The translational use of Kisspeptin and kisspeptin receptor agonists in humans

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Declaration of Originality

I hereby declare that this thesis is a presentation of my original research work and was written by myself. Contributions of others have been acknowledged and referenced. This work was carried out under the guidance of Professor Waljit Dhillo and Dr. Ali Abbara, Imperial College London.

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ABSTRACT

Kisspeptin is a neuropeptide that is known to be a critical regulator of hypothalamic gonadotrophin releasing hormone (GnRH). Physiological studies of kisspeptin have examined the effects of different kisspeptin isoforms (Kisspeptin-10, kisspeptin-54) on GnRH release in men and women. These studies have not only produced new insights into kisspeptin physiology but also paved the way for future studies using kisspeptin as a physiologic, diagnostic and therapeutic tool in individuals with reproductive disorders.

The human kisspeptin peptides, Kisspeptin-10, -13, -14, -54 are named according to their constituent amino acids. Kisspeptin-10, being smaller and easier to synthesize, offers a far more economically viable therapy compared to the longer Kisspeptin-54 peptide. However, due to Kisspeptin-10's shorter half-life, it requires repeated dosing or prolonged infusion. Kisspeptin receptor agonist (MVT-602) is a modified form of kisspeptin-10 with a longer half-life compared to native kisspeptin-54 (1.5-3.5 hrs vs 0.5 hrs). However, effects of MVT-602 in women have never been determined. By investigating the effects of MVT-602 in healthy women and women with reproductive disorders, I uncovered novel understanding of the effects of MVT-602 in women and demonstrated for the first time that the prolonged and sustained rise of gonadotrophins with MVT-602 can be used as a therapeutic tool in women with reproductive disorders.

Having established the effects of MVT-602 in women, I proceeded to investigate the utility of Kisspeptin-54 to interrogate hypothalamic function in men with Congenital Hypogonadotrophic Hypogonadism (CHH). I have shown that a kisspeptin-54 test, unlike the currently used GnRH test, could offer significant clinical benefit for the accurate diagnosis and management of patients with hypogonadism.

In summary, the data conceived from this thesis have important implications for the development of kisspeptin as a potential therapeutic and diagnostic tool for reproductive pathologies.

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LIST OF ABBREVIATIONS

A	Anosmia
ACMG	American College of Medical Genetics
AMH	Anti-Mullërian Hormone
ANOVA	Analysis of Variance
ARC	Arcuate Nucleus
AUC	Area Under the curve
AuROC	Area under the Receiving Operating Characteristic Curve
AVPV	Anteroventral periventricular nucleus of hypothalamus
BMI	Body Mass Index
CDGP	Constitutional Delay in Growth and Puberty
CHH	Congenital Hypogonadotrophic Hypogonadism
DAG	Diacylglycerol
ERα	Oestrogen receptor alpha (α)
ERβ	Oestrogen receptor beta (β)
ERK	Extracellular signal regulated kinases
FISH-1	Fluorescence in situ hybridization
FSH	Follicle Stimulating Hormone
GnIH	Gonadotrophin Inhibitory Hormone
GnRH	Gonadotrophin Releasing Hormone
GnRHR	Gonadotrophin Releasing Hormone Receptor
GPCR	G-protein coupled receptor
GPCR54	G-protein coupled receptor 54
HA	Hypothalamic Amenorrhoea
hCG	Human Chorionic Gonadotrophin

HPG	Hypothalamic-pituitary-gonadal
ICV	Intracerebroventricular
IHB	Inhibin B
IP3	Inositol 1,4,5-triphosphate
IQR	Interquartile range
IR	Immunoreactivity
IV	Intravenous
IVF	*In vitro* fertilisation
KISS1/Kiss1	Kisspeptin gene (see note below)
KISS1R/Kiss1r	Kisspeptin receptor (see note below)
KNDY	Kisspeptin/Neurokinin B/Dynorphin
KP	Kisspeptin
KP10	Kisspeptin-10
KP54	Kisspeptin-54
LepRb	Leptin receptor b
LH	Luteinising Hormone
LP	Likely Pathogenic
M	Microsmia
MAF	Minor Allele Frequency
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
ME	Median Eminence
MUA	Multi-unit Activity
N	Normosmia

n/a	Not applicable
NKA	Neurokinin A
NKB	Neurokinin B
NMDA	N-methyl-D-L-aspartic acid
NPFFR	Neuropeptide FF Receptor
NPY	Neuropeptide Y
NT	Not Tested
OHSS	Ovarian Hyperstimulation Syndrome
PCOS	Polycystic Ovarian Syndrome
pEC50	Negative logarithm of half maximal effective concentration (EC50)
PLC	Phospholipase C
POA	Preoptic area
RIA	Radioimmunoassay
RP3V	Rostral periventricular zone
SEM	Standard Error of Mean
SD	Standard Deviation
SHBG	Sex Hormone Binding Globulin
SC	subcutaneous
SP	Substance P
TAC3	Tachykinin 3 (also known as Neurokinin 3)
TACR3	Tachykinin receptor 3 (also known as Neurokinin receptor 3)
TG	Testosterone Gel
TP	Testosterone Propionate
TU	Testosterone Undecanoate (Nebido)
TV	Testicular Volume

UPSIT-40	University of Pennsylvania 40 Item Smell Test
VUS	Variance of uncertain Significance

Note: this thesis follows the agreed nomenclature for the key molecules in the kisspeptin signalling system: capitalised terms signify in humans (KISS1 and KISSR) and non-capitalised terms denote non-human species (Kiss1 and Kiss1r) (Gottsch, Clifton and Steiner, 2009).

DECLARATION OF CONTRIBUTORS

Declaration of originality: The work described in this thesis is my own. Any collaborations and assistance are described below:

Chapter 2: The study protocol was designed by Dr Ali Abbara and Professor W.S. Dhillo. I recruited and performed the human studies with the assistance of Dr. Sophie Clarke, Dr. Maria Phylactou. Reproductive hormones were analysed by Dr. Maria Phylactou and myself. The peptide product, MVT-602 was provided by Myovant Sciences Ltd.

Chapter 3: The study protocol was designed by Dr. Ali Abbara and Professor W.S. Dhillo. I recruited and obtained consent from all the patients with the assistance of Dr. Maria Phylactou. I performed the studies with the assistance of Dr. Maria Phylactou. The peptide product MVT-602 was provided by Myovant Science Ltd.

Chapter 4: The study protocol was designed by Dr. Ali Abbara and Professor W.S. Dhillo. I recruited and obtained consent from all the patients with the assistance of Dr. Maria Phylactou and Dr. Sophie Clarke. I performed the studies with the assistance of Dr. Maria Phylactou, Dr. Sophie Clarke.

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

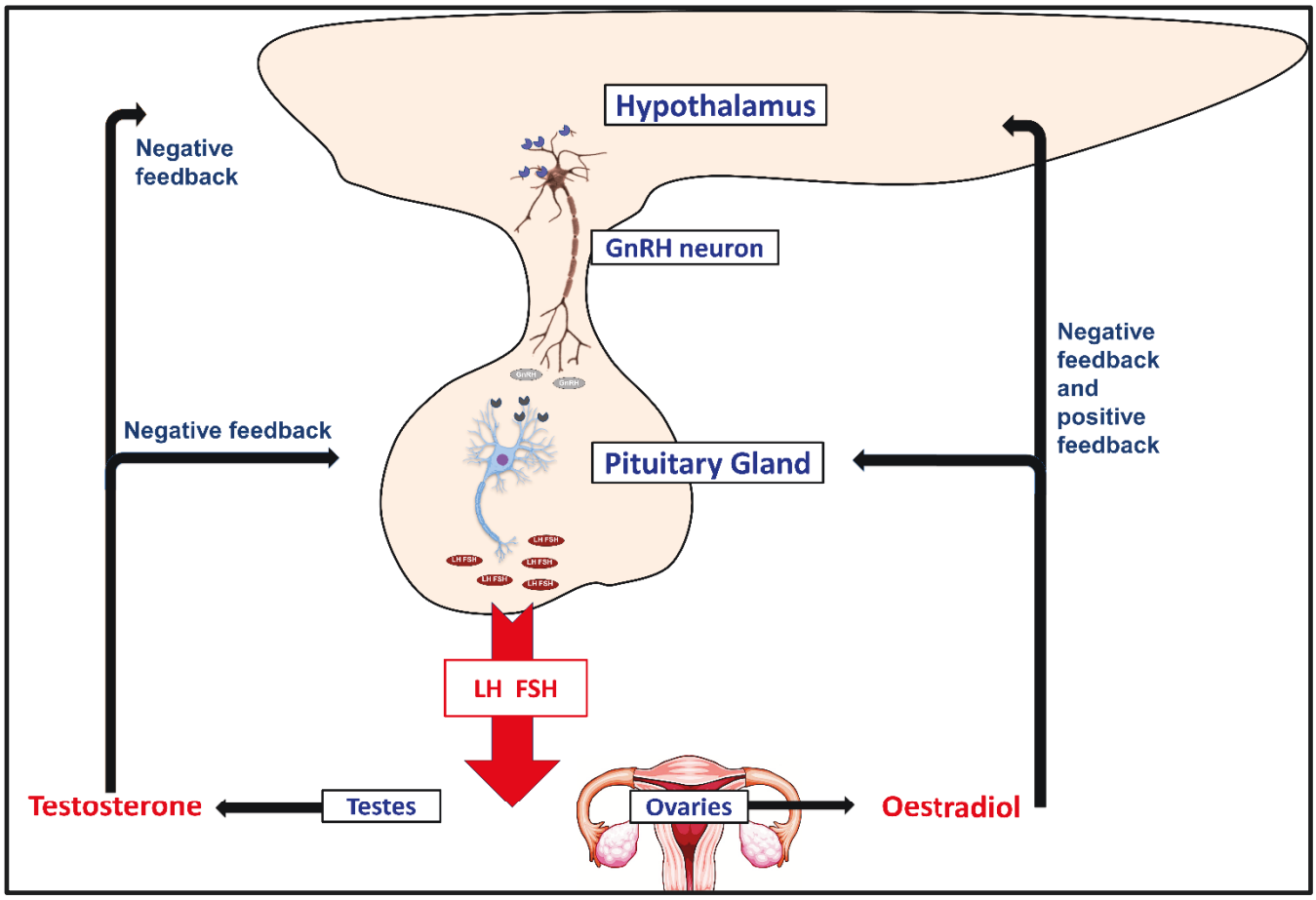
1.1 The hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis consists of a highly organised set of endocrine tissues that function as a coordinated unit in the regulation of fertility. Regulation of the reproductive axis begins at the hypothalamus, where neurosecretory cells at the median eminence (ME) and preoptic area (POA) synthesise and release the decapeptide gonadotrophin-releasing hormone (GnRH) in a pulsatile manner into the hypothalamic-hypophyseal portal circulation (Schally *et al.*, 1971; Bliss *et al.*, 2010; Brown and Roberson, 2017). GnRH reaches the anterior pituitary gland and binds to the GnRH receptor (GnRHR) on pituitary gonadotrophs to trigger synthesis and secretion of gonadotrophins (follicle stimulating hormone (FSH) and luteinising hormone (LH) (Bliss *et al.*, 2010; Stamatiades and Kaiser, 2018). In males, FSH stimulates Sertoli cell maturation and LH binds to LH receptors on Leydig cells to stimulate testosterone synthesis (Plant and Marshall, 2001). In females, FSH promotes growth of ovarian follicles, whereas LH stimulates oestrogen production by granulosa cells (Plant and Marshall, 2001). The gonadal steroids, oestrogen and testosterone, act on the hypothalamus and pituitary gland to inhibit further serum LH release (Conn *et al.*, 1994). However, in females, increasing oestradiol levels trigger a switch from negative to positive feedback at the mid ovarian cycle, resulting in an LH surge for ovulation. Serum gonadotrophin levels decrease after ovulation and the declining levels of gonadotrophins results in formation of the corpus luteum. High levels of progesterone produced by the corpus luteum inhibit further FSH and LH secretion to prevent the growth of a new follicle (Conn *et al.*, 1994; Bulun, 2011).

In summary, the proper functioning of the reproductive system depends on a coordinated network of endocrine, paracrine and autocrine feedback loops between the hypothalamus, pituitary gland and the gonads. Failure at any point in the HPG axis may cause a range of pathologies including impaired puberty and subfertility (Bulun, 2011) (Figure 1).

Figure 1: The hypothalamic-pituitary gonadal (HPG) axis before the identification of kisspeptin

The HPG axis is controlled in a classical endocrine feedback loop. Gonadotrophin releasing hormone (GnRH) is synthesised and secreted by specialised neurons located in the preoptic and arcuate nucleus of the hypothalamus. GnRH neurons have projections to the median eminence (ME), where GnRH is released into the hypophyseal-portal circulation to act on the anterior pituitary gland. This stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH), which in turn act on the gonads to activate gametogenesis and sex steroid production. Sex steroids provides both positive and negative feedback to regulate gonadotrophin release.



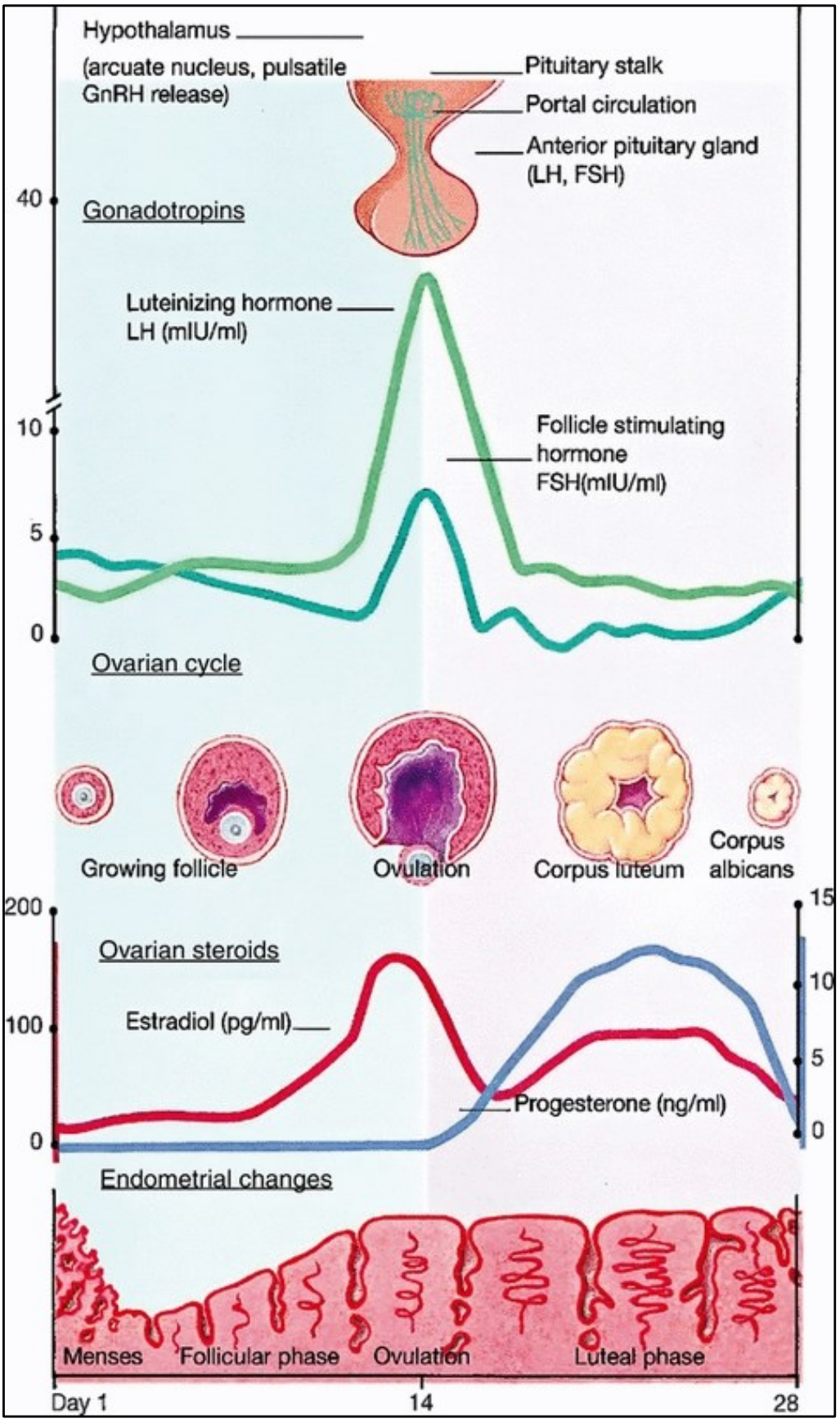
1.2 The female reproductive cycle

The menstrual cycle can be divided into two parts: the follicular phase and the luteal phase (Figure 2) (Bulun, 2011; Bates *et al.*, 2013). The follicular phase begins on the first day of menstruation and generally lasts for 10 to 14 days until ovulation occurs. During the follicular phase, FSH induces proliferation of granulosa cells and expression of LH receptors. Under the influence of FSH, aromatase enzymes are activated, causing the granulosa cells to secrete oestrogen. The rising oestradiol levels increase the sensitivity of granulosa cells to gonadotrophins leading to further rise in serum LH eventually resulting in the mid-cycle LH surge.

Throughout the follicular phase, rising oestrogen levels stimulate growth of the endometrium in preparation for the luteal phase (Pauerstein *et al.*, 1978). Following ovulation, the corpus luteum forms from the remnant of the follicle, and progesterone production becomes predominant. High levels of progesterone inhibit further FSH and LH secretion to prevent the growth of a new follicle. In the absence of fertilisation, the corpus luteum degenerates and the fall in oestrogen and progesterone levels initiates a new menstrual cycle (Pauerstein *et al.*, 1978; Bates *et al.*, 2013).

Other steroidal hormones such as inhibin, activin and follistatin also play a role in the female menstrual cycle (Kitaoka *et al.*, 1988; Besecke *et al.*, 1997; McCartney *et al.*, 2002). Serum FSH from the pituitary gland stimulates the granulosa cells to produce inhibin, which in turn inhibits further FSH secretion to enable selection of a dominant follicle (Luisi *et al.*, 2005). Activin produced in the granulosa cells and pituitary gland stimulates FSH release and sensitises the ovary to FSH actions (Kitaoka *et al.*, 1988). Lastly, follistatin binds to activin and inactivates it to inhibit the effects of FSH (Besecke *et al.*, 1997).

Figure 2: The female reproductive axis (Bates *et al.*, 2013)



1.3 Pulsatile GnRH secretion

Pulsatile GnRH secretion is a well-established feature of the HPG axis. Dierschke *et al.* first observed the varying levels of serum LH in ovariectomized rhesus monkeys (Dierschke *et al.*, 1970). Clarke *et al.* later reported a temporal relationship between GnRH release and LH secretion in ewes (Clarke and Cummins, 1982). The importance of the episodic release of GnRH on LH and FSH secretion was demonstrated in rhesus monkeys, where endogenous pituitary gonadotrophin secretion was abolished by hypothalamic lesions (Belchetz *et al.*, 1978). Intermittent administration of GnRH (at once per hour) successfully reinstated gonadotrophin secretion in these animals, whereas a constant infusion of exogenous GnRH led to decreased gonadotrophin secretion (Belchetz *et al.*, 1978).

The pulse and amplitude of GnRH secretion vary throughout the human life span (Marques *et al.*, 2000). Pulsatile GnRH secretion begins in mid-gestation and is silenced towards the end of gestation. The restraint is removed at birth leading to reactivation of the axis (also called "mini puberty") and gonadotrophin secretion continues until infancy (6 months of age in males and 2 years in females) but soon becomes quiescent in early childhood (Tsutsumi and Webster, 2009). GnRH secretion is reawakened again at the time of puberty onset, initially with nocturnal pulses and then with additional day time pulses (Plant, 2015). This pre-pubertal suppression of the HPG axis is well established in humans and primates, suggesting the presence of GnRH modulators in the post-natal quiescence of the reproductive axis (Abreu and Kaiser, 2016).

In females, GnRH pulsatility varies depending on the menstrual cycle (Tsutsumi *et al.*, 2009; Tony M. Plant, 2015). Early follicular and luteal phases are predominated by low frequency pulses which favour FSH secretion, whereas LH release is stimulated by increased GnRH pulsatility during the late follicular and ovulatory phase (Karsch, 1987; Marshall *et al.*, 1991). Low levels of oestradiol in the early follicular phase exert negative feedback on pulsatile GnRH secretion, however, as oestradiol levels increase towards the end of follicular phase, there is a switch from negative to positive feedback (Marshall *et al.*, 1991; Kirk *et al.*, 1994). Following ovulation, luteinisation of granulosa cells increases progesterone

production which slows the GnRH pulse frequency and amplitude and favours FSH production to stimulate the next round of ovulation (Petersen, Ottem and Carpenter, 2003).

In addition to oestrogen, GnRH secretion is also modulated by a multitude of central and peripheral cues including stress, metabolic state, nutritional status, and photoperiod (Marques *et al.*, 2000). Interestingly, GnRH neurons do not appear to express oestrogen receptor (ERα) and this suggests the existence of an intermediary signal for mediating gonadal steroid feedback. Kisspeptin neurons and the peptide product, kisspeptin have been recognised as a common conduit to transmit essential information to GnRH neurons to regulate the reproductive axis (Yeo *et al.*, 2014; Dubois *et al.*, 2015).

1.4 Source of GnRH Pulsatility

For many years, researchers debated the source of pulsatility in GnRH neurons. Cultures of a clonal GnRH neuronal cell line, GT1 cells, autonomously secrete GnRH episodically in the absence of other cell types (Martínez de la Escalera, Choi and Weiner, 1992; Wetsel *et al.*, 1992). This led to the suggestion of an intrinsic mechanism of pulse generation within the GnRH neuronal network. Moreover, studies investigating the electrical patterns of networked GT1 cells using microelectrodes plated at multiple sites revealed that GnRH neurons exhibited firing frequency at an interval similar to pulsatile GnRH release (inter-pulse interval: 20 to 40 minutes) (Nunemaker *et al.*, 2001). Further work to obtain a pure population of GnRH neurons from monkey foetus came from the Terasawa group (Terasawa *et al.*, 1999). The group found that episodic increases in intracellular calcium levels in cultured GnRH neurons were synchronised to the activity of the GnRH neurons *in vitro* (Terasawa *et al.*, 1999; Funabashi *et al.*, 2000; Duittoz *et al.*, 2004). Overall, these findings concluded that GnRH can be released in fixed intervals from the GnRH neuronal terminals.

Though several studies have supported the intrinsic pulsatile action of the GnRH nerve terminals, the mechanistic process to ensure a coordinated activation of the network of GnRH neurons is not known. It was thought that contact via GnRH cell bodies might allow coordinated neuronal activation to occur

(Witkin *et al.*, 1985). However, experimental observations have shown that it was possible for hypothalamic tissue without GnRH cell bodies to exhibit pulsatile secretion for several hours (Rasmussen, 1993; Purnelle *et al.*, 1997). Furthermore, during the time of the mid-cycle LH surge, when the activity of GnRH neurons was enhanced, basal multiple unit activity (MUA) in the medial basal hypothalamus did not change (O'byrne *et al.*, 1991; Tanaka *et al.*, 1995). This led researchers to propose that GnRH cells may be synchronized by an external group of cells (O'byrne *et al.*, 1991; Tanaka *et al.*, 1995). However, the identity and exact location of these cells were not known.

When MUA was measured in goats using electrodes placed in the posterior arcuate nucleus (ARC), the rhythmic pattern of MUA seemed to coincide with LH pulses (Wakabayashi *et al.*, 2010). The frequency of the MUA was also profoundly decreased in the presence of progesterone, suggesting a likely feedback action of steroids on GnRH pulsatility (Wakabayashi *et al.*, 2010). Conceivably, the ARC kisspeptin neurons may represent the source of GnRH pulse generator.

1.5 Kisspeptin and its peptide products

Kisspeptins refer to a family of neuropeptides encoded by the *KISS1* gene. The *KISS1* gene was originally discovered in 1996 for its metastasis-suppressor activity in human melanoma cells (Lee *et al.*, 1996). However, it was not until 2001 that the peptide products of *KISS1* were identified (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). As the gene was originally discovered in Hershey, Pennsylvania, the gene and the peptide products were globally named after the famous local Hershey 'kisses' chocolate.

The *KISS1* gene is mapped to the long arm of chromosome 1, in the region of 1q32-q41 and consists of a total of four exons, although the first two exons are not translated (West *et al.*, 1998). The peptide products of the *KISS1* gene were first isolated from human placenta and were derived from a precursor 145-amino acid pre-polypeptide. The pre-polypeptide is further processed into shorter peptide fragments: KP54, KP14, KP13 and KP10 (suffix signifying the number of amino acids), with KP54 being the most abundant peptide product in humans (Figure 3) (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). All kisspeptin isoforms share a conserved C-terminal decapeptide sequence, where they harbour an Arg-Phe-NH_2 motif required for biological activity (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). This unique arginine-phenylalanine residue also make them distinctive of the RF-amide family of peptides (Tsutsui *et al.*, 2010).

Figure 3: The amino acid structure of kisspeptins

The kisspeptin hormones all share a common carboxy-terminal decapeptide sequence highlighted in grey. This sequence is necessary for biological activity (Kotani *et al.*, 2001).

Kisspeptin-54 (KP54)

GTSLSPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH2

Kisspeptin-14 (KP14)

DLPNYNWNSFGLRF-NH2

Kisspeptin-13 (KP13)

LPNYNWNSFGLRF-NH2

Kisspeptin-10 (KP10)

YNWNSFGLRF-NH2

1.6 The Kisspeptin Receptor and its characteristics

Coincident with the characterisation of the *KISS1* peptides, the gene coding for the human kisspeptin receptor (*KISS1R*) was identified. It was initially termed as the G-protein coupled receptor 54 (GpR54) and was first cloned from a rat in 1999 as an orphan receptor (Lee *et al.*, 1999). Subsequently, the human orthologue of GpR54 was cloned and named as AXOR12 or hOT7T175 (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001).

