal audit will be completed at the end of the study.

Statistics and Data Analysis

Mr. Paul Bassett (independent statistical consultant) calculated the sample size required. For Group A, based on our previous work, we estimate the difference in LH concentration between gut hormone infusion and vehicle will be 2.5IU/L with an SD of 3IU/L. Therefore the sample size required for a study with 90% power and a significance level of 0.05, is 18 participants. For Group B, based on our previous work, the anticipated difference in insulin concentration following a glucose challenge between reproductive hormone infusion and vehicle is 20mU/L with an SD of 22.5mU/L. Therefore the sample size required for a study with 90% power and a significance level of 0.05, is 16 participants. We estimate there may be up to a 20% drop-out rate (based on our experience with volunteers recruited into studies investigating gut and reproductive hormones), therefore we plan to recruit 22 volunteers for Group A, 20 volunteers to receive kisspeptin in Group B and 20 volunteers to receive NKB in Group B. As a result, the total number of participants will be 62 people.

Sample & data handling and storage

The samples taken during the screening visit are labelled with the subject's name and hospital number, in a manner similar to other NHS samples. The samples taken during the study visits will be coded. Only the researchers involved in the study will have access to the codes. This is necessary to identify samples in order to correlate levels of hormones and glucose found within the samples to clinical details of the donors. The samples will be transferred to Department of Investigative Medicine at Imperial College for analysis. Blood samples will be stored in the Department of Investigative Medicine, Commonwealth Building, Hammersmith Hospital, Du Cane Road, London. Only members of the research team will have access to the samples. Data will be stored and analysed on university computers and will be password-protected. Data will only be accessed by the researchers involved in the study.

Regulatory issues

Ethics approval: The Chief Investigator has obtained approval from the west London Research Ethics Committee (reference no 16/LO/0391). The study has been submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the Trust R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

Consent: Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent will be obtained. The right of the participant to refuse to participate without giving reasons will be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

Confidentiality: The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

Indemnity: Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

Sponsor: Imperial College Academic Health Science Centre will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

Funding: NIHR is funding this study. Participants will receive £150 per completed study visit to cover expenses including travel costs, time off work and lost earnings. The investigators do not receive any payment for this study.

Audits: The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

Study Management

The day-to-day management of the study will be co-ordinated through Dr Chioma Izzi-Engbeaya and Dr Alexander Comninos who are both experienced in physiological study management.

Publication Policy

We aim to disseminate data generated during the study via publication in peer reviewed medical journals, presentation at conferences and publication in the lay press as appropriate. During publication the data will be completely anonymised and no personal information will be published. Participant confidentiality will be maintained throughout. Participants and their GPs (with the participant's consent) will be provided with a copy of published data should they wish.

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Appendix 1

STANDARD OPERATING PROCEDURE FOR PREPARATION OF PEPTIDES FOR HUMAN VOLUNTEER STUDIES

Over the last 30 years our group has infused human sequence hormone peptides to a large number of volunteers (over 1000 in total) with no untoward side effects, e.g. GLP-1: Neary et al. 2005 Endocrinology vol. 146, pp. 5120-7: OXM: Cohen et al. 2003 J. Clin. Endocrinol. Metab. vol. 88, pp. 4696-701; PYY3-36: Batterham et al. 2002 Nature vol. 418 pp. 650-4; kisspeptin: Dhillo et al. 2005 J Clin Endocrinol Metab. 90(12):6609-15, Dhillo 2007 J Clin Endocrinol Metab. 92(10):3958-66 and neurokinin B: 2014 J Clin Endocrinol Metab. 99(1):E19-27. Peptide infusions in the Department of Investigative Medicine follow protocols established after long experience investigating the physiological role of numerous peptides in humans.

For similar studies investigating the physiological effects of hormones, which does not involve administration of a medicinal product but involves administration of hormones and combinations thereof, we will comply in full with the standard procedures outlined below. The procedures were developed at the behest of the Hammersmith and Queen Charlotte's (formerly the RPMS) ethics committee following extensive discussion with a number of experts and their purpose is to ensure safety and efficacy in peptide preparation and administration. All steps required in the ethics committee guidance are listed below:

1. Peptides will be synthesised by CROs e.g. Bachem, Poly Peptide Ltd, or equivalent facility. Following initial high fidelity synthesis, peptides undergo purification by high resolution HPLC. Following synthesis, peptide compositions and purity are verified by quantitative amino acid analysis. Any problems or inconsistencies will result in that batch being completely discarded.
2. Peptides are aliquoted into sterile glass vials under aseptic conditions and freeze dried. Although the material is packed and stored under vacuum, which destroys any active aerobic microorganisms, the freeze dried peptide is also sent to the Imperial College Healthcare NHS Trust microbiology department for culture of all common pathogenic microorganisms as a further safety check.
3. Absence of pyrogen contamination of every batch of peptide is confirmed by highly sensitive measurement of endotoxin content using a Limulus Amoebocyte Lysate (LAL) test, performed by Associates of Cape Cod International, Deacon Park, Moorgate Road, Liverpool, United Kingdom.
4. For each batch of peptide aliquots, toxicity studies are carried out in mice. Greater than 10 times the maximum dose to be given to man (in pmol/kg body weight) is administered to a minimum of 20 mice and compared to a saline injected control group. The animals are observed and, if no adverse effects are seen, one group of 10 mice will be killed by a schedule 1 method after 48 hours observation and the remaining animals will be killed at 14 days. Full necropsies with histological examination of internal organs, including lungs, heart and kidneys, will be performed under the supervision of an independent experienced rodent pathologist (Prof Gordon Stamp, Dept of Histopathology at the Royal Marsden Hospital) and a formal report issued. The histopathologist will remain blinded to the treatment received by the mice.
5. Finally, participants are monitored at each peptide administration and the procedure will be stopped immediately if any unexpected side effects are observed.
6. Peptides will be stored at -20°C in a dedicated, designated freezer in a secured laboratory. Day to day access is allowed only to study investigators. A central record is kept of vial use and disposal.

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