The KISS1R (or GpR54 receptor) belongs to the rhodopsin-gamma family of the G-protein coupled receptor and is coupled to the class A Gαq/11 subunit. Though it shares significant structural homology to the galanin receptor (40%), it lacks galanin binding affinity *in vitro* (Muir *et al.*, 2001). Upon ligand-receptor binding, major intracellular signalling pathways are stimulated including hydrolysis of phospholipase C (PLC) and formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Kotani *et al.*, 2001). This results in an increase in calcium mobilisation, arachidonic acid release and protein kinase (e.g. p38 MAP kinases and ERK1/2) phosphorylation, which further stimulates opening of potassium and non-selective cation channels and facilitates kisspeptin activity (Kotani *et al.*, 2001). More recent studies have shown that continuous exposure of the KISS1R to kisspeptin elicits a biphasic increase in intracellular calcium signaling, with an acute first-phase response lasting 5mins, followed by a more sustained second-phase response lasting more than 30minutes (Min *et al.*, 2013). The slower second phase is maintained by dynamic internalization and recycling of the kisspeptin receptor to prevent receptor desensitisation (Min *et al.*, 2013).

1.7 Kisspeptin expression and distribution

1.7.1 Kisspeptin expression in the brain

Several studies have examined the distribution of *KISS1* expression in rodents and humans. The standard nomenclature is Kiss1 in non-human species and KISS1 in humans. If both KISS1 and Kiss1 are referred in a single sentence, then just KISS1 will be used for simplicity.

The distribution of kisspeptin in the hypothalamus varies between species and gender. Using the techniques of *in situ* hybridization and immunohistochemistry in rodents, researchers were able to map kisspeptin neurons to two discrete regions: the anterior region of hypothalamus called the anteroventral periventricular nucleus (AVPV) and the posterior region called the arcuate nucleus (ARC) (Smith *et al.*, 2005; 2007). In addition to these areas, *Kiss1* mRNA is also present in small populations in the areas governing reproductive behaviour such as the ventromedial hypothalamus and paraventricular nucleus (Clarkson *et al.*, 2009). The expression of *Kiss1* mRNA in the rodent AVPV exhibits clear sexual dimorphism, with females having a much larger population of Kiss1 positive neurons than males, a phenomenon that might have important physiological implications, which will be described further in subsequent sections (Kauffman *et al.*, 2007; Clarkson *et al.*, 2009).

In mammals such as sheep and primates, kisspeptin neurons are found to reside within the infundibular nucleus and preoptic area (POA) (equivalent to the rodent ARC). An equivalent anatomical area to rodent AVPV kisspeptin neurons has not yet been identified in humans, but kisspeptin neurons mediating positive feedback by sex steroids have been identified in the rostral periventricular zone (RP3V) in female monkeys (Rometo *et al.*, 2007). This population of kisspeptin neurons is also not identified in men or agonadal male monkeys suggesting a physiological role for these neurons to generate the positive feedback during ovulation (Ramaswamy *et al.*, 2008; Hrabovszky *et al.*, 2010). Unlike the AVPV kisspeptin neurons in rodents, the ARC kisspeptin neurons do not display sexually dimorphic differences in neuron density or distribution (Clarkson *et al.*, 2006; Kauffman *et al.*, 2007; Clarkson *et al.*, 2009). However, in sheep, the ARC is sexually dimorphic with a larger number of neurons observed in ram compared to ewes (Smith *et al.*, 2008).

Outside the hypothalamus, scattered populations of kisspeptin cells and fibres were also reported. Clarkson *et al.* used a more specific rat polyclonal antibody that targets the ten amino acid C-terminal of murine kisspeptin and identified kisspeptin neurons within the dorsomedial nucleus, medial amygdala, the bed nucleus of striae terminalis and around the locus coeruleus in the mouse brain (Clarkson *et al.*,

2009). The presence of kisspeptin neurons in these areas suggest a possible potential involvement of kisspeptin in functions beyond reproduction.

1.7.2 Peripheral expression of kisspeptin

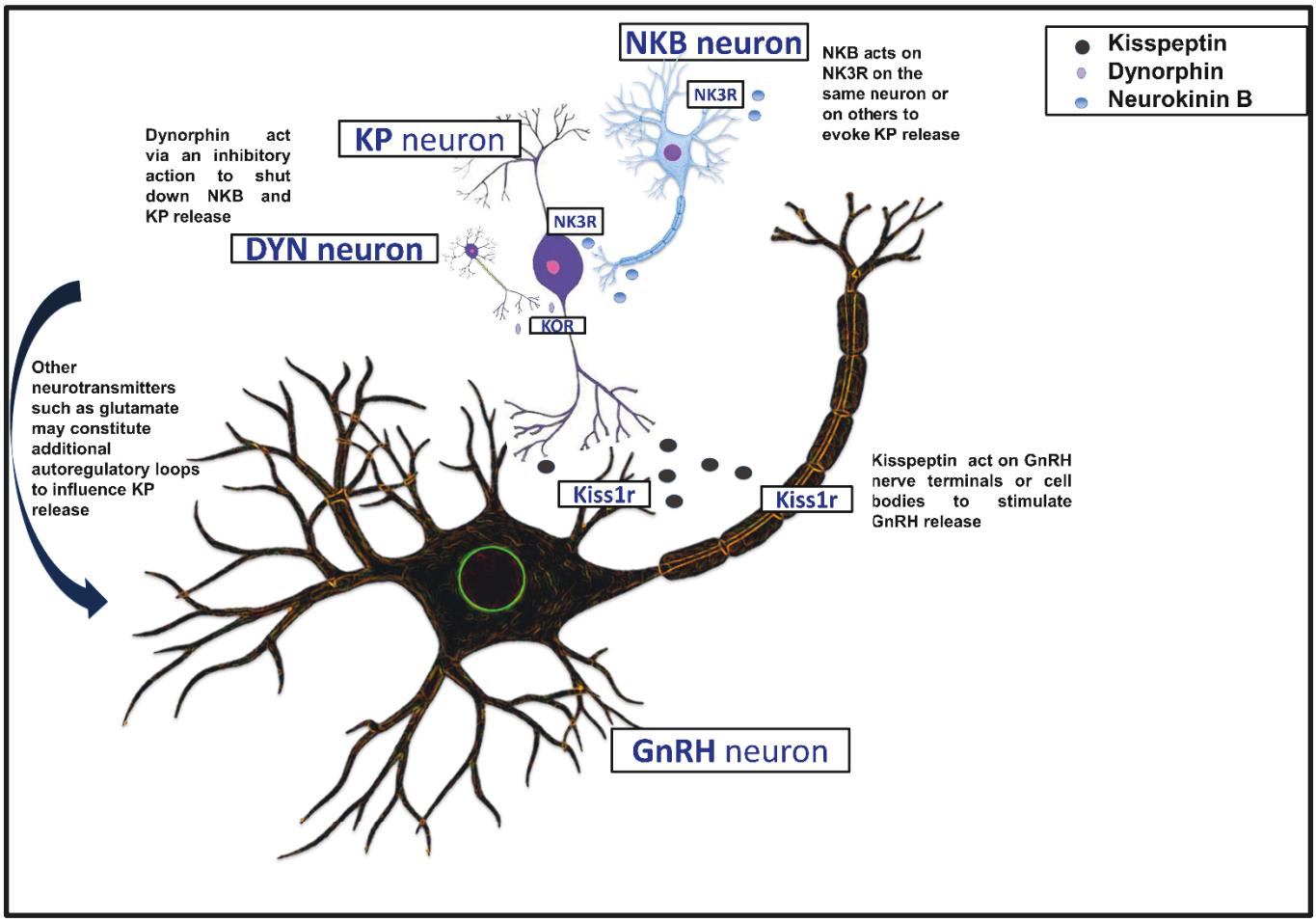
In the periphery, kisspeptin expression has also been reported in the theca layers of growing follicles, corpora lutea, testis, pancreas, liver, small intestine and carotid bodies (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Terao *et al.*, 2004; Cejudo Roman *et al.*, 2012; Owens *et al.*, 2018). The highest expression of *KISS1* was found in the syncytiotrophoblast cells of the placenta. Human maternal kisspeptin levels increase significantly throughout pregnancy and can reach to up to 7000-fold greater than in non-pregnant woman in the third trimester. The physiological role of high levels of kisspeptin in pregnancy is presently unknown but many have suggested a possible involvement in embryonic implantation and progression of uterine decidualisation (Reynolds *et al.*, 2009; Jayasena *et al.*, 2015).

1.8 The KNDY neurons as GnRH pulse generator

Shortly after identifying kisspeptin as a pivotal player of GnRH secretion, another neuropeptide, Neurokinin B (NKB) has found itself in the spotlight. Neurokinin B (NKB) is a hypothalamic neuropeptide that shares a common C-terminal amino acid motif (Phe-X-Gly-Leu-Met-NH_2, X can be either NKA, NKB or an aromatic substance P residue) as the tachykinin family of peptides. NKB and kisspeptin along with another peptide called dynorphin (DYN) were first observed to be co-localised in hypothalamic ARC neurons in sheep (also known as the KNDY neurons) (Goodman *et al.*, 2007). Following that, other researchers reported similar populations of KNDY peptides in the ARC of the rat, mouse, sheep, goat and in human infundibular nucleus (equivalent to the ARC in rodents) (Navarro *et al.*, 2009; Rance, 2009; Hrabovszky *et al.*, 2010; Y. Wakabayashi *et al.*, 2010; Skrapits *et al.*, 2014). This led to the hypothesis that arcuate neurons expressing KP, NKB and DYN form a synchronized network to drive episodic GnRH/LH release. Complementary to this proposal, optogenetic studies in mice demonstrated that selective activation of KP neurons in the ARC exhibit synchronised activity that correlated with pulsatile LH secretion (Han *et al.*, 2015), whereas inhibition of ARC kisspeptin neurons

suppress pulsatile LH (Clarkson *et al.*, 2017). In ewes, infusion of NKB or DYN receptor antagonists suppress or enhance pulsatile LH, respectively, lending credence to the notion that KNDY neurons may serve as a central node governing GnRH secretion (Goodman *et al.*, 2013). In humans, patients bearing inactivating mutations in the genes encoding NKB (TAC3) or its receptor, NK3R (TACR3) present with hypogonadotrophic hypogonadism (Topaloglu *et al.*, 2009; Young *et al.*, 2010) which is similar to the phenotype in patients with defects in kisspeptin signaling.

Figure 4: Schematic representation of a KNDY neuron depicting the possible autoregulatory loops and interactions with the GnRH neuron. As shown in the figure below, NKB acts on the receptors to evoke kisspeptin release. Kisspeptin neurons act on GnRH nerve terminals or cell bodies to stimulate GnRH release. Dynorphin acts via an inhibitory action to suppress NKB and KP release. KP: Kisspeptin, NKB: Neurokinin B; DYN: Dynorphin, GnRH: Gonadotrophin releasing hormone; NK3R: Neurokinin 3 Receptor, KOR: κ-opioid receptor; Kiss1r: Kisspeptin 1 receptor



1.9 The role of Kisspeptin in cancer biology

The first uncovered role of kisspeptin was its anti-metastatic effect in melanoma and breast cancer cell lines (Lee *et al.*, 1996; 1997). Subsequently, increasing evidence was presented on its suppressive role in other types of malignancy such as urogenital cancer, ovarian cancer and renal cancer (Ohtaki *et al.*, 2001). However, in some types of cancer such as pancreatic and endometrial cancers, both positive and negative associations with tumour progression have been reported (Papaoiconomou *et al.*, 2011). These discrepancies are not surprising as the relationship between peptide molecules and microenvironment and steroid receptor status of cancer cells is incredibly complex. Furthermore, discrepancies could be related to the different experimental procedures or different reagents/antibodies used (Papaoiconomou *et al.*, 2011). At present, the controversial role of kisspeptin in cancer biology remains to be elucidated (Harms *et al.*, 2003; Tena-Sempere, 2006).

1.10 Kisspeptin's role in reproductive endocrinology

The pivotal role of kisspeptin in the regulation of reproductive hormone secretion was recognised in 2003 when two landmark studies reported an absence of kisspeptin signaling via its G protein coupled receptor resulted in hypogonadotrophic hypogonadism (HH) (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Subsequently, various transgenic mouse models of kisspeptin receptor inactivation were generated and these mice displayed a complete phenocopy of patients with impaired kisspeptin receptor: namely low serum gonadotrophins, underdeveloped gonads and inability to progress through puberty (Funes *et al.*, 2003; Seminara *et al.*, 2003; Lapatto *et al.*, 2007). Notably, administration of GnRH restores gonadotrophin release in *Kiss1r* knock out mice, suggesting disruption of kisspeptin signaling does not impair GnRH neuronal migration, synthesis or pituitary gonadotrophin response (Seminara *et al.*, 2003). Conversely, activating mutations in *KISS1/KISS1R* caused extended kisspeptin receptor activation leading to central precocious (early) puberty (Teles *et al.*, 2008).

1.11 Acute administration of kisspeptin has a potent effect on gonadotrophin release

The above studies set the basis for an indispensable role of kisspeptin in the neuroendocrine control of reproduction. Further experiments were conducted in laboratory animals to determine the gonadotrophin-releasing effects of kisspeptin. Indeed, intracerebral doses as lows as 1fmol evoked significant LH response in the rat (Gottsch *et al.*, 2004; Matsui *et al.*, 2004). FSH appeared to be 100-fold less sensitive to the stimulatory effect of kisspeptin than LH (Gottsch *et al.*, 2004). These powerful gonadotrophin stimulatory effects were observed following peripheral (intravenous, intraperitoneal or subcutaneous) administration of KP10 or KP54 (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Matsui *et al.*, 2004; Navarro, Castellano, *et al.*, 2004; Messager *et al.*, 2005; Shahab *et al.*, 2005). KP10 exerts a potent depolarizing effect on GnRH neurons and can stimulate GnRH release from nerve terminals *in situ* without the need for GnRH cell bodies (Han *et al.*, 2005; d'Anglemont de Tassigny *et al.*, 2008). KP54, on the other hand, has been shown to cross the blood brain barrier (BBB) and increase c-fos activity (marker of cellular activation) in GnRH cell bodies resulting in a sustained LH release (De Tassigny *et al.*, 2017). Administration of GnRH antagonists abolished the stimulatory effect of KP10 on gonadotrophin release (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Shahab *et al.*, 2005). Collectively, these data indicate that kisspeptin exerts its reproductive effects directly and specifically on GnRH neurons, either via the terminals or through effects on GnRH neuronal cell bodies.

1.12 Kisspeptin effects on the pituitary

Kisspeptin receptors are also expressed in the pituitary, therefore it remains possible that kisspeptins could act directly on pituitary gonadotrophs (Kotani *et al.*, 2001; Muir *et al.*, 2001). However, *in vitro* studies on rat pituitary explants have not consistently demonstrated a robust GnRH response. Navarro *et al*. incubated rat pituitary cultures in KP10 (doses of 1nM to 10000nM) and reported a dose-dependent increase in LH after 60 and 180 minutes of incubation (Navarro *et al.*, 2005). However, the relative contribution of the direct pituitary effects is likely to be minor as *in vitro* doses of kisspeptin required to generate LH release are much higher in comparison to *in vivo* kisspeptin doses (Navarro *et al.*, 2005).

Other researchers found no changes in gonadotrophin release when rat pituitary cells were incubated with KP10 (doses 10nM to 1000nM) (Thomson *et al.*, 2004) or KP54 (Matsui *et al.*, 2004). This might be explained by the different experimental settings used (cell culture versus static incubation and the developmental stages of the pituitary explants (adult versus pubertal). Whilst kisspeptin receptors are present on the pituitary gland, the available data suggests that the pituitary is not the site of action for kisspeptin *in vivo*.

1.13 Chronic kisspeptin administration is associated with receptor desensitisation

The HPG axis is exquisitely sensitive to the pattern of sensitisation and continuous exposure of the GnRH receptor to kisspeptin paradoxically suppresses the HPG axis and reduces LH release. Seminara and colleagues reported that a continuous intravenous infusion of KP10 (100ug/hour) in agonadal juvenile rhesus monkeys led to an initial acute flare of LH followed by a reduction of LH to levels similar to controls at 12 hrs (Seminara *et al.*, 2006). Administration of single boluses of kisspeptin, N-methyl-D-L-aspartic acid (NMDA) and GnRH in the last 3 hrs of the infusion showed that the latter two were still able to elicit LH pulses, suggesting that tachyphylaxis occurred at the level of kisspeptin receptor and not at the pituitary (Seminara *et al.*, 2006). The authors carried out the same protocol in gonad intact rhesus monkeys and infusion of KP10 resulted in a peak LH at 2 to 3 hrs followed by a decline in serum LH by 24 hrs (Ramaswamy *et al.*, 2007). The difference in time to desensitisation is likely to be a consequence of the higher dose of kisspeptin infusion (400μg/day) used in the latter study and a possible feedback effect contributed by the higher plasma testosterone concentrations in the gonadal intact monkeys.

Receptor desensitisation is a characteristic shared by many G-protein coupled receptors (GPCR) (Kaiser, Conn and Chin, 1997). Sustained stimulation of GPCR typically causes phosphorylation of the receptor within carboxyl terminal tails, which promotes high affinity binding of the arrestin family of proteins. This binding sterically hinders further interaction between GPCR and G-protein and simultaneously sequesters the desensitised GPCR to clathrin-mediated vesicles for endocytosis (Luttrell and Lefkowitz,

2002). The desensitised GPCR is degraded, rather than being continuously recycled to the cell surface and this contributes to the termination of the signalling process. The kisspeptin receptor, being part of the GPCR family, shares similar desensitisation mechanisms. This process of desensitisation also involves suppression of downstream signalling pathways such as the uncoupling of IP3 or the MAPK cascades or a failure to maintain a sustained influx of extracellular calcium (Bianco *et al.*, 2011; Babwah AV *et al.*, 2012). The net outcome is the failure for the kisspeptin cell to effectively maintain dynamic trafficking and recycling of a pool of signalling competent kisspeptin receptor to the cell surface (Min *et al.*, 2013). In summary, chronic kisspeptin administration causes desensitisation of the reproductive axis and the time taken from stimulation to suppression is dependent on the animal model, metabolic status, developmental stage, as well as mode of administration, isoform, dose and duration of kisspeptin treatment.

1.14 Kisspeptin's role in puberty

As described earlier, pulsatile GnRH secretion begins at birth and continues until infancy but subsequently becomes quiescent until the onset of puberty (Tsutsumi and Webster, 2009). At puberty, the "brake" that holds back GnRH secretion is released resulting in reactivation of the HPG axis.

Interestingly, hypothalamic expression of *Kiss1* mRNA is elevated around the time of puberty in male and female rats (Navarro *et al.*, 2004). Increases in hypothalamic *KISS1* mRNA levels are also observed in association with puberty in female and agonadal male rhesus monkeys (Shahab *et al.*, 2005). In the case of the male monkey, the pubertal increase in *KISS1* mRNA seems to occur in the absence of testicular steroids. Though the female monkeys used in the study were ovarian intact, increased levels of oestradiol were not expected at this stage of development. Therefore, it is possible that the developmental increase in kisspeptin levels occurs independently of gonadal steroids (Shahab *et al.*, 2005). In addition to the increase in kisspeptin peptides, increase in expression of *Kiss1r* mRNA in the GnRH neurons is also reported in non-primates. Herbison *et al.* reported a 30% increase in GnRH

neurons expressing *Kiss1* mRNA in mice developing from postnatal day 5 to postnatal day 20 (Herbison *et al.*, 2010).

Taking the findings together, it could be proposed that increased kisspeptin signaling at KISS1R occurs at the time of puberty and reduced kisspeptin tone contributes to hypogonadotrophic state during childhood. Indeed, repeated administration of kisspeptin to prepubertal rats advances vaginal opening by ~4 days (Navarro *et al.*, 2004). Likewise, kisspeptin administration to agonadal prepubertal male monkeys primed with GnRH is able to elicit a sustained GnRH-dependent LH response similar to that in an adult (Shahab *et al.*, 2005; Plant *et al.*, 2006). Conversely, blockade of kisspeptin by an antagonist inhibited pulsatile GnRH release in pubertal monkeys and delayed puberty in rats (Shahab *et al.*, 2005). Overall, these studies support the assertion that kisspeptin-signalling is requisite for puberty.

1.15 Kisspeptin mediates positive and negative feedback of sex steroids.

Sex steroids exerts both negative and positive feedback on GnRH secretion, which is mediated via oestrogen receptor alpha (ERα). However, GnRH neurons lack ERα and it has been suggested that oestrogen binds to ERα on kisspeptin neurons in the ARC or AVPV to modulate GnRH release.

Several lines of evidence have supported this hypothesis:

(1) Gonadectomy significantly increases kisspeptin cell numbers and mRNA expression in the ARC in female rodents but downregulates kisspeptin expression in the anteroventral periventricular nucleus (AVPV) (Navarro, Castellano, *et al.*, 2004). Interestingly, in response to oestradiol, this configuration is reversed with a notable oestradiol stimulation of AVPV kisspeptin neurons and inhibition of ARC kisspeptin neurons. This suggests a differential regulation of kisspeptin in different regions of the forebrain following oestradiol replacement (Smith, Dungan, *et al.*, 2005).

(2) Kisspeptin neurons exhibit a female dominant sexually dimorphic distribution, particularly in the rodent AVPV (Smith, Cunningham, *et al.*, 2005), with females having drastically more kisspeptin neurons than males. *Kiss1* mRNA and c-fos expression are further increased in the

AVPV at the time of ovulation and this may be the driving force in the pre-ovulatory LH surge (Smith, Popa, *et al.*, 2006).

(3) Functional studies in female rats confirm that the LH surge is induced by kisspeptin and cannot occur after administration of kisspeptin antagonists or in Kiss1R/Kiss1 knock out mice (Clarkson *et al.*, 2009; Roseweir *et al.*, 2009).

(4) Mice with kisspeptin cell-specific ERα ablation fail to mount an LH response to an oestradiol challenge and exhibit much reduced kisspeptin expression in the AVPV compared to the wild type. By contrast, kisspeptin expression in the ARC is not significantly affected in the ERα knock out mice and responsiveness to the negative effects of oestradiol is maintained (Dubois *et al.*, 2015).

Overall, the evidence suggests that ERα - expressing kisspeptin neurons in the AVPV are required for positive feedback regulation of LH secretion, whereas negative feedback actions via ER signalling in the ARC is not a complete requirement and other pathways independent of kisspeptin cells might have been implicated.

1.16 Kisspeptin effect is mediated by metabolic status

Reproductive processes are sensitive to the magnitude of energy reserves of an organism. Conditions of metabolic distress associated with energy insufficiency such as in hypothalamic amenorrhoea (HA) are linked to impaired fertility (Welt *et al.*, 2004; Gordon *et al.*, 2017a; Thurston *et al.*, 2019). Conversely, excess metabolic states such as morbid obesity are associated with precocious puberty in females (Castellano *et al.*, 2011) and hypogonadism in males (Tajar *et al.*, 2010). Clearly, metabolic status has an impact on the attainment of reproductive capability, but the underlying regulatory mechanisms are complex and may involve multiple regulatory pathways. Kisspeptin has recently emerged as one of the major conduits in relaying information regarding body metabolic status to GnRH neurons (Tena-Sempere, 2007; Sanchez-Garrido *et al.*, 2013).

Among the numerous neuroendocrine modulators, the adipocyte hormone, leptin, has been recognised as an essential modulator linking body fat stores to the reproductive system (Fernandez-Fernandez *et al.*, 2006). Leptin is secreted by white adipose tissue in proportion to the amount of energy stores. In many species, negative energy balance results in decreased levels of circulating leptin (Smith *et al.*, 2006; True *et al.*, 2011). Humans with low levels of leptin due to defects in leptin signaling/leptin receptor fail to progress through puberty (Farooqi *et al.*, 1999; 2014). Furthermore, women with hypothalamic amenorrhoea (HA) have significantly lower levels of circulating leptin when compared with healthy female subjects (Welt *et al.*, 2004).

GnRH neurons do not physiologically express leptin receptors (LepRb) (Quennell *et al.*, 2009). Additionally, electrophysiological recording from hypothalamic slices reveals no direct action of leptin on GnRH neurons (Qiu *et al.*, 2011). Therefore, the effects of leptin, seems to be conducted indirectly via inter-neuronal pathways. About ~40% of the kisspeptin neurons in the ARC express leptin receptors (Smith *et al.*, 2006). Thus, it is not surprising to hypothesise that leptin is able to lower GnRH release via its effect on the hypothalamic kisspeptin system. Experimental studies demonstrating the effect of leptin on kisspeptin are summarised as follows:

(1) Rodent models of leptin deficiency (e.g. Ob/Ob mice) display lower *Kiss1* mRNA expression in the ARC and fewer kisspeptin neurons in the RP3V (Smith *et al.*, 2006; Quennell *et al.*, 2011). Leptin administration in these rodent models of leptin deficiency results in an increase expression of hypothalamic *Kiss1* gene (Smith *et al.*, 2006).

(2) Leptin was shown to depolarize 82% of the ARC *Kiss1* neurons in the guinea pig via activation of transient receptor potential cation (TRPC) channels (Qiu *et al.*, 2011). These findings support the hypothesis that kisspeptin neurons are direct targets in mediating leptin action on the HPG axis.

Other than the direct effect of leptin on kisspeptin, the hypothalamic kisspeptin system is also reported to be highly sensitive to changes in energy states. Experimental data highlighting the sensitivity of kisspeptin to metabolic state are summarized as follows:

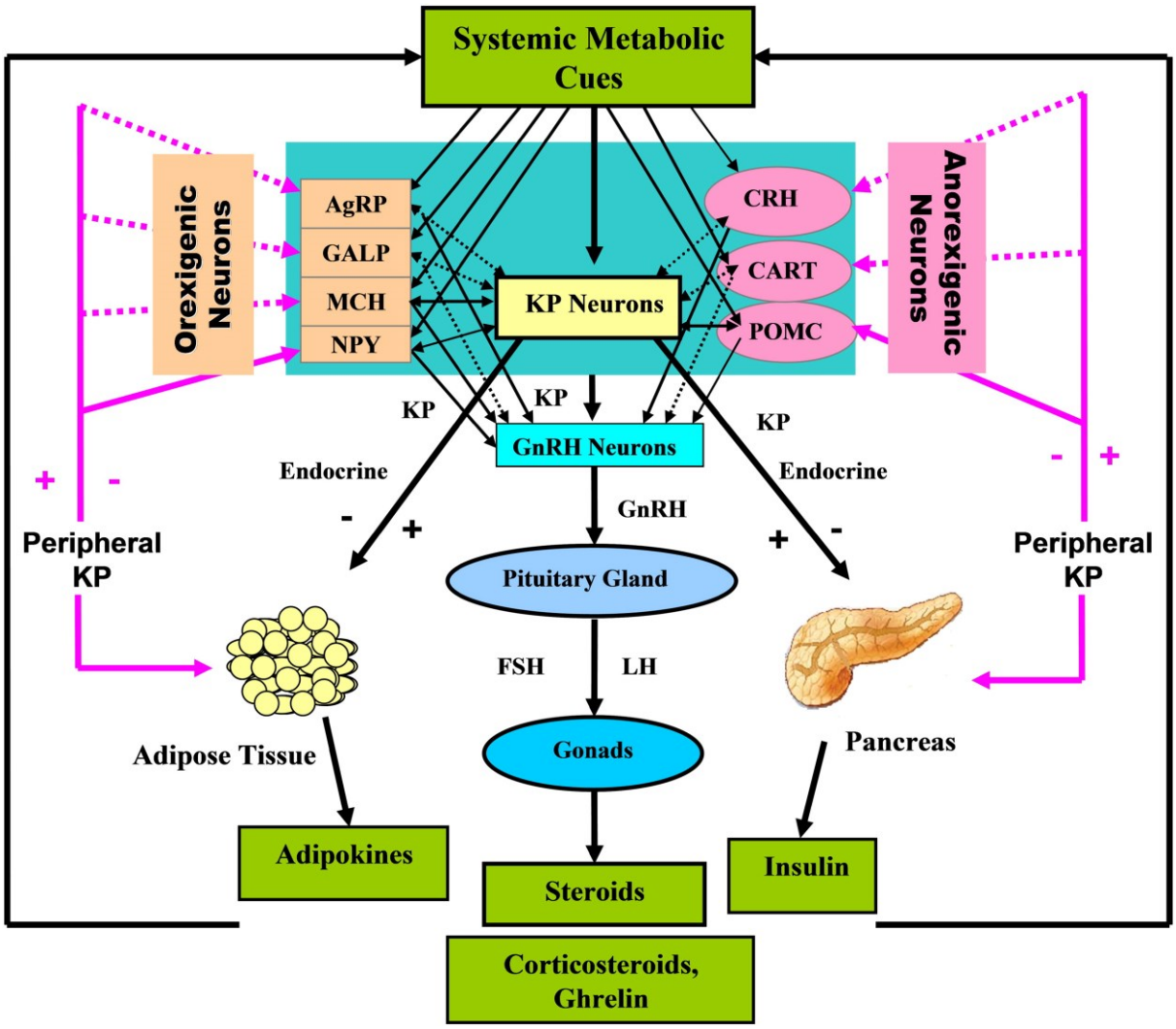
(1) Studies in pubertal male and female rats subjected to 72 hours (hrs) of fasting showed a decrease in hypothalamic expression of *Kiss1* mRNA (Castellano *et al.*, 2005). A similar response was also observed in adult male and female rats at 12 hrs after food restriction (Luque *et al.*, 2007; Brown *et al.*, 2008) and in female rats subjected to prolonged state of negative energy balance in lactation (True *et al.*, 2011). Notably, suppression of *Kiss1* mRNA levels were also observed in the ARC in rodent models subjected to negative energy balance such as in streptozotocin - induced diabetic rats (Castellano *et al.*, 2006) or in diet-induced obese DBA/2J female mice (Quennell *et al.*, 2011).

(2) Repeated central injections of kisspeptin in models of negative energy balance can ameliorate reproductive failure. For example, administration of KP10 to immature female rats subjected to 30% of chronic sub-nutrition resulted in potent gonadotrophin and oestrogenic responses (Castellano *et al.*, 2005). Moreover, administration of KP10 to hypothalamic fragments taken from fasted pubertal rats induced a greater gonadotrophin response when compared to those from *ad libitum* fed rats, suggesting a greater sensitivity to kisspeptin in states of energy insufficiency (Castellano *et al.*, 2005). The duration of LH responses to KP10 appeared to be augmented in female rats subjected to chronic sub-nutrition, suggesting a state of hyper-responsiveness in negative energy balance (Roa and Tena-Sempere, 2007), likely related to a compensatory increase in kisspeptin receptors.

Therefore, based on current available evidence, kisspeptin neurons can sense extreme metabolic information and transmit information to GnRH neurons. The central role of kisspeptin in relaying metabolic information to the GnRH system make them attractive drug targets for treatment of reproductive problems associated with metabolic disorders.

Figure 5 Possible ways by which metabolic information is relayed to the kisspeptinergic neurons. Adapted from (Wahab *et al.*., 2013).

A schematic representation of the possible ways by which the hypothalamic anorexigenic, orexigenic, and kisspeptin neurons interact with each other, systemic metabolic cues, and reproductive axis through the GnRH neuronal network. Broad arrows show interactions for which evidences are available while dotted arrows show hypothetical potential links. This figure above is also showing endocrine actions of KP on adipose tissue, hypothalamic neuronal systems, and pancreas which will not be discussed in the thesis.



1.17 Human studies of Kisspeptin

Since its discovery, kisspeptin has been acknowledged as the most potent secretagogue for GnRH release. The potent effect of kisspeptin on GnRH release, along with its strategic central positioning within the HPG axis offers an alternative novel way of modulating the reproductive axis. Therefore, it seems logical to explore the physiological effects of kisspeptin in humans.

1.17.1 Kisspeptin in Healthy men and Women

In 2005, Dhillo *et al.* conducted the first-in-human study of kisspeptin and observed a dose-dependent increase in gonadotrophin secretion following a 90-minutes infusion of KP54 at 4 pmol/kg/min in healthy men. As observed in the animal studies, serum FSH was also increased (by about 18%), but the effects were less marked compared to serum LH rise. The increase in gonadotrophins contributed to a 13% increase in serum testosterone at 180 minutes (Dhillo *et al.*, 2005). Other than KP54, intravenous bolus administration of KP10 (at a dose range of 0.01-3ug/kg) also induced acute LH responses with the highest LH rises observed after 1 μg/kg (0.77 nmol/kg) (George *et al.*, 2011). In the same study, a persistent rise of serum LH was also seen after a continuous intravenous infusion of KP10 at 4 μg/kg/hr (George *et al.*, 2011).

Whilst kisspeptin reliably stimulates gonadotrophin release in men, the effects of kisspeptin in women varies depending on the phase of their menstrual cycle (Dhillo *et al.*, 2007). Chan *et al*. observed that healthy women were least sensitive to an intravenous bolus of KP10 during the follicular phase of the menstrual cycle (Chan *et al.*, 2012). Our group also reported similar findings in which healthy women demonstrated the lowest inducible response to KP54 in the follicular phase, but a greater LH-rise was observed during the pre-ovulatory phase when oestradiol levels were highest (Dhillo *et al.*, 2007a; Jayasena *et al.*, 2013). These findings suggested that oestradiol might enhance the gonadotrophin response to exogenous kisspeptin. The findings were consistent with *in vitro* studies that showed an augmented GnRH secretion to kisspeptin in GnRH neuronal cell lines in the presence of oestradiol (Tonsfeldt *et al.*, 2011).

1.17.2 Kisspeptin resets GnRH pulse generator in healthy men and women

Pulsatile release of GnRH is crucial for normal reproductive function and abnormalities in GnRH pulsatility can cause reproductive disorders. A single subcutaneous bolus of kisspeptin was shown to increase LH pulsatility in healthy women during the follicular phase (Jayasena *et al.*, 2013). In men, an intravenous bolus of KP10 could increase the LH amplitude and reset the GnRH pulse generator by inducing an intermediate LH pulse (Chan *et al.*, 2011). In another study, a 22.5-hours continuous infusion of KP10 triggered large LH pulses and increased LH pulse frequency (George *et al.*, 2011).

In summary, the above studies validated the data from animal studies and confirm the extraordinary potency of kisspeptin in stimulating gonadotrophin release in healthy men and women. Moreover, a new understanding of kisspeptin physiology is unravelled such as the potential to reset the GnRH pulse generator in men and the variable responses to kisspeptin administration in different menstrual phases in women. More importantly, these studies have reported no adverse events, no changes in blood pressure or other vital cardiovascular parameters in humans (Nijher *et al.*, 2010).

1.17.3 Therapeutic use of kisspeptin in disorders of reproduction

Kisspeptin administration stimulates an endogenous pool of GnRH, which in turn stimulates physiological levels of gonadotrophin secretion. Therefore, kisspeptin stimulates the reproductive axis in a more physiological and self-limited manner when compared to direct pituitary or gonadal stimulation with GnRH receptor agonists or gonadotrophin preparations. Furthermore, the excellent safety profile of kisspeptin in human studies offers novel potential for using kisspeptin as a tool in the treatment of reproductive studies. Notably, stimulation or suppression of the gonadotrophic axis, by use of kisspeptin or their receptor agonists, have been explored as a viable treatment option in a number of conditions such as hypogonadism, anovulation, infertility and specific cancers (e.g. prostate cancer) (Matsui and Asami, 2014).

11.7.3.1 Kisspeptin use in In Vitro Fertilisation (IVF)

Kisspeptin signalling is requisite for physiological ovulation. In female rats, infusion of a kisspeptin neutralising antibody in the preoptic area abolishes the midcycle LH surge (Adachi *et al.*, 2007). Furthermore, kisspeptin administration to super-ovulated rats induces ovulation to the same extent as human chorionic gonadotrophin (hCG) (Matsui *et al.*, 2004). As described in the section above, data in healthy women showed that kisspeptin dramatically increased gonadotrophin secretion in the pre-ovulatory phase of the menstrual cycle (Dhillo *et al.* 2007; Jayasena *et al.*, 2011; Chan, 2013). This augmented rise in serum LH suggested the potential for kisspeptin to be considered as an agent to induce ovulation.

Indeed, in 2004, our group conducted a "proof of concept" study demonstrating that a single subcutaneous bolus of KP54 (1.6-12.8nmol/kg) induced an LH-surge with a peak of ~40iU/L lasting 12-14 hrs (Jayasena *et al.*, 2014). The magnitude and duration of LH-rise was sufficient to mature oocytes in 51 of the 53 (96.2%) subfertile women during *in vitro* fertilisation (IVF) treatment (Jayasena *et al.*, 2014). Furthermore, kisspeptin matured oocytes without causing the dangerous complication OHSS, even in women at increased risk of OHSS (Abbara *et al.*, 2015). Ovarian hyperstimulation syndrome (OHSS) is a life-threatening condition of IVF treatment and is predominantly a consequence of excessive stimulation of ovaries caused by hCG resulting in release of vascular endothelial growth factor (VEGF) causing increased vascular permeability and serosal fluid accumulation. More recently, *in vitro* studies have shown that kisspeptin could have a direct effect in reducing oestradiol-induced VEGF production and is less likely to induce OHSS, as compared to hCG (Zhai *et al.*, 2017). This complements data obtained from clinical studies suggesting that extending the duration of the LH surge with a second dose of kisspeptin, further improved oocyte maturation compared to one dose, but still did not increase the occurrence of OHSS (Abbara *et al.*, 2017). In addition, Owens and colleagues observed that KP54 augmented gene expression for steroidogenesis pathways (e.g. Lh/hCG and FSH receptors, oestrogen receptors and aromatase enzyme) in granulosa lutein cells when compared to those cells triggered by

traditional ovulation maturation agents (Owens *et al.*, 2018). The increased expression of receptors and steroidogenic enzymes potentially creates an ovarian environment favourable for progesterone synthesis and luteal phase implantation (Owens *et al.*, 2018).

1.17.3.2 Kisspeptin in Hypothalamic amenorrhoea (HA)

Functional hypothalamic amenorrhoea (HA) is a condition characterised by a functional failure of secretion of GnRH owing to a physiological insult such as low body weight, excessive exercise, or psychological stress (Gordon *et al.*, 2017b). Previous work from our laboratory has shown that women with HA were far more responsive to exogenous kisspeptin than healthy females in the follicular phase of the menstrual cycle. This was thought to be secondary to increased kisspeptin receptor expression as shown by a rodent model of hypogonadotrophic hypogonadism caused by undernutrition (Castellano *et al.*, 2005; Jayasena *et al.*, 2009). Thus, HA could be considered as a state of hypothalamic kisspeptin deficiency and restoring GnRH secretion by using kisspeptin would offer a novel way of treating this disease. Our group had previously trialed a twice daily KP54 injection (6.4nmol/kg) for two weeks in women with HA (Jayasena *et al.*, 2010). Whilst robust increases in gonadotrophin levels were observed on day 1 of administration, these were reduced within 7 days. However, when a twice weekly kisspeptin protocol was used, gonadotrophin stimulation was restored but the levels were not sufficient to increase oestradiol secretion to restore ovulation (Jayasena *et al.*, 2010). Tachyphylaxis in response to kisspeptin has been observed most frequently following high dose and frequent/continuous administration (Abbara *et al.*, 2013), thus fine-tuning the dose and frequency of administration is important in this cohort of amenorrhoeic patient who were extremely sensitive to kisspeptin. Therefore, it would be of interest to determine if a longer acting kisspeptin receptor agonist or a continuous infusion of low dose kisspeptin, could restore physiological hormonal secretion without tachyphylaxis. In the subsequent chapters, I have shown that a single subcutaneous injection of a longer acting kisspeptin receptor agonist, MVT-602, significantly increases serum LH and serum FSH in women with HA and could have huge translational potential in the treatment of this disorder (Chapter 3).

1.17.3.3 Kisspeptin in Hypogonadotrophic Hypgonadism secondary to Type 2 Diabetes Mellitus

Up to half of the men with type 2 diabetes exhibit a subnormal testosterone level with an inappropriately low gonadotrophin concentration (Dandona and Dhindsa, 2011). As the gonadotrophin response to GnRH in these men was often observed to be normal, it had been hypothesised that the neuroendocrine mechanisms contributing to diabetes-related hypogonadism occurred at the level of, or upstream of kisspeptin neurons (George *et al.*, 2011). In a proof-of-concept study involving 5 men with T2DM (mean age of 34 years) and biochemical hypogonadism, a continuous intravenous infusion of KP10 at 4µg/kg/hr over 11 hours stimulated serum LH, increased LH pulse frequency and testosterone secretion (George *et al.*, 2011). In another study, responses to kisspeptin in healthy older men were also maintained (Abbara *et al.*, 2018). This suggests a preserved GnRH neuronal population in age-related hypogonadism or in diagnosis related to secondary hypogonadism. Thus, kisspeptin based therapeutics could be developed for the treatment of age, obesity or diabetes related hypogonadism and could potentially restore testosterone level to a more physiological range as opposed to exogenous testosterone therapy (Swerdloff *et al.*, 2000).

1.17.3.4 Kisspeptin in Hypogonadotrophic Hypogonadism due to TAC3 or TACR3 Mutations

Neurokinin B (NKB) is a hypothalamic neuropeptide that shares a common C-terminal amino acid motif (Phe-X-Gly-Leu-Met-NH_2) with the tachykinin family. Several lines of evidence had established that NKB, kisspeptin and dynorphin co-localise in the ARC in sheep, rats, mice, goats and in the infundibular nucleus in humans and monkeys (Navarro *et al.*, 2009; Rance, 2009; Y. Wakabayashi *et al.*, 2010; Skrapits *et al.*, 2014). Patients bearing inactivating mutations in the genes encoding NKB (TAC3) and its receptor, NK3R (TACR3) had low GnRH pulse frequency, low serum LH but relatively preserved FSH levels. These patients displayed a phenotype of hypogonadotrophic hypogonadism (Topaloglu *et al.*, 2009; Young *et al.*, 2010), similar to that observed in individuals with inactivating mutations of kisspeptin signaling. Pulsatile GnRH administration reverses the gonadotrophin deficiency observed in patients with TAC3 or TAC3R mutations (Young *et al.*, 2010). A continuous kisspeptin infusion

(1.5µg/kg/hour) also restored LH pulse frequency and mean LH concentrations in patients with TAC3 or TAC3R mutation, indicating that NKB pathways regulate GnRH secretion, but its action is likely to be upstream of kisspeptin signaling (Young *et al.*, 2013).

1.17.3.5 NKB antagonists in menopausal symptoms

In humans, Jayasena *et al.* demonstrated that NKB administration to healthy men and women at doses of up to 5.12 nmol/kg/hour did not alter reproductive hormone secretion or LH pulsatility (Jayasena *et al.*, 2014). However, some of the participants in the study reported symptoms of flushing and feeling hot at doses of NKB up to 10.2nmol/kg/hour (Jayasena *et al.*, 2014). This was investigated further in ten healthy women who received a 30 minute infusion of either NKB or vehicle in random cross over design (Jayasena *et al.*, 2015). Interestingly, NKB was found to induce flushing, a characteristic feature of menopausal symptoms during NKB administration. Following that, clinical trials conducted to investigate the potential of NK3R antagonists as a novel therapy to improve hot flush symptoms showed a significant reduction of severity of flushes by 41% to 45% (Prague *et al.*, 2017).

1.17.3.6 NKB antagonists in Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is a condition characterised by anovulation, hyperandrogenism and polycystic ovary syndrome (Teede *et al.*, 2014). Women with PCOS usually display increased GnRH pulsatility along with increased serum LH levels. Kisspeptin and NKB are hypothalamic neuropeptides that regulate the pattern and frequency of GnRH release. Therefore, kisspeptin-NKB signaling pathways could be targeted to treat women with PCOS. Indeed, George and colleagues investigated the use of pharmacological blockade of NKB action to reduce GnRH pulsatility in women with PCOS (George *et al.*, 2016). Women in the study were randomised to receive an oral NK3R antagonist (at dose of 20, 40 or 80 mg/day) or placebo for 28 days. The study showed a 52% reduction in area under the curve (AUC) of LH levels and reduction in LH pulsatility by 3.6 pulses per 8 hrs ($P < 0.05$) after 7 days of treatment with 80mg/day of NK3R antagonist. Total testosterone levels were also reduced by 28.7% in the

treatment group (George *et al.*, 2016). Further longer-term studies are indicated to evaluate the efficacy of this novel approach in women with PCOS.

Summary: The translational use of Kisspeptin and kisspeptin receptor agonists

Since the discovery of the kisspeptin gene almost two decades ago, an impressive amount of data in kisspeptin research have been collected. To date, the role of kisspeptin as a hypothalamic regulator of reproductive function is well-established. This pivotal discovery makes kisspeptin an appealing target to modulate GnRH release. However, clinical applications of kisspeptin-based therapies still face several challenges. First, there is a need to specify the form of kisspeptin peptide that could be reliably used in clinical practice. Secondly, the appropriate dose, frequency and route of administration of kisspeptin to overcome the risk of tachyphylaxis and yet achieve a stimulatory gonadotrophin response optimal for treatment of reproductive disorders needs to be fine-tuned. Thirdly, the effects of kisspeptin in certain metabolic states such as in PCOS or in obesity hypogonadism needs to be clarified. Human studies usually generate greater impact as they can truly identify the physiological hormonal profile in humans and reveal any safety issues regarding kisspeptin administration. Hence, the overall goal of my thesis is to delve into the application of kisspeptin/kisspeptin receptor agonists in humans and explore the potential utility of these various forms of kisspeptin peptides in clinical practice.

A) **To investigate the effects of the kisspeptin receptor agonist, MVT-602, in healthy women**

MVT-602 (previously known as TAK448) is a kisspeptin receptor agonist that has a longer half-life than native KP54 (1.5-3.5hr vs 0.5hrs) (MacLean *et al.*, 2014). Several modifications were introduced to the KP10 peptide to create MVT-602. Previous studies have shown that a single dose of MVT-602 resulted in elevated and sustained LH-rise with peak levels observed between 8-12hrs before returning to baseline by 72hrs post dose (MacLean *et al.*, 2014). This sustained elevation of LH (~24 to 48 hrs) greatly mimics the physiological LH surge and would have great potential in promoting oocyte maturation in women who are infertile. However, MVT-602 has never been studied in healthy women. Therefore, in this

chapter of my PhD, I explored the endocrine profile of MVT-602 in the follicular phase of healthy women and following oestrogen supplementation.

HYPOTHESIS:

MVT-602 will promote a more potent effect on gonadotrophin release resulting in an extended physiological serum LH increase when compared to that of KP54 during the early follicular phase of menstrual cycle in healthy women. The greater increase in duration of LH surge would make MVT-602 a far more useful trigger agent in IVF therapy.

B) To investigate the effects of the kisspeptin receptor agonist, MVT-602 in women with hypothalamic amenorrhoea and polycystic ovarian syndrome.

Subfertility affects up to 10% of couples worldwide (Farquhar *et al.*, 2019; Thurston *et al.*, 2019). The two most common female causes of subfertility are polycystic ovarian syndrome (PCOS) and hypothalamic amenorrhoea (HA) (National Institute for Health and Care Excellence, 2013; Thurston *et al.*, 2019). Our group has previously investigated the hormonal profile following KP54 administration in women with HA (Dhillo *et al.*, 2005; Jayasena *et al.*, 2010; Jayasena *et al.*, 2014) and demonstrated persistent stimulation of serum gonadotrophins levels with twice weekly kisspeptin administration. MVT-602 is a potent kisspeptin receptor agonist with improved *in vivo* stability (Asami *et al.*, 2013; Matsui and Asami, 2014). The effects of MVT-602 in women with subfertility have never been explored. In this study, I investigated the endocrine profile of a single subcutaneous dose of MVT-602 and a single dose of KP54 9.6nmol/kg in women with HA and PCOS and compared their response to the hormonal profile in healthy women in the follicular phase.

HYPOTHESIS:

The prolonged duration of gonadotrophin stimulation by MVT-602 in healthy women could be used as a potential therapy in women with anovulatory disorders (HA or PCOS).

C) To investigate the use of kisspeptin as a novel clinical test in the diagnosis of hypogonadotrophic hypogonadism

Hypogonadotrophic hypogonadism (HH) is characterised by hypogonadism due to congenital or acquired diseases that affect the hypothalamus and or the pituitary gland (Boehm *et al.*, 2015). Patients with HH present with low sex steroid levels and low/inappropriately normal gonadotrophin levels. A subset of patients with HH are those with Congenital Hypogonadotrophic Hypogonadism (CHH). Typically, patients with CHH have mutations in genes involved in GnRH neuron migration or in the maturation of GnRH neuronal network and do not progress through puberty without medical assistance (Seminara *et al.*, 1998; Boehm *et al.*, 2015). Whilst a specific test of pituitary function e.g. gonadotrophin releasing hormone (GnRH test) is widely used to identify patients with CHH clinically, no diagnostic test of hypothalamic function is currently available (Boehm *et al.*, 2015). Kisspeptin is a hypothalamic neuropeptide that directly stimulates GnRH release. Thus, it would be of great interest to determine the potential utility of kisspeptin-54 as a specific test of hypothalamic function in patients with CHH. In this final chapter, I compare the performance of a KP54 test to that of a GnRH test in interrogating the hypothalamic GnRH neuronal integrity in patients with CHH.

HYPOTHESIS:

Healthy men with a functional HPG axis would exhibit a normal response following KP54 and GnRH stimulation tests. On the contrary, patients with genetic mutations leading to reduced or absent GnRH neurons would exhibit a reduced or attenuated response to KP54 but a normal response to GnRH test indicating abnormal hypothalamic GnRH function. I hypothesise that an intravenous bolus of KP54 can more reliably differentiate men with CHH from healthy men than the current GnRH test.

CHAPTER 2

THE EFFECT OF KISSPEPTIN RECEPTOR AGONIST (MVT-602) ON GONADOTROPHIN SECRETION IN THE FOLLICULAR PHASE OF HEALTHY WOMEN

CHAPTER 2: THE EFFECT OF KISSPEPTIN RECEPTOR AGONIST (MVT-602) ON GONADOTROPHIN SECRETION IN THE FOLLICULAR PHASE OF HEALTHY WOMEN

2.1 INTRODUCTION

2.1.1 Development of kisspeptin receptor agonists

KP10 and KP54 were the two peptides commonly used in a vast majority of animal and human studies. However, limiting the utility of kisspeptin peptides are their susceptibility to proteolytic degradation in blood circulation. KP10 has a short half-life of 4 minutes due to its greater susceptibility to enzymatic cleavage (Dhillo *et al.*, 2005; Jayasena *et al.*, 2015a). In contrast, KP54, being a longer peptide, has a longer half-life (27.6 ±1.1 min), but are more expensive to produce. Moreover, tachyphylaxis has been reported with more frequent and continuous administration (Abbara *et al.*, 2013). Therefore, investigating the pharmacological profile of a longer acting kisspeptin receptor agonist would be of great interest in the development of kisspeptin-based therapeutics.

Kisspeptin receptor agonists have been developed through modifications of human KP10, as this is the smallest fragment needed to bind and activate the receptor (Orsini *et al.*, 2007; Matsui and Asami, 2014; Nishizawa *et al.*, 2016) (**Table 1** and **Figure 1**). A peptide product arising from these modifications is a nano-peptide kisspeptin receptor agonist called MVT-602 (previously known as TAK448) which has a half-life that is longer than native KP54 (1.5-3.5 hrs vs 0.5 hrs) (MacLean *et al.*, 2014). MVT-602 was initially created to downregulate the reproductive axis, with the aim of inducing chemical castration in the treatment of prostate cancer (MacLean *et al.*, 2014). Indeed, the preclinical data were convincing and demonstrated that the subcutaneous injection of MVT-602 stimulates gonadotrophin release, whereas continuous infusion over several days results in a sustained reduction of hormone levels (Matsui *et al.*, 2012). In healthy males, a single dose of MVT-602 caused an elevated and sustained LH-rise with peak levels observed between 8-12 hrs before returning to baseline by 72 hrs post dose (MacLean *et al.*, 2014). This LH profile greatly mimics the normal physiology of LH surge (~24 to 48 hrs) in healthy ovulatory women, suggesting that kisspeptin receptor agonists, like MVT-602, could have huge

translational potential for the treatment of female reproductive disorders. However, the effects of MVT-602 have not been previously investigated in women. Therefore, in this chapter, I explored the endocrine profile of MVT-602 in the early follicular phase (day 1 to 4) in healthy women. As the response to kisspeptin can be altered by sex-steroid milieu; I also investigated the impact of oestrogen supplementation on the endocrine response to MVT-602 in healthy women to mimic the oestrogenic state leading to pre-ovulatory LH surge.

Table 1: Amino acid sequence of KP10 and MVT-602

Peptides	Amino acid sequence											
	Position (N-terminal)	1	2	3	4	5	6	7	8	9	10	C-terminal
KP10		Tyr-	Asn-	Trp-	Asn-	Ser-	Phe-	Gly-	Leu-	Arg-	Phe-	NH_2
TAK-448 (MVT-602)	Ac-	D-Tyr		Hyp-	Asn-	Ser-	Phe-	Aza Gly-	Leu-	Arg (Me)-	Phe-	NH_2

KP10: Tyr, tyrosine; Asn, asparagine; Gly, Glycine; Ser, serine; Leu, Leucine; Thr, threonine; Trp, tryptophan; Arg, Arginine.

TAK-448: Ac, acetylated group (CH_3CO-); Ala, alanine; Arg(Me), methyl-arginine; Asn, asparagine; AzaGly, aza-glycine; D-Tyr, D-tyrosine; Gly, glycine; Hyp, hydroxyproline; Ser, serine; Leu, Leucine; Thr, threonine; Trp, tryptophan

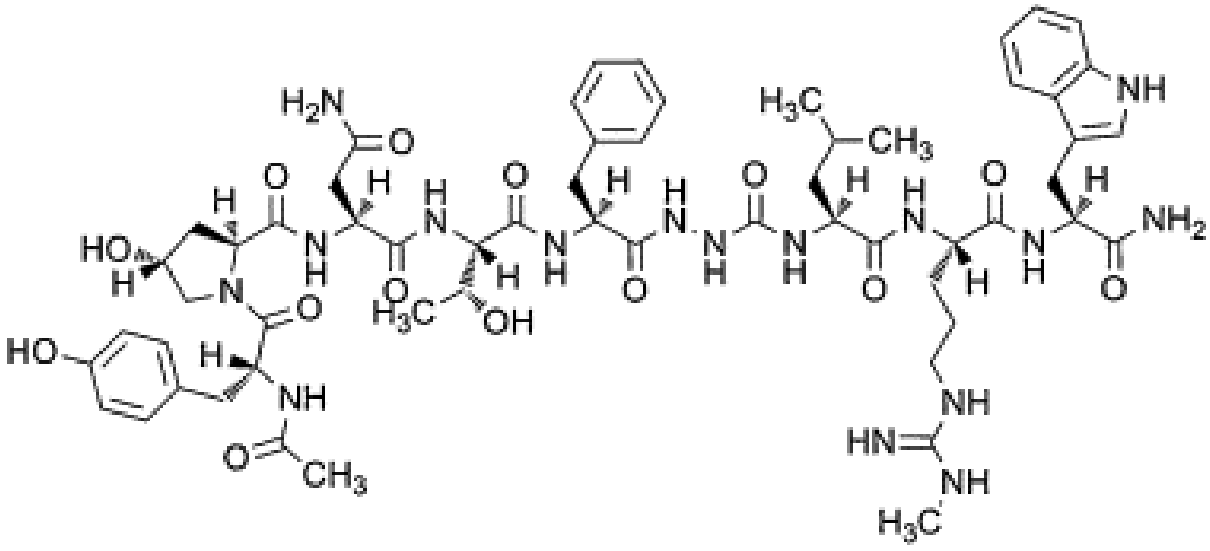


Figure 6: Chemical structure of MVT-602 (Kuze *et al.*, 2013)

2.2 AIMS AND HYPOTHESIS

2.2.1 Aims

(1) To investigate the effect of MVT-602 on gonadotrophin secretion in the early follicular phase of healthy women and to compare the gonadotrophin response following administration of a single dose of MVT-602 to native KP54 (9.6nmol/kg). This dose of KP54 was chosen as it had been previously shown to induce a robust gonadotrophin rise and safely induce oocyte maturation during IVF treatment (Jayasena *et al.*, 2014).

(2) To investigate the impact of oestrogen supplementation on the gonadotrophin secretion following MVT-602 in the early follicular phase of the healthy women given that the response to kisspeptin is greatest in the pre-ovulatory phase (Jayasena *et al.*, 2011).

2.2.2 Hypothesis

(1) A more extended duration of LH secretion would be induced with MVT-602 in comparison to that of KP54 in women in the early follicular phase of the menstrual cycle.

(2) The LH and FSH secretion in response to MVT-602 in the presence of oestrogen would be augmented.

2.3 MATERIALS AND METHOD

2.3.1 Ethical approval

The study was conducted with approval from the West London Research Ethics committee, London, UK (reference: 12/LO/0507) in accordance with the Declaration of Helsinki. Recruitment was via advertisement sin the local press. Full written consent was obtained from all participants.

2.3.2 Subjects

Healthy ovulatory women aged 18-35 years, with a menstrual cycle length <35 days, BMI 18-30kg/m^2, not taking any medications or hormonal contraception were included in the study. All women underwent a detailed evaluation of their medical history, menstrual history and clinical examination. The following blood tests were assessed during the screening visit: full blood count; renal profile; liver profile; bone profile; random glucose; thyroid profile; LH, FSH, oestradiol, progesterone, anti-Müllerian hormone (AMH), testosterone, prolactin and sex hormone binding globulin (SHBG).

2.3.3 Study protocol

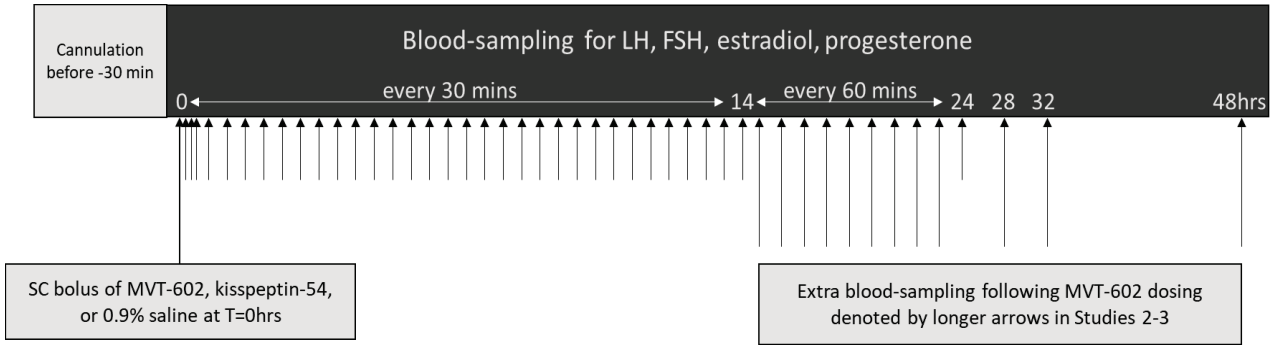
All study visits were carried out at the Clinical Research Unit, Imperial College Healthcare NHS Trust and commenced in the morning (~8am). Pregnancy was excluded using a urine pregnancy test at the beginning of each study visit. Blood pressure and pulse were recorded throughout each study visit. The occurrence of symptoms was recorded following each intervention and during the following week. Participants were free to eat and drink as normal before and during each study visit. The study was single blinded with participants being unaware of the intervention that was administered at each study visit **(Figure 7).**

2.3.3.1 Study 1: Preliminary dose-finding study for MVT-602 in healthy women.

As MVT-602 has not been previously administered to women, I carried out a preliminary broad dose-finding study to determine the hormonal profile of a subcutaneous bolus of MVT-602 in the early follicular phase (menstrual cycles days 1-4) in healthy ovulatory women (n=3). Women received a

subcutaneous bolus of MVT-602 at the following doses: MVT-602 at 0.003nmol/kg, 0.03nmol/kg, 0.1nmol/kg, 0.3nmol/kg and 1.0nmol/kg during the early follicular phase of successive menstrual cycles. Each woman received one study intervention each month in random order. The doses were selected based on a previous study conducted in healthy men (MacLean *et al.*, 2014). Following a single bolus of MVT-602, serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30, 0, 5, 15 minutes post injection and then every 30 minutes until 14 hrs and additionally at 24 hrs and 48 hrs. MVT-602 levels were also measured at 0, 5, 15, 30, 60, 120, 150, 240 and 360 minutes post injection.

Figure 7: Protocol diagram of healthy females recruited for MVT-602 study



2.3.3.2 Study 2: Comparison of MVT-602 with native KP54 in healthy women

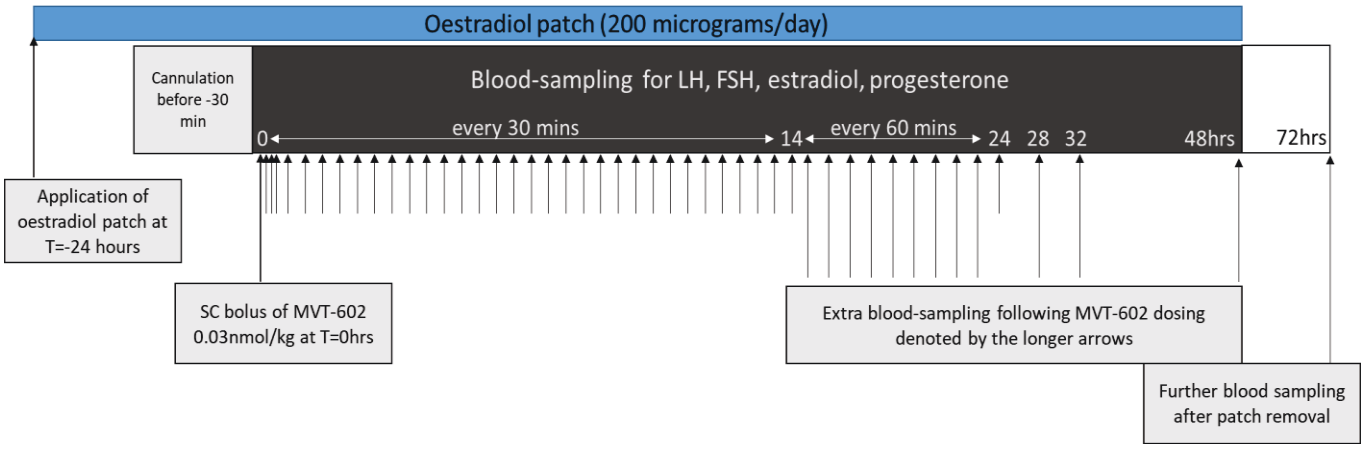
Following the analysis of study 1, two doses of MVT-602 (0.01nmol/kg and 0.03nmol/kg) were selected for comparison with native KP54 (9.6nmol/kg) and 0.9% saline (control) in the early follicular phase (Day 1 to day 4) in a further 9 healthy women. The dose of 9.6nmol/kg of KP54 was selected as this was previously shown to induce a robust gonadotrophin response and to safely induce oocyte maturation during IVF treatment (Abbara *et al.*, 2015). Each woman received one of the following interventions in each month (MVT-602 0.01 nmol/kg, MVT-602 0.03 nmol/kg, KP54 9.6nmol/kg and 0.9% saline) in the early follicular phase of the menstrual cycle in random order. Serum reproductive hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 minutes for the first 30 minutes, and then every 30 minutes until 10 hrs, and additionally at 24 hrs following each intervention (KP54, 0.9%

saline). Based on the LH-profile observed following MVT-602 observed in study 1, the duration of 30 minutely blood-sampling for measurement of reproductive hormones was extended to 24 hrs, and additionally at 28 hrs, 32 hrs and 48 hrs for the two MVT-602 arms (0.01 and 0.03nmol/kg) (see **Figure 7**: Additional blood sampling denoted by longer arrows).

2.3.3.3 Study 3: The effect of oestrogen supplementation on the response to kisspeptin receptor agonist MVT-602

To investigate the effect of sex-steroid milieu on the response to MVT-602, five healthy women had a repeat of their study visit with MVT-602 during the follicular phase of their menstrual period, but with additional estrogen supplementation. A transdermal oestradiol patch (200µg/day), was administered for 72 hrs commencing from 24 hrs prior to the start of each study visit. Thereafter, a single subcutaneous bolus of MVT-602 0.03nmol/kg was administered on the day of the study visit. Study visits were as described in study 2, but with an additional blood test before the application of the oestradiol patch and an additional blood test after removal of the patch at 72 hrs **(Figure 8)**.

Figure 8 Protocol diagram of healthy females (n=5) given MVT-602 with oestrogen supplementation.



2.3.4 Hormone Assays

Blood samples for serum LH, FSH, oestradiol and progesterone measurement were collected in simple Vacutainer tubes (Becton Dickinson, Franklin Labs, New Jersey). Samples were allowed to clot prior to centrifugation for 10 minutes at 1210 g and then separated. Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Becton Dickinson, Franklin Labs, New Jersey) containing 5000 Kallikrein inhibitor units of aprotinin (0.2 ml Trysalol; Bayer, Newbury, UK) and were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 4 minutes at 2150 g and then separated and immediately frozen at -20ºC. Blood for MVT-602 levels was collected in chilled K2EDTA tubes (Becton Dickinson, Franklin Labs, NJ) and was spun immediately for 10 minutes at 194 g at a temperature of 4ºC and stored at -20ºC until analysis. Serum LH, FSH, oestradiol and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Interassay coefficients of variation were as follows: LH, 2.7%; FSH, 3.0%; oestradiol, 3.0%; progesterone, 2.9%. Limits of detection for each assay were as follows: oestradiol, 37pmol/liter; FSH, 0.05iU/L; LH 0.05iU/L; progesterone, 0.318nmol/L. Reference ranges were as follows: LH in iU/L, 2-10 (follicular), 20-60 (mid cycle), 4-14 (luteal); FSH in iU/L, 1.5-8 (follicular and luteal), 10-50 (mid cycle) and oestradiol in pmol/L, less than 300 (early follicular), 400-1500 (mid cycle), 200 -1000 (luteal). MVT-602 levels were measured using liquid chromatography with tandem mass spectrometry by PPD laboratories in Virginia, USA. Plasma kisspeptin immunoreactivity (IR) was measured using an established RIA. The antibody cross-reacted 100% with human KP54, kisspeptin-14, and KP10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF.

2.3.5 Statistical methods

Analyses were conducted using Graphpad Prism version 7.0. Parametrically distributed data are presented as mean$\pm$SD, whereas non-parametric data are presented as median$\pm$IQR. Statistical

comparison across intervention groups were performed using one-way ANOVA with post hoc Tukey's multiple comparison test or Kruskal-Wallis test with post hoc Dunn's test, as appropriate. Categorical data were compared using or logistic regression. A p-value <0.05 was considered to be statistically significant. Area under curve analysis (AUC) was used to assess quantitative size of effect.

2.4 RESULTS

Following screening, three women were recruited into Study 1 (preliminary dose-finding study) and an additional nine women were recruited into Study 2. The baseline characteristics of all the healthy women recruited in Study 1 and Study 2 (total n=12) are summarised in Table 2 below. Hormonal levels were taken in the follicular phase of the menstrual cycle.

Table 2 Baseline characteristics of the healthy women (n=12) recruited into the study.

Clinical Characteristics	Healthy women (n =12)
Age (years)	27±5.1
Weight (Kg)	64.4±12.4
Body Mass Index (Kg/m^2)	23.8±2.8
Menstrual cycle length (Days)	28.1±1.2
Serum LH (iU/L)	3.8±1.4
Serum FSH (iU/L)	5.1±1.7
Serum Oestradiol (pmol/L)	102.7±38.4
Sex Hormone Binding Globulin (nmol/L)	76.1±38.1
Serum AMH (pmol/L)	24.6±15.4
Serum FSH-LH (iU/L)	1.3±1.9

Mean±SD are presented for parametrically distributed data. Hormonal values presented were baseline values of the first study visit. Reference ranges for serum LH for healthy women were 2.0–10.0iU/L (Follicular phase), serum FSH 1.5-8.0iU/L (Follicular phase) SHBG, serum oestradiol (Early follicular phase: 25–75pg/mL, late follicular phase: 200-600pg/mL)

2.4.1 Study 1: Preliminary dose-finding study for MVT-602 in healthy women.

2.4.1.1 Change in reproductive hormones

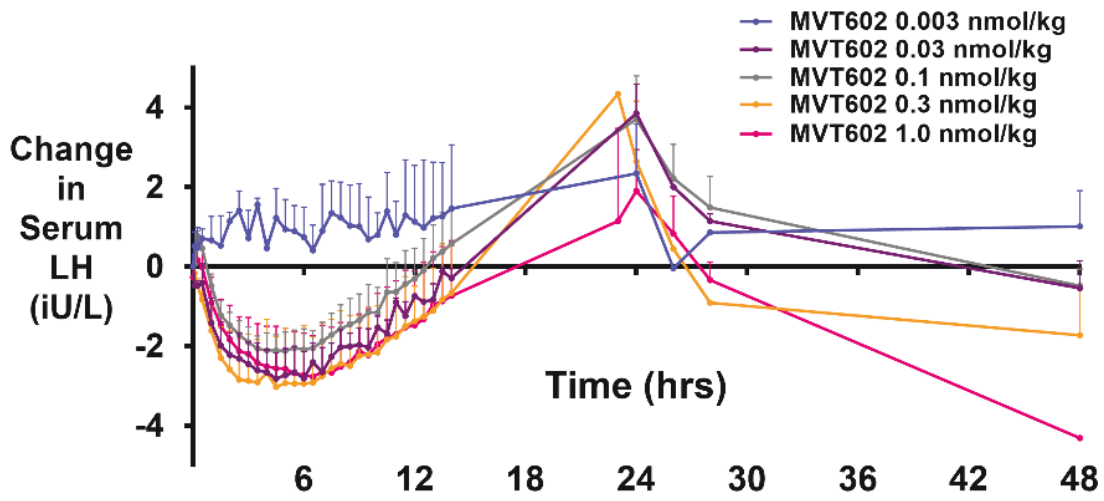
With the lowest dose of MVT-602 at 0.003nmol/kg, a gradual increase in serum LH was observed which was predominantly driven by one individual's LH response. However, with the next highest dose, MVT-602 0.03 nmol/kg; an initial delay of rise in serum LH up to ~5.4 hrs was observed (mean change in serum LH reduction from baseline: 2.89±1.2iU/L) before gradually rising to a peak that likely occurred between 14 and 24 hrs. Administration of the remaining doses (0.1nmol/kg, 0.3nmol/kg, 1.0nmol/kg) revealed a consistent profile whereby the rise in LH appeared to be delayed. Of note, no further increase in change in serum LH was observed at 24 hrs beyond 0.03nmol/kg (Table 3). The dose reflecting the greatest change in serum LH at 24 hrs was 0.03nmol/kg (mean change in serum LH: 3.86±1.1iU/L) and thus, this dose was taken forward to the next part of the study **(Figure 9A and 10A)**.

The serum FSH response mirrored the serum LH profile with a gradual rise observed with the lowest dose of MVT-602 at 0.003nmol/kg and a delayed rise with the next dose MVT-602 0.03nmol/kg and no further rise in FSH was observed at doses higher than 0.03nmol/kg **(Figure 9B, 10B**). The lowest dose of MVT-602 exhibited a more pronounced rise of serum oestradiol than the higher doses, but at doses higher than 0.03nmol/kg, change in serum oestradiol level were consistently lower in the three healthy women **(Figure 9C, 10C).** As expected, no changes in serum progesterone was observed.

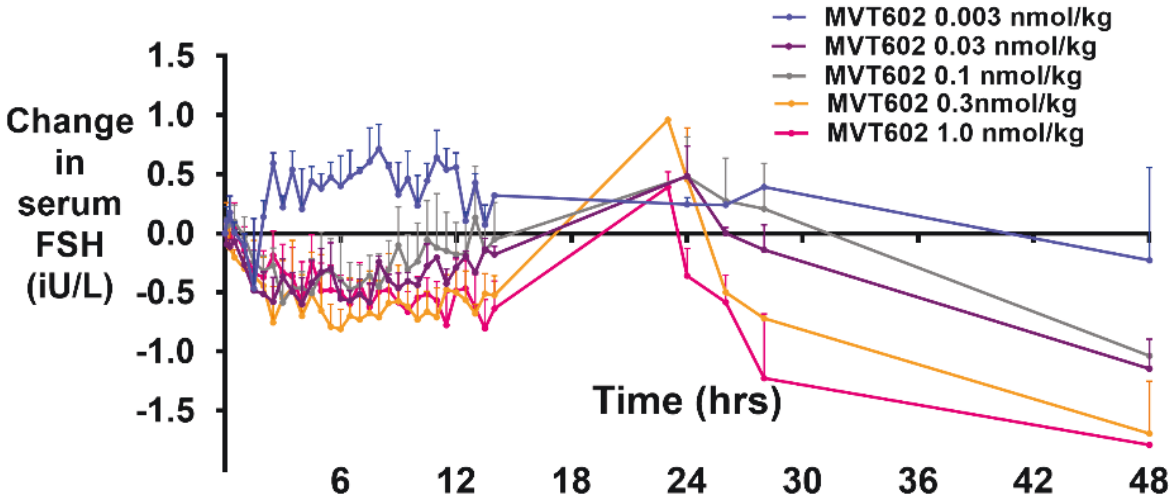
Dosing regimen (n=3)	**Mean change in serum LH at 24 hrs (iU/L)**
MVT-602 0.003nmol/kg	3.32±1.1
MVT-602 0.03nmol/kg	3.86±1.3
MVT-602 0.1nmol/kg	3.71±1.9
MVT-602 0.3nmol/kg	2.65±2.2
MVT-602 1.0nmol/kg	2.56±2.9

Table 3 Mean±SD are presented for parametrically distributed data. Hormonal values presented were change in serum LH at 24 hrs following intervention.

A


MVT602 0.003 nmol/kg
MVT602 0.03 nmol/kg
MVT602 0.1 nmol/kg
MVT602 0.3 nmol/kg
MVT602 1.0 nmol/kg
Change in Serum LH (iU/L)
4
2
0
-2
-4
Time (hrs)
6
12
18
24
30
36
42
48

B


MVT602 0.003 nmol/kg
MVT602 0.03 nmol/kg
MVT602 0.1 nmol/kg
MVT602 0.3nmol/kg
MVT602 1.0 nmol/kg
Change in serum FSH (iU/L)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Time (hrs)
6
12
18
24
30
36
42
48

C

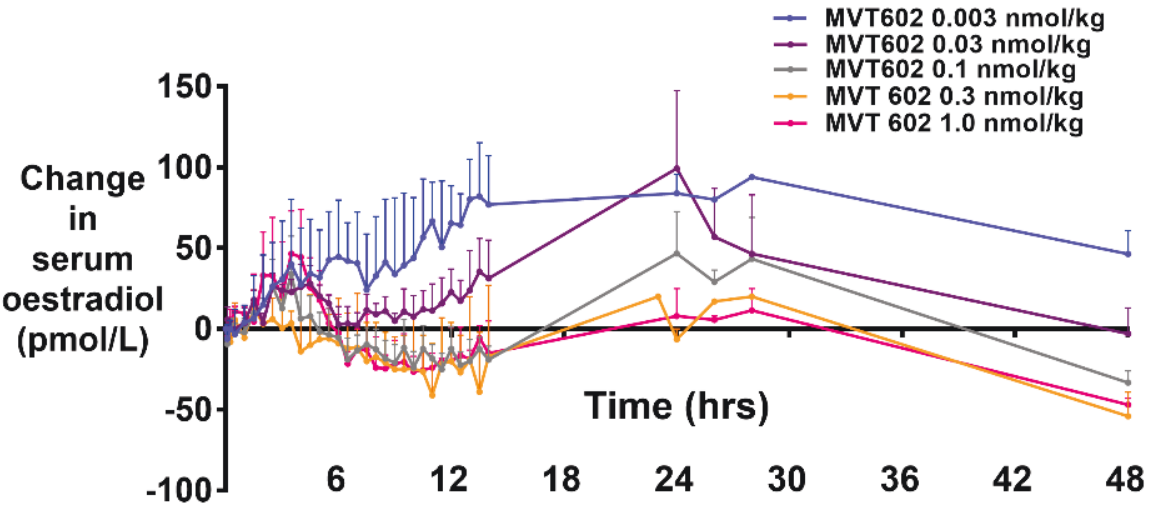
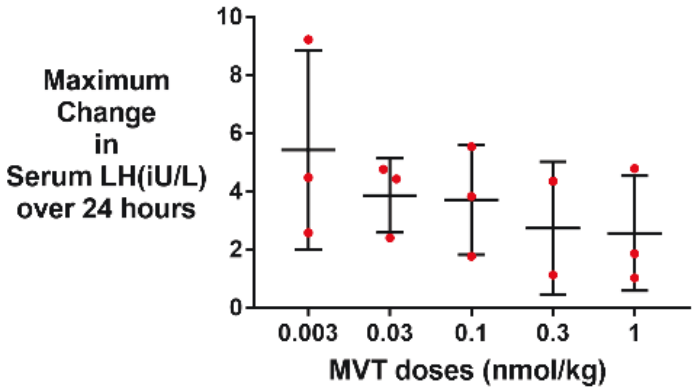
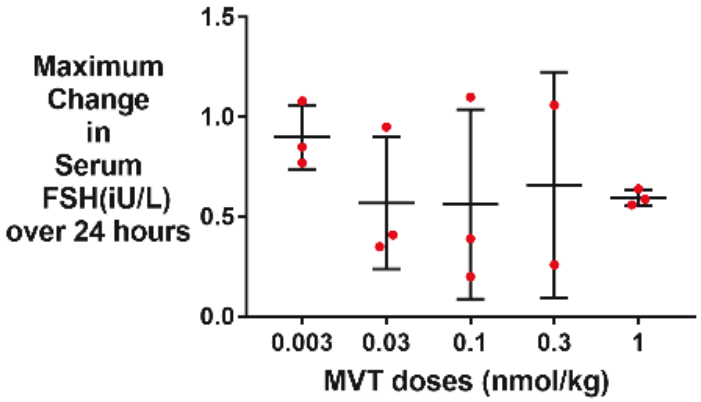

MVT602 0.003 nmol/kg
MVT602 0.03 nmol/kg
MVT602 0.1 nmol/kg
MVT 602 0.3 nmol/kg
MVT 602 1.0 nmol/kg
Change in serum oestradiol (pmol/L)
150
100
50
0
-50
-100
Time (hrs)
6
12
18
24
30
36
42
48

Figure 9: Mean±SEM of change from baseline levels of (A) serum LH (iU/L) (B) serum FSH (iU/L) and (C) serum oestradiol (pmol/L) in healthy women (n=3) over 48 hrs receiving a single dose of MVT-602 0.003nmol/kg (blue), 0.03nmol/kg (purple), 0.1nmol/kg (grey), 0.3nmol/kg (orange) and 1.0nmol/kg (pink).

A



B



C

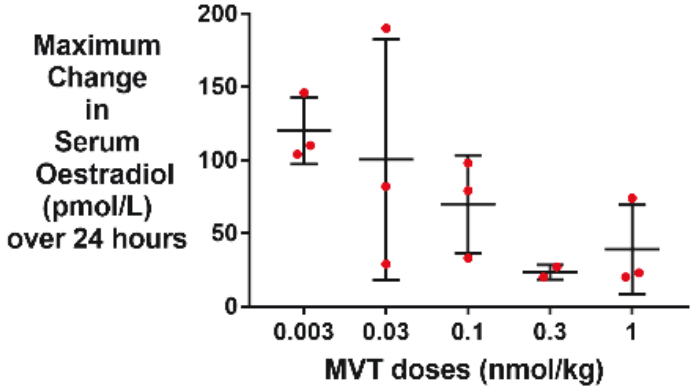


Figure 10: Scattergram (Median±IQR) of maximum change from baseline in (A) serum LH (iU/L) (B) serum FSH (iU/L) (C) serum oestradiol (pmol/L) over the first 24 hrs in healthy women (n=3) receiving the different doses of MVT-602.

2.4.1.2 Plasma MVT-602 levels

MVT-602 was rapidly absorbed from injection sites and reached maximum plasma levels within 15 minutes in all three healthy volunteers during the follicular phase of the menstrual cycle. A mean peak of ~65pg/mL was reached with MVT-602 0.03nmol/kg, which was significantly higher compared to the lowest dose of MVT-602 at 0.003nmol/kg (P-value<0.0001 by two-way ANOVA). Plasma-MVT-602 were less than 3pg/mL at 6 hrs beyond injection **(Figure 11)**.

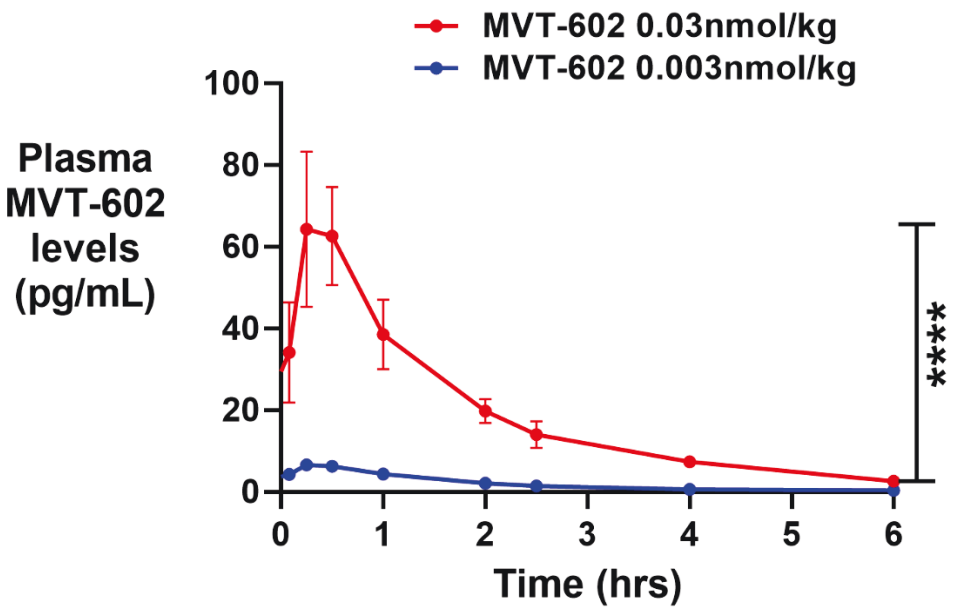


Figure 11: Mean±SD of the plasma concentration of MVT-602 (pg/mL) vs time (hrs) in healthy women (n=3) receiving a subcutaneous bolus of MVT-602 at 0.003nmol/kg in blue and 0.03nmol/kg in red in the first 6 hrs of the study. Groups were compared by repeated measures of two-way ANOVA (****P-value<0.0001).

In summary, the peak LH response is likely to occur between 14 and 24 hrs following administration of MVT-602 and no further increase in serum oestradiol and serum gonadotrophin levels were observed at doses beyond 0.03nmol/kg. The lowest dose of MVT-602 resulted in a more pronounced rise of serum oestradiol but only one of the three individuals showed a dramatic response to serum LH **(Figure 9A).** Taking all these data into consideration, MVT-602 0.003mol/kg appeared to result in a minimal LH

response, whereas MVT-602 0.03nmol/kg resulted in greatest stimulation of LH release. As there was no notable additional benefit to LH release beyond doses of 0.03nmol/kg, a lower dosing regimen of 0.01nmol/kg, was selected for the next phase of study, along with 0.03nmol/kg.

2.4.2 Study 2: Comparison of MVT-602 with native KP54 in healthy women

Following the analysis of study 1, two doses of MVT-602 (0.03nmol/kg and 0.01nmol/kg) were selected for comparison with native KP54 (9.6nmol/kg) and 0.9% saline in the early follicular phase in a further 9 healthy women. Based on the LH-profile observed following MVT-602 observed in study 1, the duration of 30 minute blood-sampling for measurement of reproductive hormones was extended to 24 hrs, and additionally at 28, 32 and 48 hrs for the two MVT-602 arms (0.01 and 0.03 nmol/kg) **(Figure 7)**. In healthy women (n=9) a maximum median change in serum LH to 7.8iU/L was observed at 5.5 hrs following a single subcutaneous dose of KP54 at 9.6nmolkg in the follicular phase of the menstrual cycle. Both MVT-602 at 0.01nmol/kg and 0.03nmol/kg potently stimulated serum LH release in healthy women (n=9) to a maximum median change of 6.2iU/L and 6.6iU/L at 24 hrs **(Figure 12A)**. The time to peak LH response occurred much later with MVT-602 at 24 hrs and thus greatly extended the area under the curve for LH release **(Figure 13A)**. As in study 1, a distinctive initial brief delay of serum LH rise was observed following subcutaneous administration of MVT-602. Notably, the serum LH response to subcutaneous bolus of KP54 is also characterised by a brief initial LH rise, followed by a plateau between 30 and 75 minutes and a second rise peaking between 4-6 hrs. Serum FSH responses following administration of KP54 and MVT-602 showed a similar profile to that of serum LH with a maximum change of 2.73iU/L for KP54 and 2.40iU/L for MVT-602 at 0.01nmol/kg and 0.03nmol/kg **(Figure 12B)**. The area under the curve for FSH release was similar following MVT-602 0.03nmol/kg and KP54 **(Figure 13B)**. However, there appeared to be a trend for a greater serum oestradiol response following MVT-602 compared to KP54 **(Figure 12C, 13C)**.

In summary, MVT-602 at 0.01nmol/kg and 0.03nmol/kg stimulated a gonadotrophin response that was of a similar amplitude to that observed following KP54 9.6nmol/kg although the peak LH occurred much later following MVT-602 than KP54, suggesting a longer duration of stimulation **(Figure 12)**.

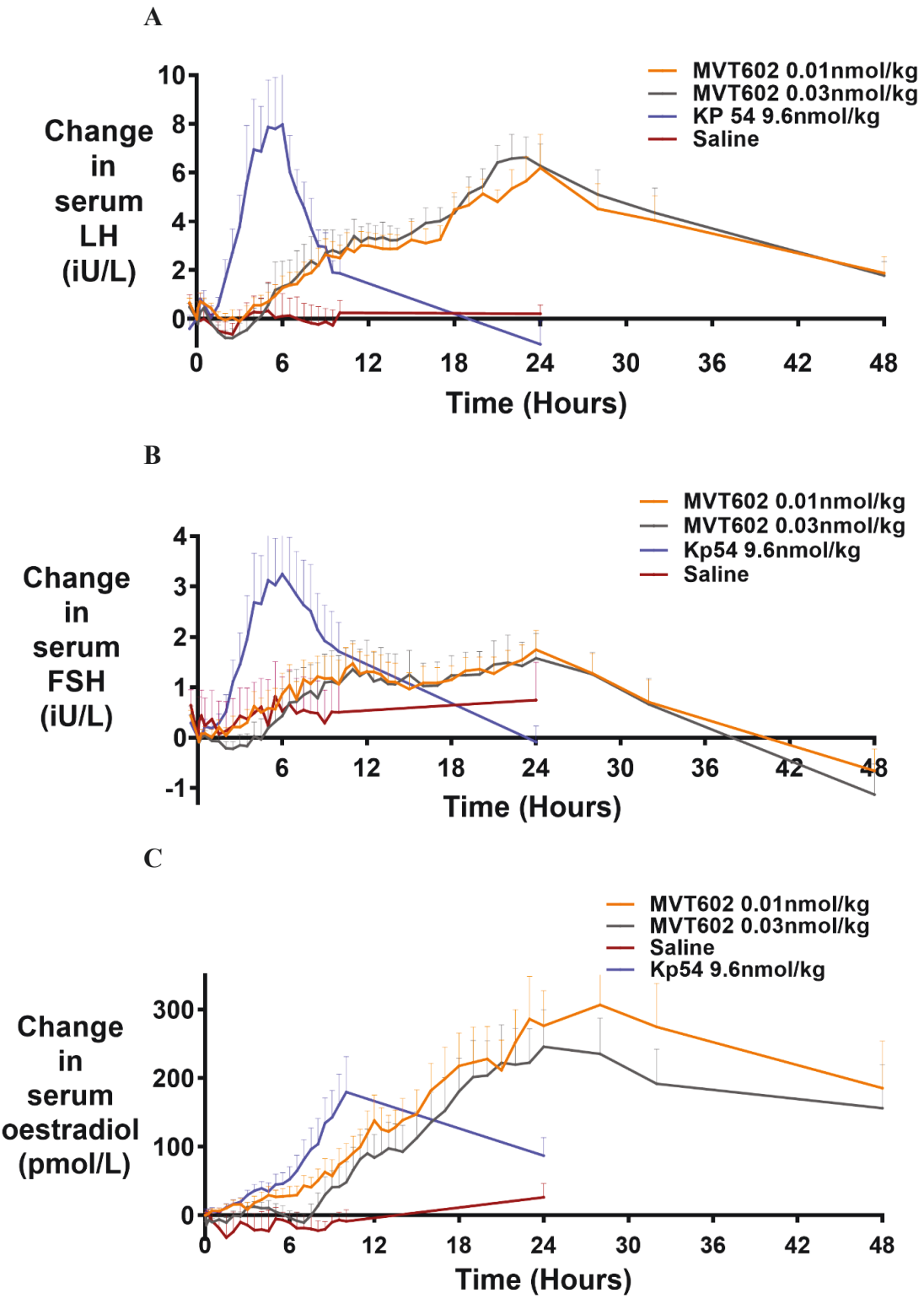


Figure 12 Mean±SEM of change from baseline levels in (A) Serum LH (iU/L) (B) Serum FSH (iU/L) and (C) serum oestradiol (pmol/L) in healthy women (n=9) receiving a subcutaneous bolus of MVT-602 at doses of 0.01nmol/kg (in orange), 0.03nmol/kg (in grey) over 48 hrs and KP54 9.6nmol/kg (in blue) and saline (in maroon) over 24 hrs.

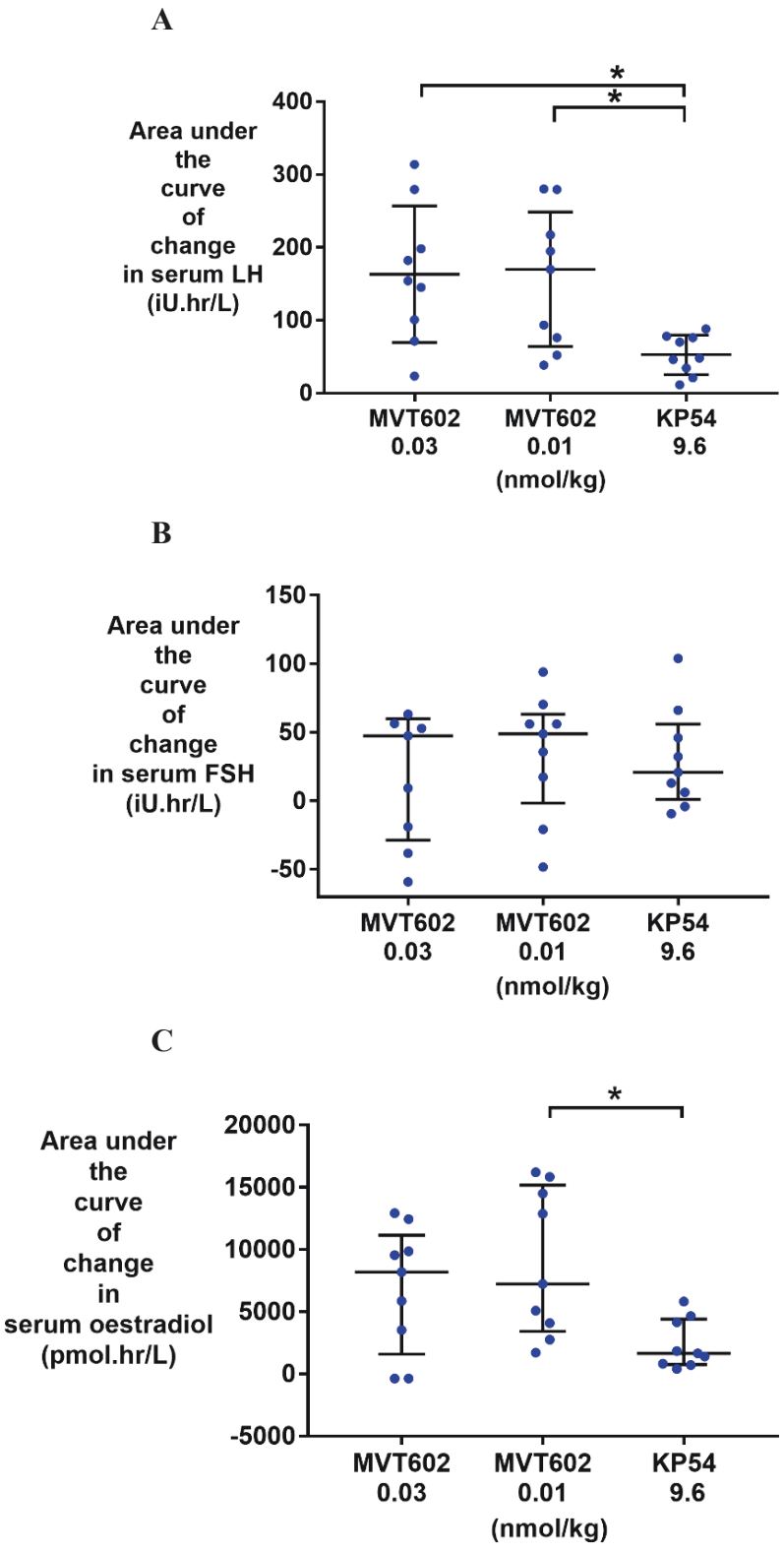


Figure 13 Scattergram (median±IQR) of the area under the curve (AUC) for change from baseline in (A) serum LH (iU/hr.L) (B) Serum FSH (iU/hr.L) (C) serum oestradiol (pmol/hr/L) in healthy women (n=9) receiving KP54 (KP54) 9.6nmol/kg, MVT-602 0.01nmol/kg and MVT-602 0.03nmol/kg. Groups were compared by Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

2.4.3 Study 3: The effect of oestrogen supplementation on the response to kisspeptin receptor agonist MVT-602

Administration of exogenous oestradiol for 24 hrs increased serum oestradiol concentrations in participants **(Figure 14 and 15)** and resulted in a standardised oestrogen exposure throughout the study period (Baird *et al.*, 1995). Following 24 hrs of oestradiol administration via a transcutaneous patch delivering 200mcg/24hrs, baseline serum LH and serum FSH concentrations were reduced. During oestrogen supplementation, a single subcutaneous bolus of MVT-602 0.03nmol/kg stimulated LH secretion to a mean peak of 25.7±11.9iU/L **(Figure 14B and 15A)**. This was 2.7-fold higher than in the group of women receiving MVT-602 without oestradiol supplementation (Mean peak LH of 9.7±4.0iU/L) **(Figure 14B and 15A)**. There was no change to the timing of peak response when MVT-602 0.03nmol/kg was given with oestradiol supplementation. Serum FSH levels were also increased by 2.7-fold in the oestradiol treated group (**Figure 15B**: *Mean peak of serum FSH with oestradiol supplementation:* 7.8±3.1iU/L; *mean peak FSH without oestradiol supplementation*: 2.9±1.7iU/L). Of note, the initial depression of serum LH was less pronounced in the oestradiol-treated group (*Mean change in serum LH reduction from baseline for MVT-602 0.03nmol/kg without oestradiol supplementation:* 0.8±0.4iU/L; *mean change in serum LH reduction from baseline for MVT-602 with oestradiol supplementation*: 0.2±0.1iU/L) **(Figure 15A)**.

2.4.4 Determinants of response to MVT-602 in healthy women

Overall, the maximum change in serum LH (P=0.005, r^2 =0.55) and maximum change in serum FSH (P=0.005, r^2 =0.56) levels predicted the stimulated oestradiol response following MVT-602 in healthy women. While there appeared to be a negative trend between baseline inhibin B and maximum change in serum FSH, statistical significance was not reached (P=0.16, r^2 =0.19) **(Figure 16)**.

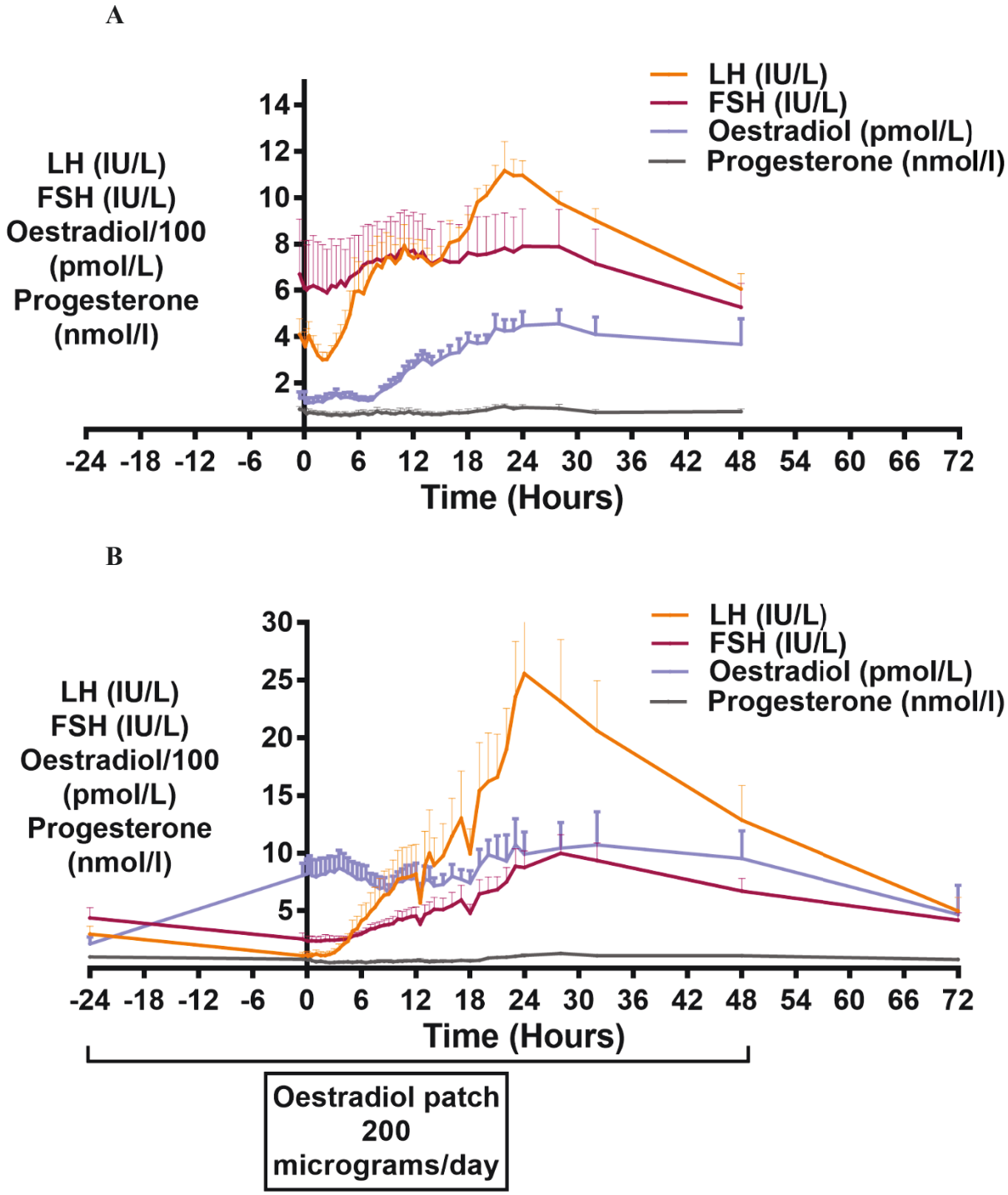


Figure 14 Mean±SEM of change from baseline levels in serum LH (iU/L) (in orange), serum FSH (iU/L) (in maroon), serum oestradiol (pmol/L) (in blue) and serum progesterone (nmol/L) in grey over 72 hrs in healthy women (n=5) (A) without oestradiol supplementation (B) with oestradiol supplementation.

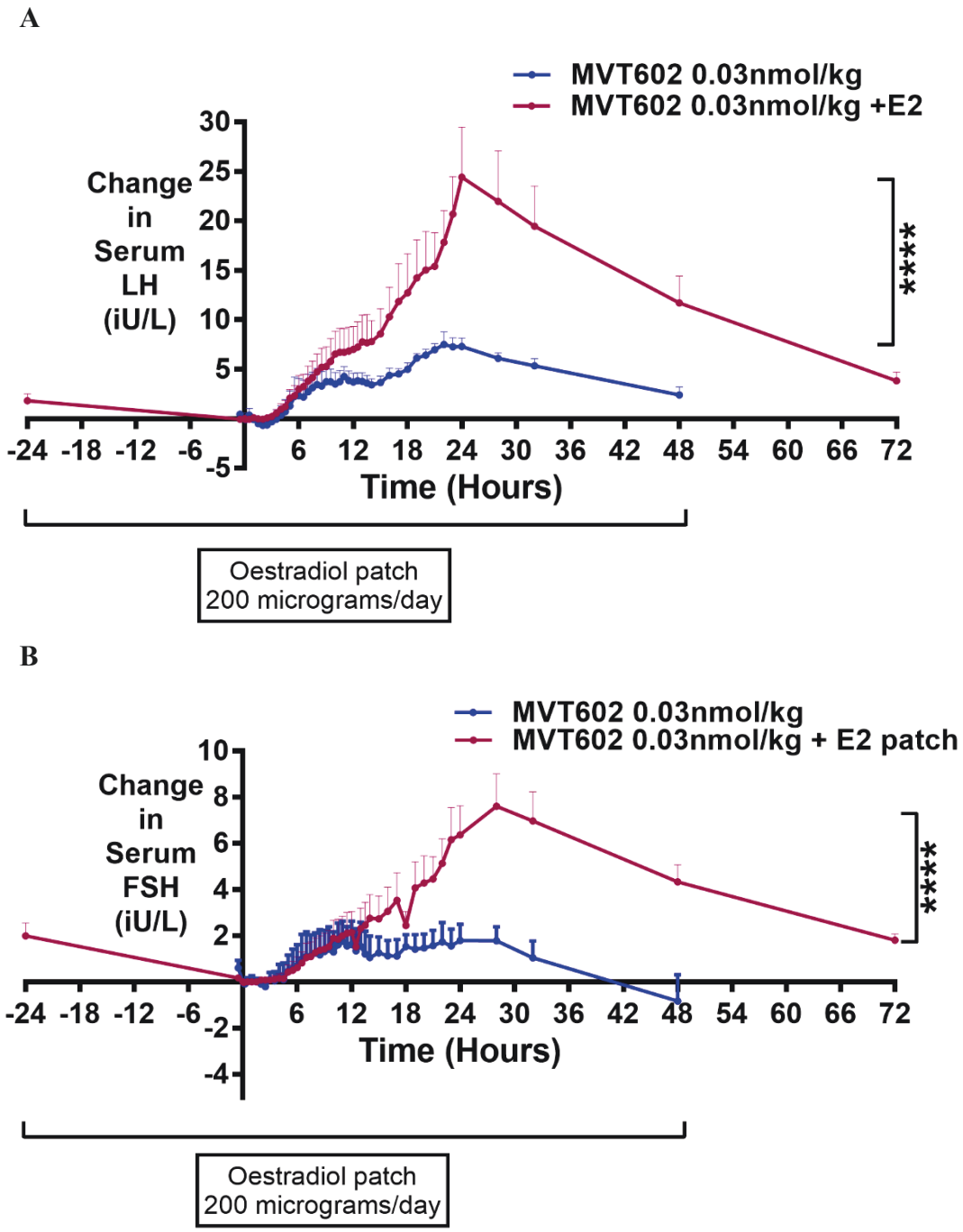


Figure 15 Mean±SEM of change from baseline levels in (A) serum LH (iU/L) in healthy women (n=5) receiving a single subcutaneous bolus of MVT-602 0.03nmol/kg with oestradiol supplementation (in maroon) and without oestradiol supplementation (in blue) for 72 hrs (B) serum FSH (iU/L) in healthy women (n=5) receiving a single subcutaneous bolus of MVT-602 0.03nmol/kg with oestradiol supplementation (in maroon) and without oestradiol supplementation (in blue). Groups were compared by two-way ANOVA. **** denotes P-value<0.0001.

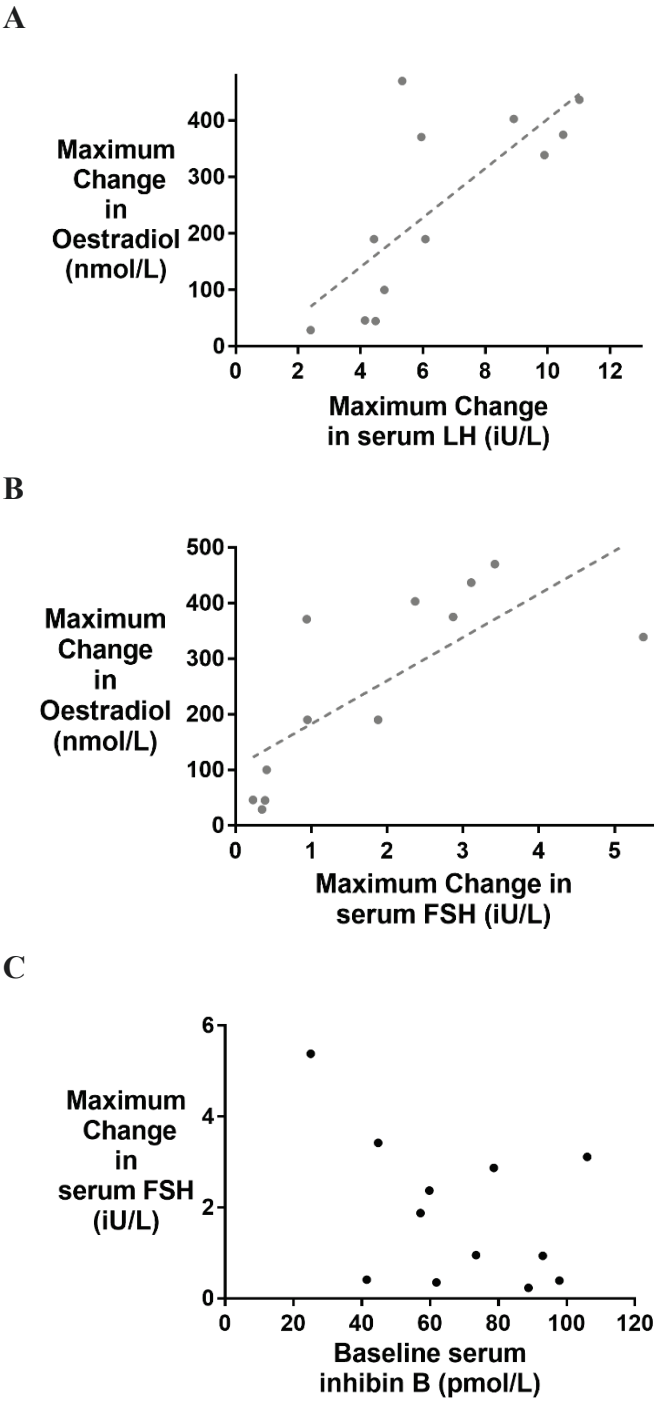


Figure 16 Correlation between maximum change in serum oestradiol (nmol/L) and (A) maximum change in serum LH (iU/L) (r^2=0.56, P-value=0.005) (B) maximum change in serum FSH (iU/L) in healthy women (n=12) (r^2=0.56, P-value=0.005) following a subcutaneous bolus of MVT-602 0.03nmol/kg (C) Correlation between maximum change in serum FSH following a single subcutaneous dose of MVT-602 0.03nmol/kg and baseline inhibin B in healthy women (n=12).

2.5 DISCUSSION

In this study, I present novel data on the endocrine profile following administration of the kisspeptin receptor agonist MVT-602 in the follicular phase of healthy ovulatory women. A single dose of MVT-602 induced a peak LH level of similar amplitude to native KP54 but at a longer duration following administration (22 hrs to peak LH response compared to 4.9 hrs for native KP54) and thus, greatly extended the area under the curve for gonadotrophin exposure. This extended duration of gonadotrophin-surge could have potential utility for the treatment of reproductive disorders in women.

As MVT-602 has never been investigated in women, I chose a broad-dosing range that would facilitate a breadth of LH responses for comparison with KP54 at 9.6nmol/kg, which is a clinically established effective dose to induce an acute stimulatory gonadotrophin response in women (Jayasena *et al.*, 2014) . Five doses of MVT-602 (0.003, 0.03, 0.1, 0.3, 1.0nmol/kg) were selected based on a previous phase 2 study in healthy men (MacLean *et al.*, 2014). KP54, rather than KP10 was used for comparison as the longer peptide KP54 was more efficacious *in vivo* (De Tassigny *et al.*, 2017) and displayed more sustained levels of kisspeptin in comparison to KP10 (Jayasena *et al.*, 2015b). The study duration was initially decided based on the data derived from the phase 2 study investigating the effects of MVT-602 in healthy men that demonstrated a peak gonadotrophin response between 6 to 12 hrs (MacLean *et al.*, 2014). Although I did not measure reproductive hormones between 14 and 24 hrs in our initial dose finding **(Study 1)** and peak hormone levels may have been missed, the peak serum LH levels measured in study 2 was strongly associated to the peak serum LH levels measured at 24 hrs in study 1 (r^2=0.64, P=0.01).

In my dose-finding study **(Study 1)**, the lowest dose of MVT-602 (0.003nmol/kg) resulted in a gradual rise in gonadotrophin response, but only in one of the three individuals. The next dose 0.03nmol/kg showed a delay of LH-rise in the first 6 hrs followed by a gradual rise in serum LH to a likely peak between 14 and 24 hrs. No further increase in mean change of serum LH at 24 hrs was observed at doses

beyond 0.03nmol/kg **(Table 3)**, suggesting that a maximal dose response had been reached at 0.03nmol/kg. As no benefit could be gained from change in gonadotrophin levels with doses beyond 0.03nmol/kg, the dose 0.03nmol/kg and a lower dose of 0.01nmol/kg were selected for comparison with KP54 in the second part of the study.

The LH profile following a single dose of MVT-602 reveals a distinctive pattern, characterised by an initial brief delay of serum LH-rise lasting up to 6 hrs. This effect is highly reproducible with doses beyond 0.03nmol/kg. This finding is contrary to the documented response in healthy males, which revealed a robust LH-rise that peaked at 6-12 hrs and remained elevated for 48 hrs before returning to baseline. Several possible hypotheses may explain this phenomenon. Firstly, it is possible that kisspeptin agonists such as MVT-602 could activate other G-protein coupled receptors (GPCR) yet retaining an equipotent bioactivity towards kisspeptin receptors. Kisspeptin belongs to the lineage of RF-amide neuropeptides, a group of peptides characterised by their Arg-Phe-NH_2 at the common carboxyl C-terminal (Lyubimov *et al.*, 2010; Oishi *et al.*, 2011; Quillet *et al.*, 2016). While each family has its own cognate receptors (e.g. NPFF1r, NPFFF2R, KISS1R), there is increasing crosstalk signalling amongst the RF-amide peptides. Indeed, studies have demonstrated that kisspeptin can bind and activate NPFF1R and NPFFR2 expressed in cell lines (Oishi *et al.*, 2011; Elhabazi *et al.*, 2013) and lower the plasma LH (Murakami *et al.*., 2008; Clarke *et al.*., 2009; Kriegsfeld *et al.*., 2010). As NPFFRs are widely expressed in the brain including in several regions where kisspeptin fibres are found (Poling *et al.*, 2013), it is possible that MVT-602 whilst having highest affinity for one receptor can simultaneously activate multiple receptors within the family. This is not unusual as electrophysiological studies by Liu and Herbison clearly showed that neuromodulatory concentrations of kisspeptin (100 to 400nM) could act both as a NPFFR agonist and cause neural firing in the absence of KISS1R (Liu and Herbison, 2015). Admittedly, further experiments in KISS1R knock out mice or using specific NPFFR antagonists (Kim *et al.*, 2015) would be critical to determine the interaction between kisspeptin and RF amide receptors in the brain. Nevertheless, this LH profile could not be totally attributed to the off-target effects on

different receptor sites as work on the KISS1R had suggested that kisspeptin stimulates a biphasic intracellular response, with an acute rapid release in intracellular calcium (lasting 5 minutes) followed by a more sustained second-phase calcium response (lasting 30 minutes) (Min *et al.*, 2013). Therefore, differences in intracellular signaling pathways may contribute to the LH profile observed.

Secondly, it is possible that the delay in serum LH-rise is due to the effect of the circulating sex steroid milieu in these female participants. Whilst in previous MVT-602 study, healthy men exhibited an LH rise of ~20iU/L with a single bolus of MVT-602 (MacLean *et al.*, 2014), in this study, healthy women demonstrated a rise in serum LH of ~7iU/L. It took ~5.4 hrs for the serum LH to rise during the follicular phase after administration of MVT-602. Plasma levels of MVT-602 are observed shortly after administration (at 0.5 hrs), therefore impaired systemic absorption is unlikely to underlie the difference in serum LH response. Women in the follicular phase are known to be the most resistant to stimulation with kisspeptin (Dhillo *et al.*, 2007; Chan *et al.*, 2012). A possible cause of the greater resistance to stimulation with kisspeptin in the follicular phase therefore is the influence of sex steroid on the LH response to kisspeptin.

Various *in vitro* studies have shown that oestradiol enhanced GnRH secretion in GnRH neuronal cell lines exposed to kisspeptin (Novaira *et al.*, 2009; Tonsfeldt *et al.*, 2011). Our group and others have also reported a higher gonadotrophin response to kisspeptin in the preovulatory phase when there are high oestradiol levels (Dhillo *et al.*, 2007; Chan *et al.*, 2012) as well as in women with higher basal oestradiol levels during the follicular phase (Narayanaswamy *et al.*, 2016). In order to avert the influence of oestradiol on gonadotrophin response, I have purposefully undertaken this study in the follicular phase (Day 1 to Day 4) of healthy women where oestradiol levels were the lowest. I later repeated the same study with oestrogen supplementation at MVT-602 0.03nmol/kg in healthy women (n=5) and observed a less pronounced initial LH suppression followed by a significantly augmented peak gonadotrophin response (Study 3). It is possible that an oestradiol threshold level needs to be reached in order to cause an elevated gonadotrophin response to MVT-602. To lend credence to this observation, I noted that there

was no clear association of gonadotrophin response to basal oestradiol levels, which is different to that observed in our previous studies (Narayanaswamy *et al.*, 2016). However, all women in our study had a basal oestradiol level less than 200pmol/L in the early follicular phase (Day 1 to Day 4) whereas healthy women in the previous study were recruited on day 2 to day 6 of the follicular phase and had a mean basal oestradiol level of more than 200pmol/L on the day of study visit.

Serum oestradiol levels progressively increased with increasing doses of MVT-602. However, at the highest doses (0.3nmol/kg and 1nmol/kg), oestradiol levels appeared to be lower without a significantly lower gonadotrophin secretion. In healthy men, MVT-602 suppressed testosterone (MacLean *et al.*, 2014) but this occurred following continuous administration as opposed to a single bolus administered to our participants. Whilst this might be an indirect consequence of the central effect of kisspeptin on gonadotrophin secretion, it is equally plausible to speculate that there is a local modulatory action of kisspeptin at the gonadal level. Indeed, kisspeptin receptors are known to be expressed on ovarian theca cells (Castellano *et al.*, 2006). Another group of researchers have also observed reduced follicular growth and a downregulation of FSH receptor in rats' ovaries treated with a 28-days infusion of kisspeptin (Fernandois *et al.*, 2016). More recently, Owen and colleagues observed increased gonadotrophin gene expression and steroidogenesis in human granulosa lutein cells after exposure to KP54 during IVF treatment (Owens *et al.*, 2018). Notably, the understanding of the physiological relevance of ovarian kisspeptin, distinct to central kisspeptin, in the modulation of ovulation and steroidogenesis, warrants further investigation.

In summary, in healthy women in the follicular phase, both MVT-602 and KP54 resulted in a robust rise in gonadotrophins that was of similar amplitude, consistent with them both activating the hypothalamic-pituitary pathway via kisspeptin receptors expressed on GnRH neurons.

2.5.1 Clinical significance of my study

Ovarian Hyperstimulation Syndrome (OHSS) is a serious complication of *in-vitro* fertilisation (IVF) treatment. OHSS is characterised by a prolonged ovarian LH receptor stimulation, leading to the release of vascular endothelial growth factor (VEGF) and resultant fluid shift into the third space, contraction of vascular volume, oliguria and ascites (Whelan *et al.*, 2000).

Data from our previous studies have concluded that a single subcutaneous injection of KP54 can be used to effectively induce oocyte maturation during IVF treatment without OHSS, even in women at increased *a priori* risk (Jayasena *et al.*, 2014; Abbara *et al.*, 2015). Indeed, a single subcutaneous dose of KP54 was able to stimulate an LH surge of approximately 40iU/L lasting for 12 to 14 hrs and reduce the odds of severe OHSS by 33.6-fold when compared to women who had hCG as a trigger for oocyte maturation during IVF treatment (Abbara *et al.*, 2015; Abbara *et al.*, 2018). Furthermore, in a further study, when the duration of the LH-surge was extended by administration of a second dose of kisspeptin 10 hrs following the first, not only was efficacious oocyte maturation observed but still OHSS was not induced in participants who were at high risk of developing the condition (Abbara *et al.*, 2018). In addition, KP54 had an intrinsic ability to reduce oestradiol-driven VEGF production without affecting ovulation (Cerrillo *et al.*, 2009; Zhai *et al.*, 2017). Apart from the safety profile, the longer acting kisspeptin receptor agonist also displayed a greater AUC of gonadotrophin exposure for up to ~22 hrs. In primates, achieving a longer duration of a low-level LH has been reported to be more efficacious in inducing ovulation than a higher level of LH for a shorter duration (Ishikawa, 1992). This indicates that a threshold value exists for LH to initiate the process of ovulation and once this threshold is reached, the duration of exposure is more critical for inducing ovulation and supporting luteal formation (Ishikawa, 1992). Female macaques receiving a number of different inductors of oocyte maturation (GnRH agonist and hCG) in gonadotrophin stimulated cycles further revealed that an LH exposure lasting at least 14 hrs to 48 hrs after injection would be needed to induce ovulatory changes in primate follicle for adequate oocyte maturation, ovulation and corpora lutea development (Chandrasekher *et al.*, 1991). Therefore,

this longer LH profile observed with MVT-602 in healthy follicular women could have potential for use as an inductor for oocyte maturation in IVF cycles, however further research would be required to fully confirm this observation.

2.5.2 Limitations

As hormonal effects of MVT-602 in women were until now unknown, the number of recruited participants was selected based on proof of concept studies done in our previous studies (Dhillo *et al.*, 2005). The recruitment number was limited by the difficulty in finding women who were willing to undergo invasive study procedure, such as overnight blood sampling within the first four days of their menstrual period every month over a 6 months duration. We were also constrained by the number of blood samples that were ethically permitted over the maximum period of 24 hrs in a human physiological study. However, I have achieved my original objectives and demonstrated the pharmacokinetic and pharmacodynamic profile following MVT-602 for the first time. Though we repeated the study in the follicular phase in the presence of oestrogen supplementation, the sex steroid milieu in superovulated IVF setting can be different. An important area for further investigation will be determining the physiological effects of MVT-602 in this context.

2.6 SUMMARY

In summary, I present novel data on the hormonal profile of MVT-602 in healthy women. I have shown that MVT-602 induces a gonadotrophin surge with a peak LH that is comparable to KP54. However, the duration of LH-surge is extended when compared to KP54. This profile could be of value as a trigger of oocyte maturation during IVF treatment as the excursions in serum gonadotrophins replicate the physiological mid-cycle LH-surge. Importantly, a single dose of MVT-602 induces robust and sustained FSH secretion sufficient to stimulate oestradiol secretion, a characteristic that could have great application in the treatment of women with anovulatory disorders. Preliminary data for these possible applications will be explored in the next chapter.

2.7 FUTURE WORK

Exogenous kisspeptin has been demonstrated to be a potential therapeutic agent in certain reproductive disorders e.g. in women with hyperprolactinaemia (Millar *et al.*, 2017) or women with hypothalamic amenorrhoea (Jayasena *et al.*, 2014). It would therefore be of interest to evaluate the therapeutic potential of MVT-602 in these disorders. MVT-602 not only induced a peak LH similar to KP54 but also extended the duration of action of gonadotrophin surge. It therefore would be of interest to determine if such an extended LH surge would be clinically beneficial in an IVF setting. Sonographic imaging of the follicular features throughout the menstrual cycle would offer great value in elucidating further the ovarian effects of MVT-602 that were not identified here.

An unexpected yet interesting finding is the delay in serum LH rise for up to 5.4 hrs in healthy women during the follicular phase. Future work to decipher the mechanisms of receptor activation following MVT-602/KP54 include investigating the level of receptor-ligand interactions and intracellular signaling mechanisms. Furthermore, a comparable design to the current study could be applied to evaluate gonadotrophin response in different phases of the menstrual cycle.

CHAPTER 3

THE EFFECT OF KISSPEPTIN RECEPTOR AGONIST (MVT-602) IN WOMEN WITH HYPOTHALAMIC AMENORRHOEA (HA) AND WOMEN WITH POLYCYSTIC OVARIAN SYNDOMRE (PCOS)

CHAPTER 3: THE EFFECT OF KISSPEPTIN RECEPTOR AGONIST (MVT-602) IN WOMEN WITH HYPOTHALAMIC AMENORRHOEA AND WOMEN WITH POLYCYSTIC OVARIAN SYNDROME

3.1 INTRODUCTION

Subfertility affects up to 10-14% of couples worldwide (Farquhar *et al.*, 2019; Thurston *et al.*, 2019). Causes of subfertility are numerous and often multifaceted, with female causes contributing to about 37% of cases. Of the female causes, anovulatory disorders such as polycystic ovarian syndrome (PCOS) and hypothalamic amenorrhoea (HA) are the most common (National Institute for Health and Care Excellence, 2013; Thurston *et al.*, 2019). While boluses of GnRH or GnRH receptor agonists are commonly used to treat reproductive disorders, it is prone to desensitisation after repeated or continuous administration (Broekmans *et al.*, 1993) and also carries a risk of ovarian hyperstimulation syndrome (OHSS) when used as a ovulation trigger in women with PCOS (Abbara *et al.*, 2018; Broekmans, 2019). Consequently, an agent that acts through existing physiological pathways to stimulate endogenous gonadotrophin secretion with a lower complication rate than existing therapies would offer valuable clinical potential.

Kisspeptin peptides have emerged as potent elicitors of gonadotrophin secretion and their central location within the HPG axis has made them novel targets for pharmacological intervention. Moreover, it has been established that kisspeptin neurons play a key role in integrating feedback from peripheral signals such as sex-steroids (Roa *et al.*, 2007; Tena-Sempere, 2010), prolactin (Brown *et al.*, 2019) and metabolic cues such as leptin (Quennell *et al.*, 2011) into the secretion of GnRH. Kisspeptin not only stimulates GnRH secretion, but also changes GnRH pulse frequency (Chan *et al.*, 2011). In healthy males, a bolus injection of KP10 delayed the next endogenous LH pulse by prolonging the interval between two consecutive endogenous LH pulses (Chan *et al.*, 2011). Furthermore, a continuous iv infusion of KP10 (0.01 to 1ug/kg) increased LH pulse frequency in men (George *et al.*, 2011). This suggests the possibility of using kisspeptin based therapies to modulate abnormal GnRH pulsatility commonly

associated with women with HA–a condition characterised by reduced gonadotrophin secretion and low pulsatility (Gordon *et al.*, 2017a) or PCOS–a condition characterised by increased pulsatility (Tsutsumi *et al.*, 2009). Analyses from short term experimental studies have demonstrated that although a single injection of KP54 or KP10 safely stimulates reproductive hormones, repeated administration in chronic studies may cause tachyphylaxis in animals (Seminara *et al.*, 2006; Thompson *et al.*, 2006; Ramaswamy *et al.*, 2007; Jayasena *et al.*, 2009) and in women with HA (Jayasena *et al.*, 2009). However, desensitisation to the effects of kisspeptin has not been consistently observed in all studies. George and colleagues reported no tachyphylaxis when a continuous infusion of KP10 was administered to healthy men for 22.5 hrs (George *et al.*, 2011). A similar effect on serum LH was also observed in men with hypogonadism due to type 2 diabetes, with a resulting restoration of testosterone levels to within normal range following administration of KP10 (George *et al.*, 2010; 2013). These findings suggest that longer acting formulations of KP10 might find utility in the treatment of reproductive disorders.

Notably, our group was one of the first to explore kisspeptin-based protocols in the treatment of women with HA (Jayasena *et al.*, 2010). Indeed, a subcutaneous injection of KP54 resulted in an exaggerated gonadotrophin response in women with HA when compared to that in healthy females in the follicular phase. However, this acute increase in gonadotrophins did not translate to an increase in oestradiol secretion which could be related to the limited effect on FSH secretion or the short timescale of the study (Jayasena *et al.*, 2010). Twice daily subcutaneous administration of KP54 (6.4nmol/kg) in women with HA resulted in a progressive loss of LH response, whereas a twice-weekly KP54 for eight weeks resulted in only partial desensitisation with restoration of reproductive hormone secretion (Jayasena *et al.*, 2010). The results of this study indicated that the time-course to desensitisation in women with HA occurred over days and this could potentially be overcome by reducing the dose and frequency of administration (Jayasena *et al.*, 2010). Further work had been carried out to elucidate whether kisspeptin could restore LH pulsatility given that the lack of hormone pulsatility is a key feature in women with HA. Indeed, an intravenous infusion of KP54 at 0.01 nmol/kg/hour (0.059 μg/kg/hour) to 1 nmol/kg/hour (5.9

μg/kg/hour) for 8 hrs not only increased both basal and pulsatile LH secretion and in women with HA but also induced a 3-fold increase in LH pulse frequency (Jayasena *et al.*, 2013). Taken together, these studies suggested that kisspeptin-based therapies can be used for treatment in women with HA but a delicate balance of dose and exposure in maintaining GnRH responsiveness to the kisspeptin neuropeptide needs to be considered. Tachyphylaxis is associated with higher and increased frequency of administration of kisspeptin (Abbara *et al.*, 2013), thus it would be of great interest to determine if a longer acting kisspeptin receptor agonist such as MVT-602 could allow for restoration of physiological hormonal secretion in women with HA who were sensitive to kisspeptin. Women in HA have slow GnRH pulsatility whereas women with PCOS have increased GnRH pulsatility. As KP54 has been shown to produce an augmented response in women with HA (Jayasena,*et al.*, 2013), it would be of great interest to explore the pharmacological profile of a longer acting kisspeptin agonist such as MVT-602 in women with PCOS.

MVT-602 is a novel short form of kisspeptin receptor agonist of KP10 with improved *in vivo* stability and potent agonistic activity largely due to its increased resistance to enzymatic degradation (Asami *et al.*, 2013; Matsui and Asami, 2014). In the previous chapter, I explored the pharmacological profile of MVT-602 in healthy women. However, effects of MVT-602 in women with subfertility have never been explored. In this chapter, I investigated the hormonal profile of a single subcutaneous dose of MVT-602 in women with HA and PCOS and compared their response to the hormonal profile in healthy women in the follicular phase (Chapter 2). As part of my exploratory study, I also compared the hormonal responses following KP54 and GnRH agonist in healthy women and women with HA and PCOS.

3.2 HYPOTHESIS AND AIMS

3.2.1 Aims

(1) To investigate the effects of MVT-602 on gonadotrophin secretion in the early follicular phase (day 1 to day 4) of women with PCOS and women with HA and to compare the gonadotrophin response to that observed in healthy women (n=9) in the follicular phase recruited in the previous study.

(2) To determine the gonadotrophin response in follicular phase of women with PCOS and women with HA following single dose of native KP54 at 9.6nmol/kg and to compare the gonadotrophin response to that observed in healthy women (n=9) in the follicular phase. This dose of KP54 9.6nmol/kg was selected as it was previously shown to induce a robust gonadotrophin rise during IVF treatment (Jayasena *et al.*, 2014).

(3) To investigate and compare the effect on hormonal responses following a single subcutaneous dose of the GnRH agonist, triptorelin (0.2mg) in healthy women (n=9), women with HA(n=6) and women with PCOS (n=6) to that of the hormonal responses induced by KP54/MVT-602.

3.2.2 Hypothesis

(1) I hypothesise that MVT-602 will produce an augmented and sustained gonadotrophin response in women with HA whereas the gonadotrophin response in women with PCOS would be lower or comparable to that observed in healthy women in the follicular phase.

(2) I hypothesise that women with HA will exhibit an augmented gonadotrophin response to KP54 compared to healthy women in the follicular phase and women with PCOS.

(3) I hypothesise that the duration of hormonal responses following triptorelin, a GnRH agonist will be shorter in duration but higher in amplitude in comparison to KP54 or MVT-602.

3.3 Material and methods

3.3.1 Ethical approval

Ethical approval for this study was granted by the West London Research Ethics committee, London, UK (reference: 12/LO/0507) and conducted in accordance to the Declaration of Helsinki. Recruitment was via advertisements in the local press. All participants provided full written consent. Six women with HA and six women with PCOS were recruited via newspaper advertisements.

3.3.2 Subjects

Women were diagnosed with HA in accordance with American Endocrine Society guidelines (Gordon *et al.*, 2017a) whereas women with PCOS were defined according to the 2018 international PCOS guidelines (Teede *et al.*, 2018). All women underwent detailed evaluation of their medical history, medication history, menstrual history and clinical examination, including an ultrasound scan to determine the antral follicle count. The following blood tests were assessed during the screening visit: full blood count, renal function, liver function, bone profile, thyroid hormone profile, luteinising hormone (LH), follicle stimulating hormone (FSH), oestradiol, progesterone, androstenedione, dehydroepiandrosterone, testosterone, sex hormone binding globulin (SHBG), prolactin, and anti-müllerian hormone (AMH).

3.3.3 Study protocol

All study visits were carried out at the Clinical Research Unit, Imperial College Healthcare NHS Trust and commenced in the morning (~8am). Pregnancy was excluded using a urine pregnancy test at the beginning of each study visit. Blood pressure and pulse were recorded throughout each study visit. The occurrence of symptoms was recorded following each intervention and during the following week. Participants were free to eat and drink as normal before and during each study visit. The study was single-blinded with participants being unaware of the intervention that was administered at each study visit.

In the previous chapter, MVT-602 at 0.03nmol/kg was shown to induce a robust LH profile in healthy women. Therefore, this dose was selected for comparison with native KP54 (9.6nmol/kg) and 0.9% saline in women with HA and women with PCOS. The dose of 9.6nmol/kg of KP54 was selected as this was previously shown to induce a robust gonadotrophin response and to safely induce oocyte maturation during IVF treatment. Triptorelin is GnRH agonist routinely used at a dose of 0.2mg in IVF treatment (Abbara *et al.*, 2018). Each woman received one of the following interventions in each month (MVT-602 0.03nmol/kg, KP54 9.6nmol/kg, triptorelin 0.2mg and 0.9% saline) in the early follicular phase (day 1 to day 4) of the menstrual cycle in random order. Women with PCOS were provided with a 7 days course of progesterone tablet (10mg twice a day for 7 days) to induce a withdrawal bleed. All women with HA had the saline study as their first intervention to ensure a reduced LH profile before being invited to study visits at a monthly interval of 28 to 30 days.

Serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30 minutes, 0 minutes, 5 minutes, 15 minutes post injection and then every 30 minutes until 10 hrs, and additionally at 24 hrs following each intervention (KP54, 0.9% saline). Measurement of reproductive hormones was extended to 24 hrs, and additionally at 28 hrs, 32 hrs and 48 hrs for the MVT-602 arm (0.03 nmol/kg) (see **Figure 7**: Additional blood sampling denoted by longer arrows).

3.3.4 Hormone Assays

Blood samples for serum LH, FSH, oestradiol and progesterone measurement were collected in plain Vacutainer tubes (Becton Dickinson, Franklin Labs, New Jersey). Samples were allowed to clot prior to centrifugation for 10 minutes at 1210 g and then separated. Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Becton Dickinson, Franklin Labs, New Jersey) containing 5000 Kallikrein inhibitor units of aprotinin (0.2 ml Trasylol; Bayer, Newbury, UK) and were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 4 minutes at 2150 g and then separated and immediately frozen at -20ºC. Plasma samples for MVT-602 levels was collected in chilled K2EDTA tubes (Becton Dickinson, Franklin Labs, NJ) and

was centrifuged immediately for 10 minutes at 194 g at a temperature of 4ºC and stored at -20ºC until analysis. Serum LH, FSH, estradiol and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Interassay coefficients of variation were as follows: LH, 2.7%; FSH, 3.0%; estradiol, 3.0%; progesterone, 2.9%. Limits of detectability for each assay were as follows: estradiol, 37 pmol/liter; FSH, 0.05iU/L; LH, 05 iU/L; progesterone, 0.318nmol/L. Reference ranges were as follows: LH in iU/L, 2-10 (follicular), 20-60 (mid cycle), 4-14 (luteal); FSH in iU/L, 1.5 -8 (follicular and luteal), 10-50 (mid cycle) and oestradiol in pmol/L, less than 300 (early follicular), 400-1500 (mid cycle), 200 -1000 (luteal). MVT-602 levels were measured using liquid chromatography with tandem mass spectrometry by PPD laboratories in Virginia, USA. Measurement of plasma kisspeptin immunoreactivity (IR) was measured using an established RIA. The antibody cross-reacted 100% with human KP54, kisspeptin-14, and KP10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF.

3.3.5 Statistical methods

Parametrically distributed data are presented as mean$\pm$SD, whereas non-parametric data are presented as median$\pm$IQR. Statistical comparison across intervention groups were performed using one-way ANOVA with post hoc Tukey's multiple comparison test or Kruskal-Wallis test with post hoc Dunn's test, as appropriate. A p-value<0.05 was considered to be statistically significant. The correlations between variables were assessed using Pearman correlation analysis for parametric distribution and Spearman rank correlations for non-parametric distribution.

3.4 RESULTS

3.4.1 Baseline Characteristics

Six women with HA and six women with PCOS participated in this part of the study. Women with HA and women with PCOS had longer menstrual cycle length than healthy women. Weight, body mass index (BMI) and serum gonadotrophin levels were similar among the women but serum sex hormone binding globulin (SHBG) was lower in women with PCOS. Serum anti-Mullërian hormone (AMH) levels were higher in women with PCOS.

Clinical Characteristics	Healthy women (n =9)	Women With HA (n=6)	Women with PCOS (n=6)	P-value
Age (years)	28.2±5.0	25 (23.0,30.8)	24.5(21,26.3)	ns
Weight (Kg)	63.3±10.9	55.3(51.5,61.0)	61.2(51.9,72.1)	ns
Body Mass Index (Kg/m^2)	23.6±2.8	20.7(19.4,23.1)	23.2(18.4,25.1)	ns
Menstrual cycle length (Days)	28.1±1.2	366(319.5,366)	150(55.5,366)	<0.0001
Serum LH (iU/L)	3.9±1.6	2.9(1.4,3.6)	4.4(1.9,9.3)	ns
Serum FSH (iU/L)	4.7±1.3	5.2(4.6,6.3)	4.3(2.9,5.0)	ns
Serum Oestradiol (pmol/L)	97.0±39.9	72.5(50.5,110.3)	81(67.3, 107)	ns
Sex Hormone Binding Globulin (nmol/L)	75.8±40.6	65(39.8,100.3)	32(31.3,48.3)	0.04
Serum AMH (pmol/L)	18.9±12.3	20.8(14.9,23.2)	70.7(29.8,104.3)	0.01
Serum FSH-LH (iU/L)	1.3±2.1	2.8(0.8,4.1)	-0.3(-4.3,1.1)	ns

Table 4 Baseline characteristics of the healthy women, women with HA and women with PCOS who participated in the study. Mean ± SD is presented for parametrically distributed values and median (25^{th} centile, 75^{th} centile) is presented for non-parametrically distributed data. Comparison between the parameters were made using Kruskal-Wallis Dunn's multiple comparison test. Hormonal levels on the day of study visit were reported**.**

3.4.2 Gonadotrophin response following MVT-602 in healthy women, women with HA and PCOS

Serum LH rise following MVT-602 0.03nmol/kg was similar in women with PCOS and healthy women in the follicular phase. However, in women with HA, the rise in serum LH occurred sooner and was increased by more than 2-fold at 6 hrs when compared to healthy women and women with PCOS. In women with HA, a second smaller peak rise at 24 hrs was observed **(Figure 17A, 18A, 19)**. The initial brief delay of serum LH rise was observed in all women following MVT-602, however, in women with HA, the delay in LH rise was shorter compared to healthy women and women with PCOS (*Mean delay in LH rise for women with HA*: 0.05±0.04 hrs, *mean delay in LH rise for women with PCOS*: 4.1±6.7 hrs, *mean delay in LH rise for healthy women in follicular phase*: 5.4±3.7 hrs) **(Figure 17A)**. In women with HA, the serum FSH rise was increased by more than 4-fold when compared to that in the healthy follicular phase and to women with PCOS leading to a significantly greater area under the curve of FSH release **(Figure 17B, 18B)**. The exaggerated rise of serum FSH in women with HA resulted in a greater increase in serum oestradiol over the 48-hrs study period **(Figure 17C, 18C)**.

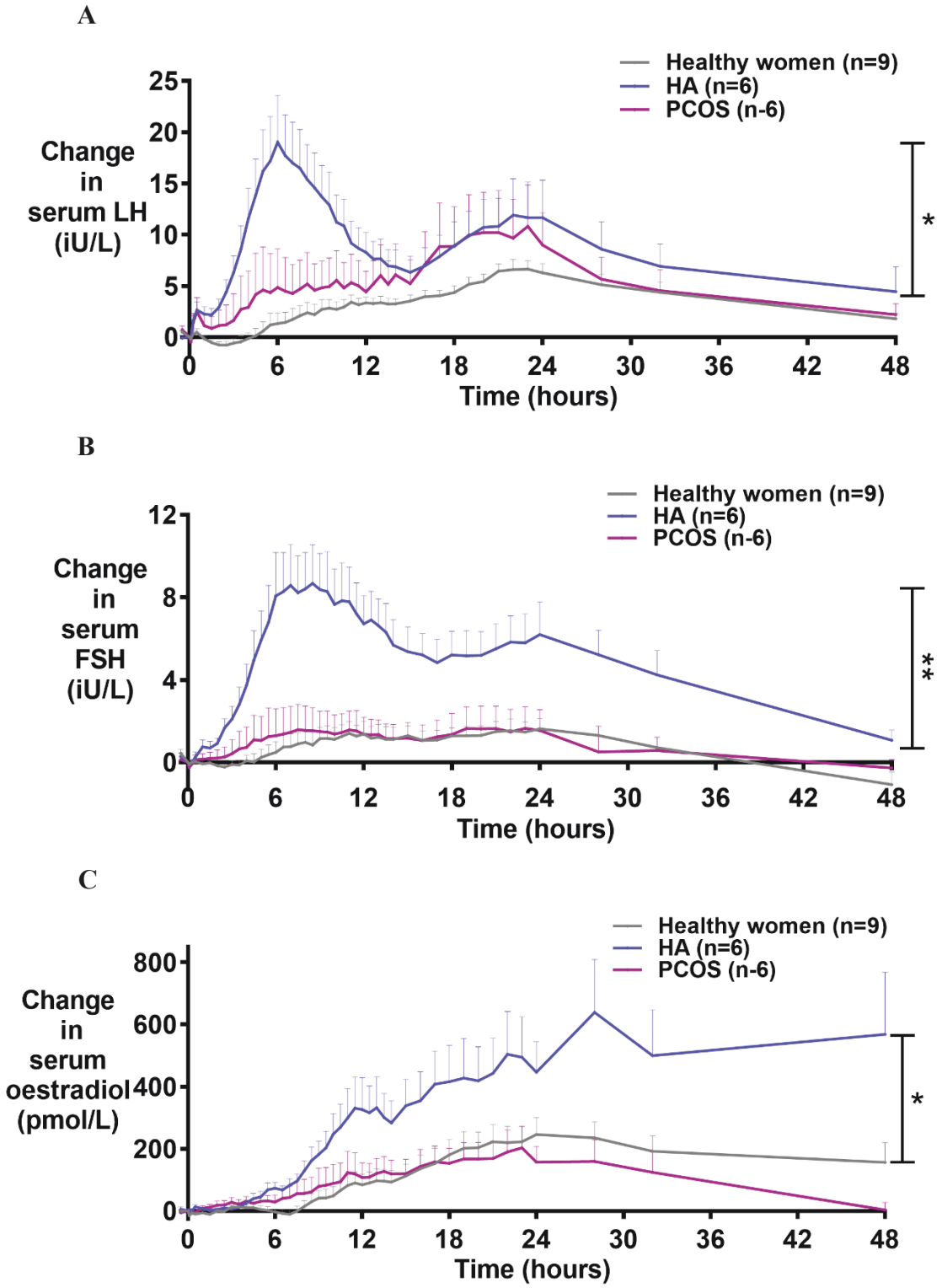


Figure 17 Mean±SEM of change from baseline levels in (A) Serum LH (iU/L) (B) Serum FSH (iU/L) (C) Serum oestradiol (pmol/L) in healthy women in grey (n=9), women with PCOS in purple (n=6) and women with HA in blue (n=6) following a single subcutaneous bolus of MVT-602 0.03nmol/kg.

Comparison between groups were made by two-way ANOVA. ** denotes P-value<0.001 and * denotes P-value<0.05.

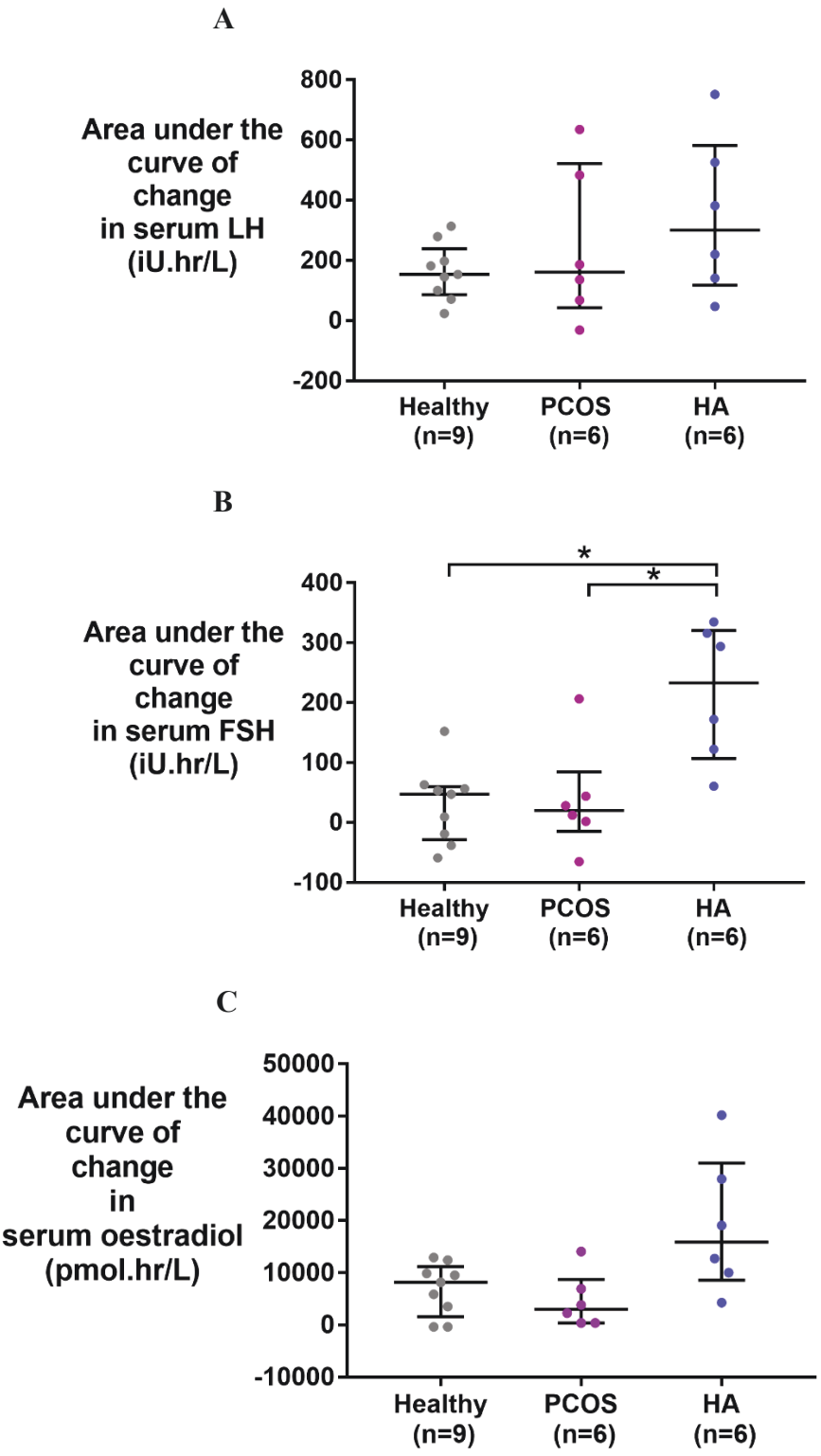


Figure 18 Scattergram (Median±IQR) of AUC of change in (A) Serum LH (iU.hr/L) (B) Serum FSH (iU.hr/L) (C) Serum oestradiol (pmol.hr/L) in healthy women in grey (n=9), women with PCOS in purple (n=6) and women with HA in blue (n=6) receiving a single subcutaneous dose of MVT-602 at

0.03nmol/kg. Comparison between the groups were made by using Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

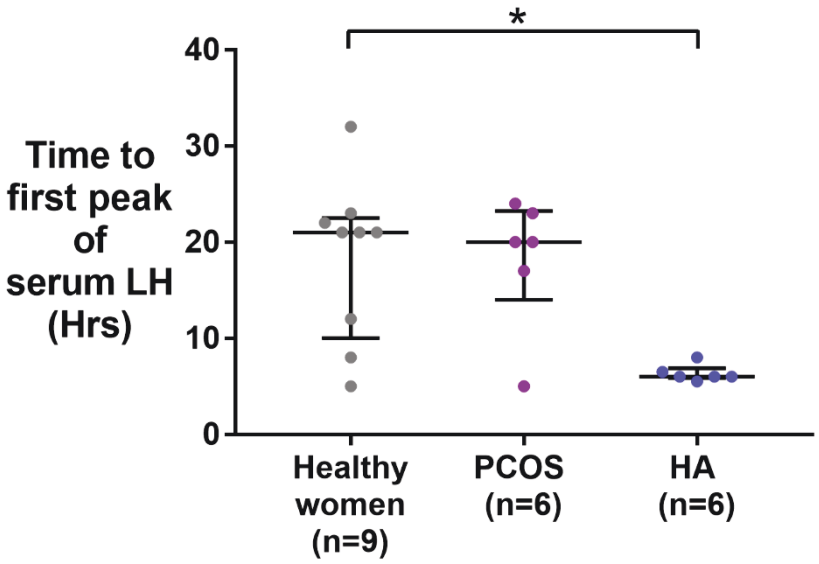
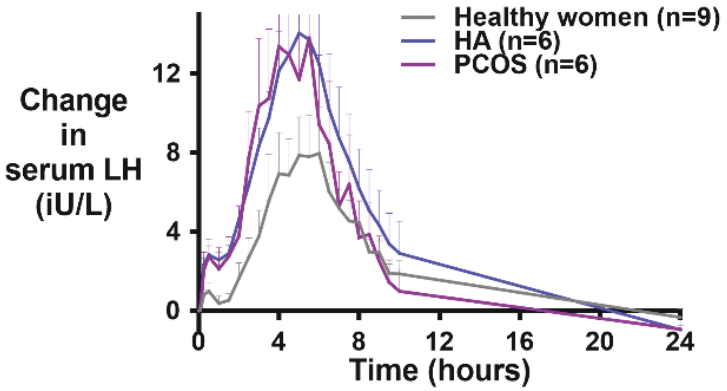


Figure 19 Scattergram (Median±IQR) of time to first peak of serum LH (hrs) in healthy women in grey (n=9), women with PCOS in purple (n=6) and women with HA in blue (n=6) receiving a single subcutaneous bolus of MVT-602 at 0.03nmol/kg. Comparison between the groups were made by using Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

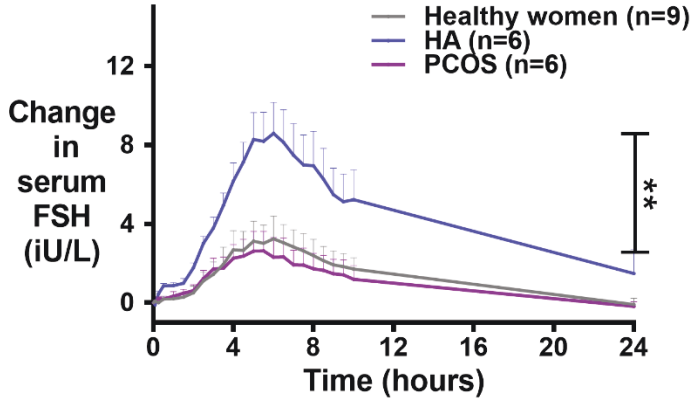
3.4.3 Gonadotrophin response following KP54 in healthy women, women with HA and PCOS

In response to a single subcutaneous dose of kisspeptin-54, serum LH peaked at 5.5 hrs in all women. Women with PCOS had a non-statistically greater median change in serum LH although this was predominantly driven by one individual. The rise in serum FSH was far more exaggerated in women with HA (P-value=0.04) whereas FSH rise in women with PCOS was similar to healthy women in the follicular phase. However, there was an overlap of serum oestradiol responses between the groups, with maximal rise in serum oestradiol as high as 544pmol/L in women with HA **(Figure 20 and Figure 21)**.

A



B



C

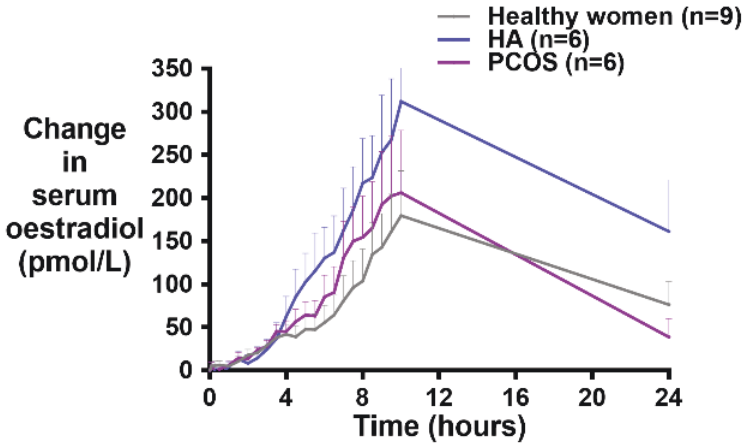


Figure 20 Mean±SEM of change from baseline levels in (A) Serum LH (iU/L) (B) Serum FSH (iU/L) (C) Serum oestradiol (pmol/L) in healthy women in grey(n=9), women with PCOS in purple(n=6) and women with HA in blue(n=6) receiving a single subcutaneous bolus of KP54 at 9.6nmol/kg. Comparison between the groups were made by two-way ANOVA. ** denotes P-value<0.01.

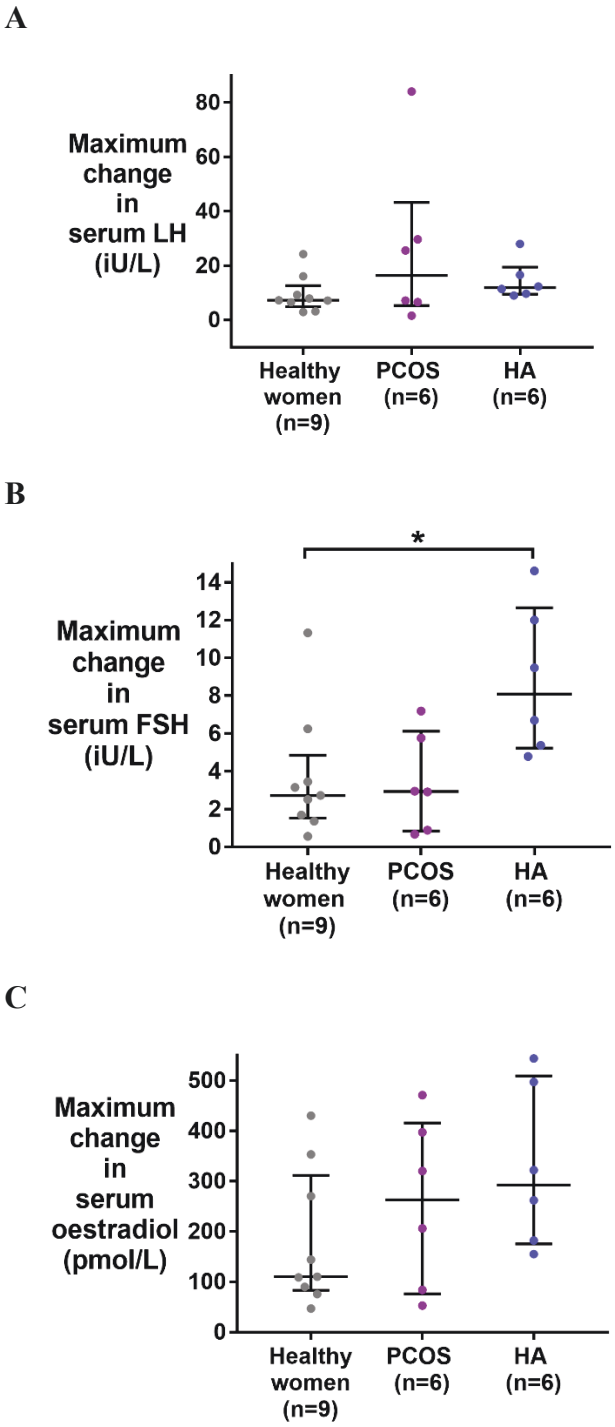


Figure 21 Scattergram (Median±IQR) of maximum change from baseline in (A) Serum LH (iU/L) (B) Serum FSH (iU/L) (C) Serum oestradiol (pmol/L) in healthy women in grey (n=9), women with PCOS in purple (n=6) and women with HA in blue (n=6) receiving a single subcutaneous dose of KP54 at 9.6nmol/kg. Comparison between the groups were made by using Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

3.4.4 Gonadotrophin response following KP54 to MVT-602 within each category of women

Within each group of women, MVT-602 0.03nmol/kg stimulated a serum gonadotrophin change that was of similar amplitude to that observed following KP54 9.6nmol/kg. Gonadotrophin rise following KP54 and MVT-602 were of similar amplitude in all three groups of women **(Figure 22)**.

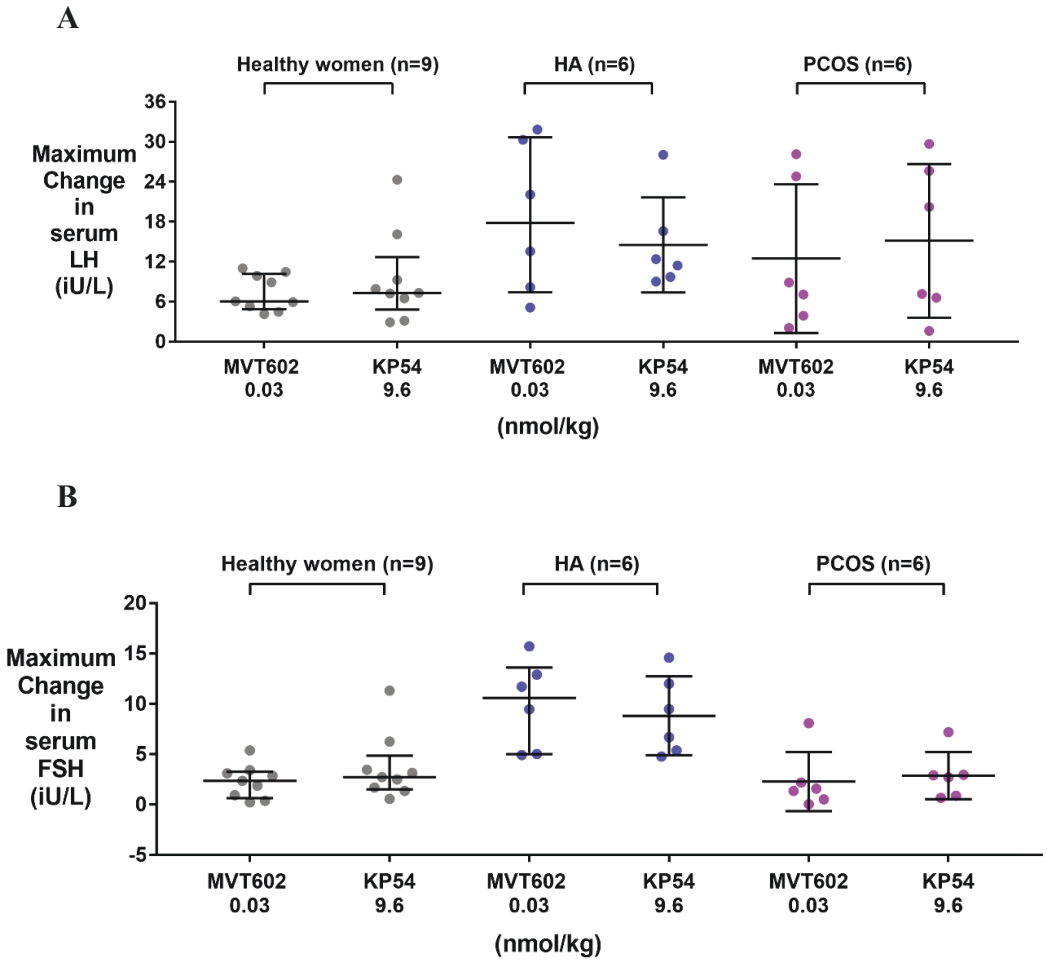
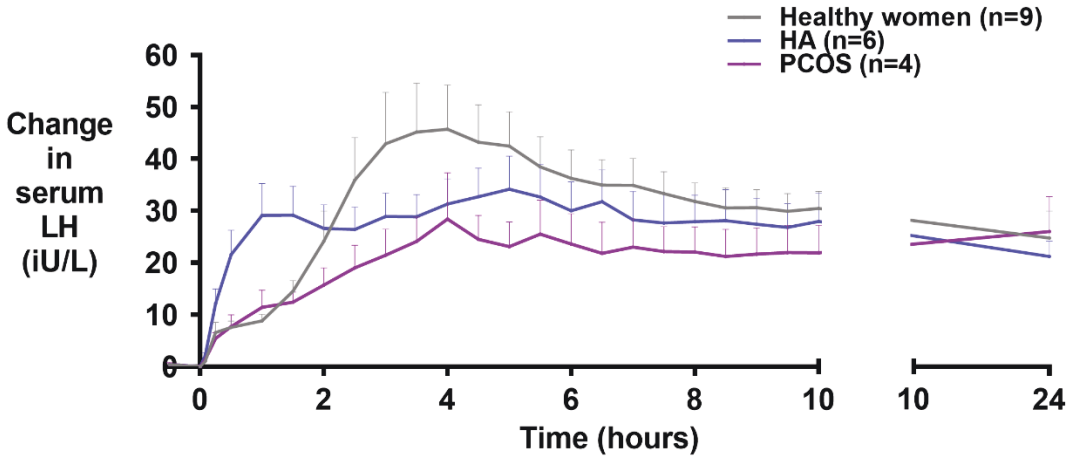


Figure 22 Scattergram (Median±IQR) of maximum change from baseline in (A) serum LH (iU/L) (B) serum FSH (iU/L) after a single subcutaneous bolus of MVT-602 0.03nmol/kg, KP54 9.6nmol/kg in healthy women in grey (n=9), women with HA in blue (n=6) and women with PCOS in purple (n=6) Comparison was made within each group of women by Mann-Whitney test and no statistical difference in gonadotrophin levels were observed in each group.

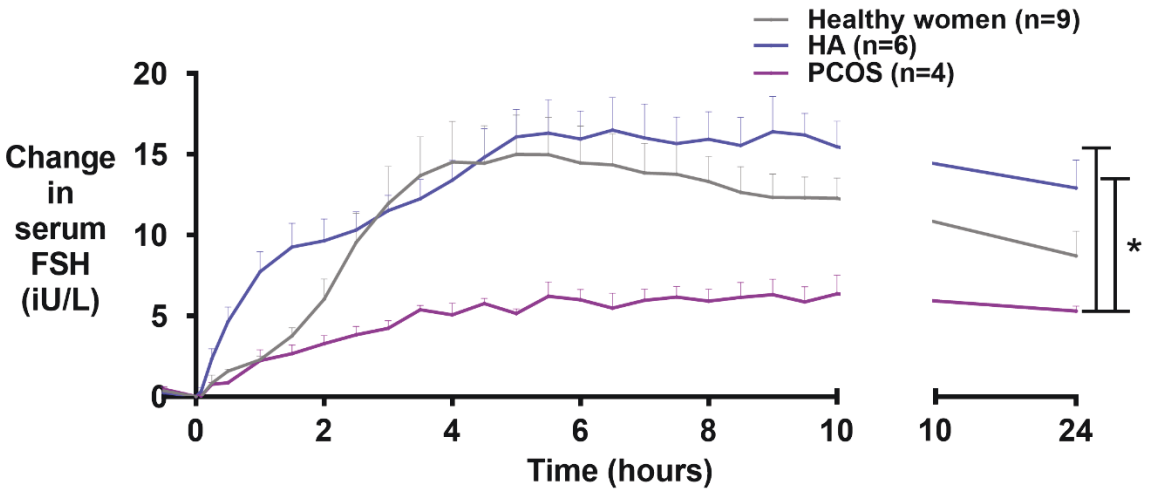
3.4.5 Hormonal profile following triptorelin in healthy women, women with HA and women with PCOS

A single subcutaneous dose of triptorelin at 0.2mg induced a serum LH response of a similar amplitude in all women. However, the rise in serum LH occurred earlier in women with HA at 2.3 hrs whereas peak serum LH occurred at ~4.5 hrs in healthy women and at ~4.3 hrs in women with PCOS. Women with PCOS had a far attenuated change in serum FSH compared to healthy women in the follicular phase and women with HA (P-value=0.01). However, the change in serum oestradiol following pituitary stimulation was similar in all women **(Figure 23 and Figure 24)**.

A



B



C

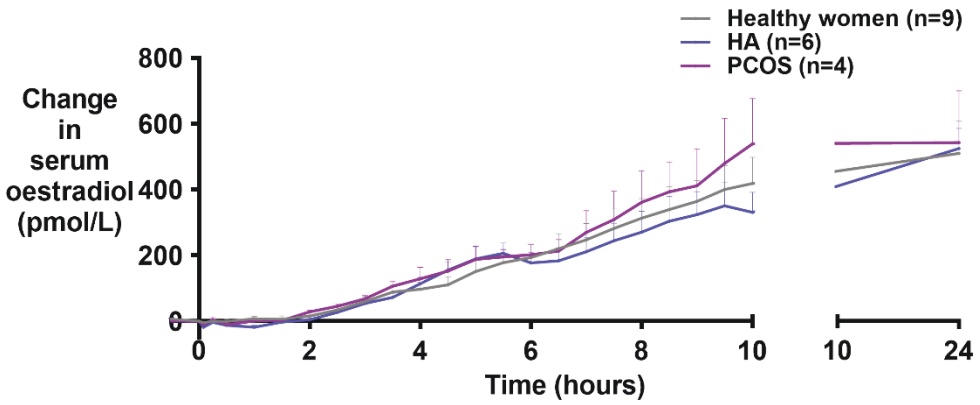
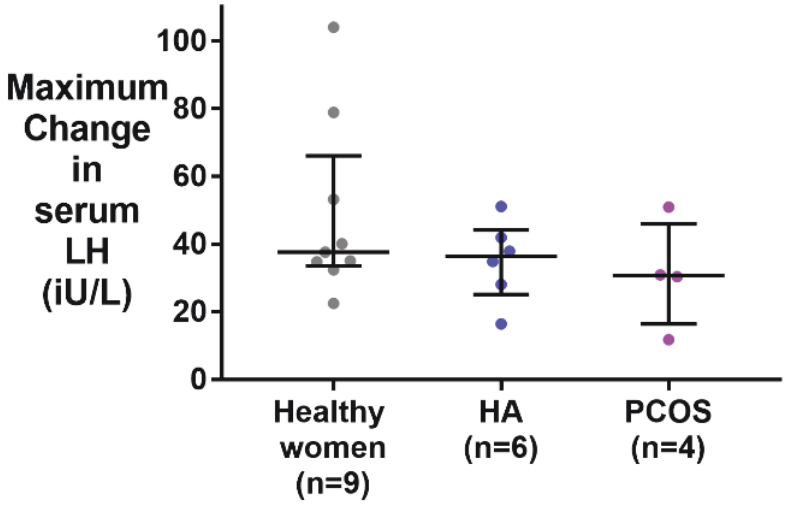


Figure 23 Mean±SEM of change from baseline in (A) Serum LH (iU/L) (B) Serum FSH (iU/L) (C) Serum oestradiol (pmol/L) in healthy women in grey (n=9), women with PCOS in purple (n=6) and women with HA in blue (n=6) after a single subcutaneous bolus of triptorelin at 0.2mg. Comparison between groups were made by two-way ANOVA. * denotes P-value<0.05.

A



B

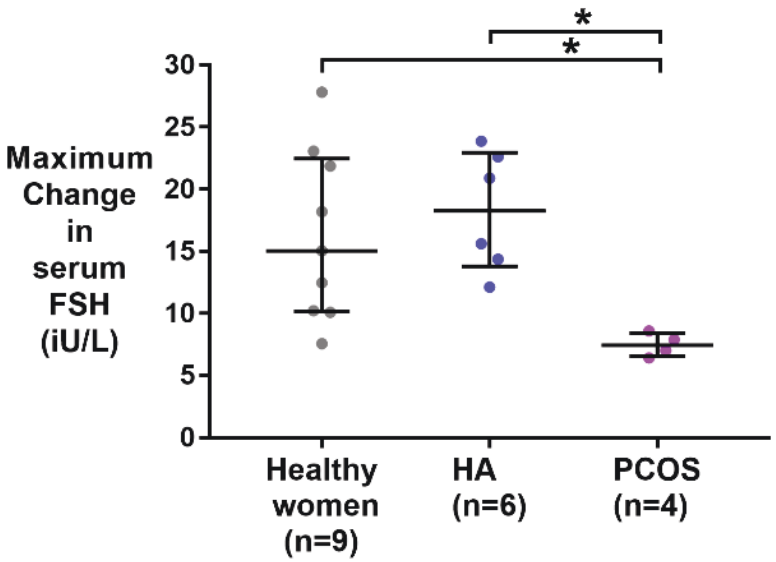


Figure 24 Scattergram (Median±IQR) of maximum change from baseline in (A) serum LH (iU/L) (B) serum FSH (iU/L) in healthy women in grey (n=9), women with HA in blue (n=6) and women with PCOS in pink (n=6) after receiving a subcutaneous of Triptorelin 0.2mg. Comparison was made by Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

3.4.6 Differential secretion of serum LH and FSH following KP54, MVT-602 and triptorelin in women

Previous data suggested that pituitary sensitivity is unlikely to be increased in women with HA **(Figure 23A, 24A)**. As the response to MVT-602 includes the pituitary response, I compared the ratio of serum LH rise following MVT-602 to that of triptorelin to determine the contribution from the hypothalamic component in the serum gonadotrophin response. The ratio of serum LH rise following MVT-602 to that of serum LH rise after triptorelin was 0.5 in women with HA and 0.1 in healthy women and 0.2 in women with PCOS **(Figure 25A)**. Similarly, the ratio of serum FSH rise after hypothalamic stimulation by MVT-602 to that of serum FSH rise after pituitary stimulation by triptorelin was significantly higher in women with HA than healthy women **(Figure 25 B)**. Notably, women with PCOS displayed a far more attenuated serum FSH response to MVT-602 0.03nmol/kg, KP54 9.6nmol/kg and triptorelin 0.2mg when compared to healthy women or women with HA **(Figure 26)**.

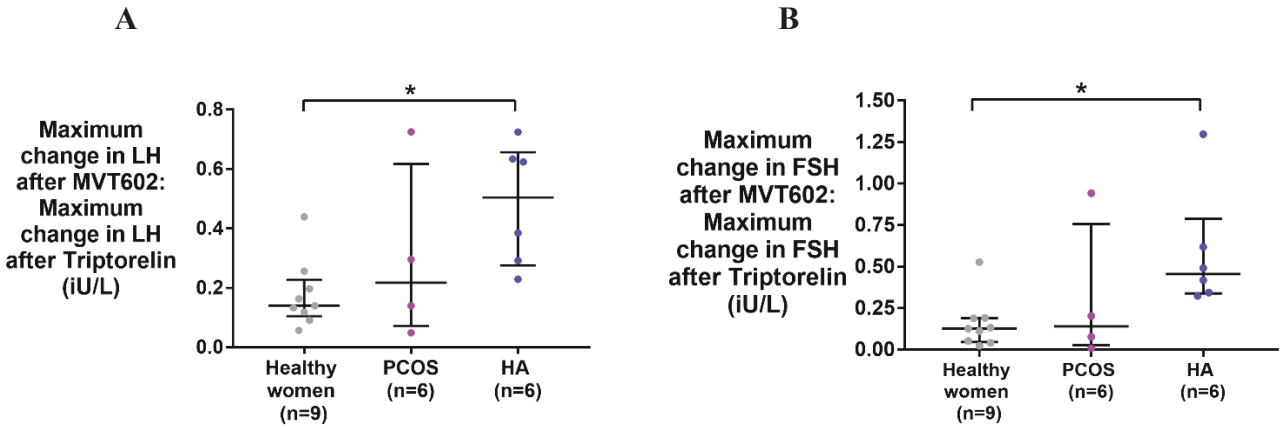


Figure 25 Scattergram (Median±IQR) of ratio of maximum change in (A) serum LH (iU/L) following MVT-602 to the maximum change in serum LH following triptorelin (B) serum FSH (iU/L) following MVT-602 to the maximum change in serum FSH following Triptorelin in healthy women in grey (n=9), women with HA in blue (n=6) and women with PCOS in pink (n=6). Comparison was made by Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

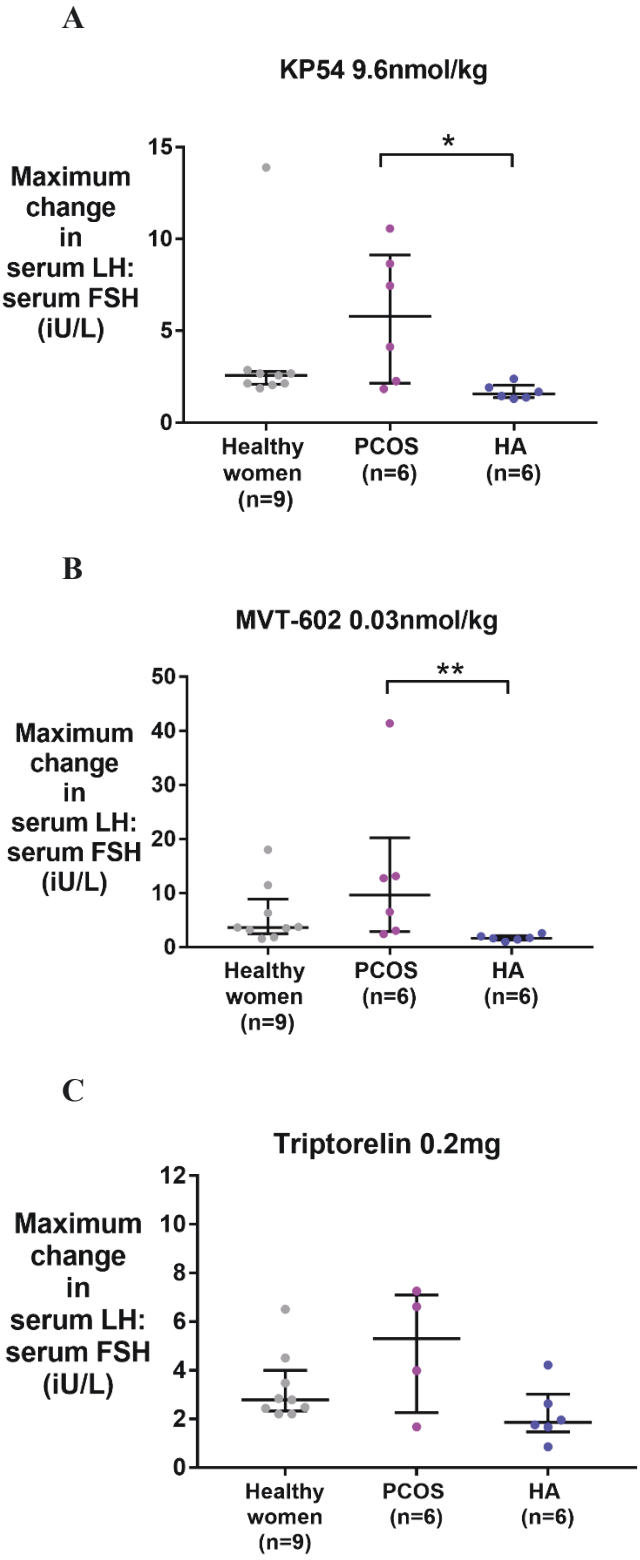


Figure 26 Scattergram (Median±IQR) of the ratio of maximum change in serum LH to the maximum change in serum FSH after (A) KP54 9.6nmol/kg (B) MVT-602 0.03nmol/kg (C) Triptorelin 0.2mg in healthy women in grey (n=9), women with HA in blue (n=6) and women with PCOS in pink (n=6). Comparison was made by Kruskal-Wallis Dunn's multiple comparison test. * denotes P-value<0.05.

3.4.7 Determinants of serum gonadotrophin responses

The change in serum LH and serum FSH following MVT-602 was significantly associated with basal oestradiol levels in women with HA (P=0.01), but not in the healthy women in the follicular phase nor in women with PCOS. Though a positive association was observed following MVT-602 administration, basal oestradiol level did not correlate with response in women following KP54 9.6nmol/kg **(Figure 27)**. Serum inhibin B and serum AMH did not associate with the change in serum FSH response **(data not shown as no correlation)**.

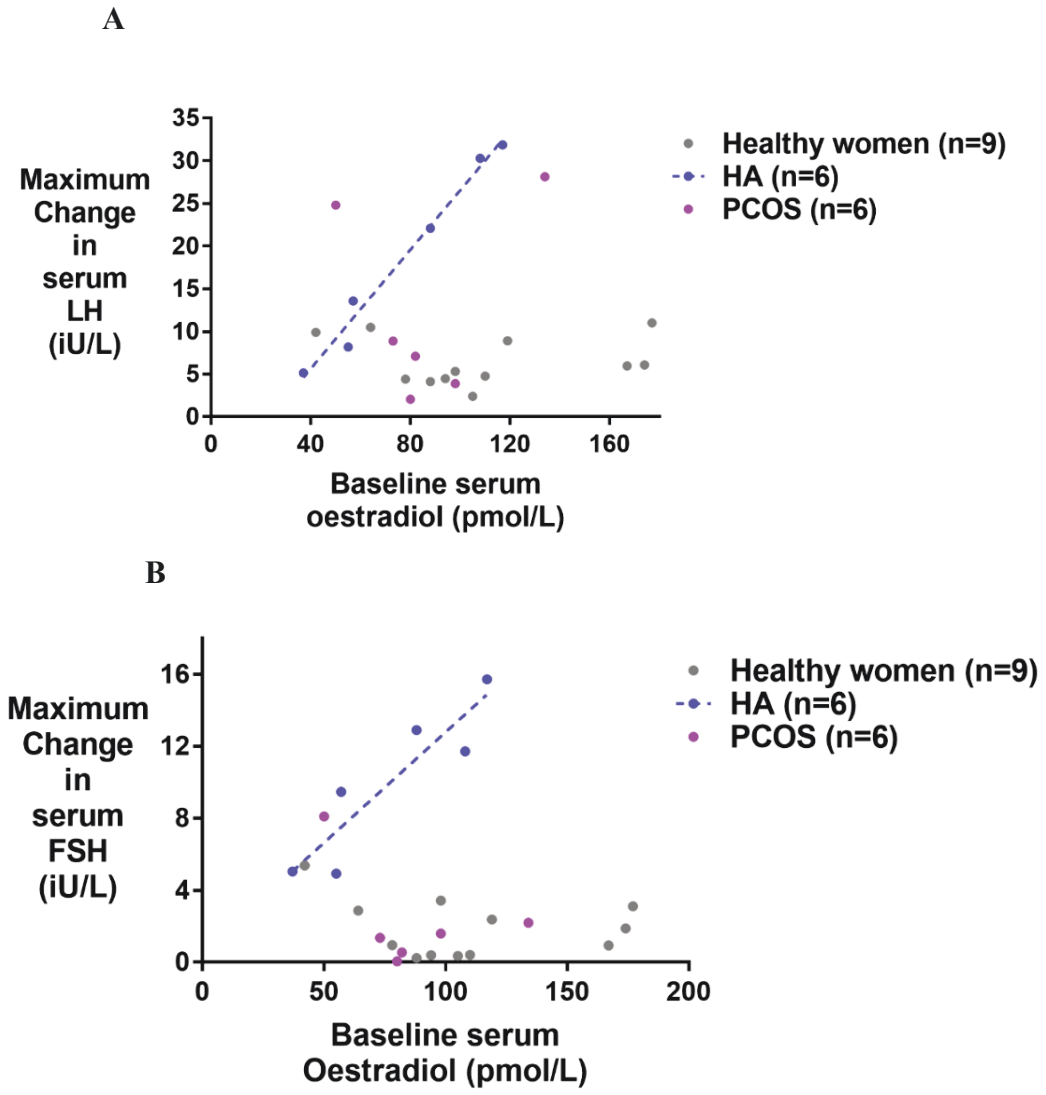


Figure 27 Linear correlation of maximum change in (A) serum LH (iU/L) (HA: P-value=0.002, r^2=0.98) (B) serum FSH (iU/L) (HA: P-value=0.01, r^2=0.83) to baseline serum oestradiol (pmol/L) following a

single dose of MVT 0.03nmol/kg in healthy women in grey (n=9), women with HA in blue (n=6) and women with PCOS in pink (n=6).

3.5 DISCUSSION

In this study, I investigated the effects of MVT-602 in women with subfertility due to HA and PCOS and compared their responses to healthy women in the follicular phase of their menstrual cycle. The study showed that acute administration of MVT-602 to women with HA resulted in an augmented stimulation of gonadotrophins and a sustained rise in oestradiol. This highlights the potential for MVT-602 to restore reproductive health in anovulatory women with HA.

My analyses revealed that the acute LH response following an equimolar weight-adjusted dose of MVT-602 was augmented by at least 2-fold in women with HA as compared to healthy females in the follicular phase. Whilst the pituitary sensitivity in women with HA was unlikely to be increased, there was a notable relative increase in hypothalamic response in women with HA. This can be seen from the higher ratio of serum gonadotrophin response after MVT-602 administration to the serum gonadotrophin response after triptorelin administration in women with HA. The higher ratio suggests that there is likely to be an additional element of enhanced GnRH neuronal sensitivity to kisspeptin in women with HA. Indeed, in our previous studies, women with HA were noted to be more sensitive to KP54 than healthy women in the follicular phase (Jayasena *et al.*, 2009). Pubertal rats subjected to conditions of negative energy balance (for up to 72 hrs) showed a significant suppression of the hypothalamic KISS1 expression and an enhanced gonadotrophin release to exogenous kisspeptin stimulation (Castellano *et al.*, 2005, 2010). Other researchers also reported a prolonged and augmented LH release following infusion of KP10 in female rats subjected to chronic subnutrition (Roa *et al.*, 2008). My findings are consistent with these results and suggest that the enhanced serum LH rise observed following kisspeptin administration could be due to a compensatory increase in KISS1 receptors in response to the prevailing metabolic state (Castellano *et al.*, 2005; Iwasa *et al.*, 2010; Matsuzaki *et al.*, 2011).

One of the most salient observations of my study was the augmented FSH secretion following MVT-602/KP54 in women with HA. Indeed, circulating levels of serum FSH were at least four-fold higher in

women with HA than healthy women (P-value=0.02) and women with PCOS (P-value=0.02) and this enhanced FSH response led to a significant increase in serum oestradiol. Our group had previously shown that once or twice daily administration of KP54 to women with HA resulted in tachyphylaxis (Jayasena *et al.*, 2009, 2010). Paradoxically, twice weekly administration of KP54 over an 8 weeks period restored gonadotrophin responses but the magnitude of FSH responses were inadequate to facilitate ovarian follicular growth (Jayasena *et al.*, 2009, 2010). Thus, the novel finding of a more pronounced serum FSH profile in women with HA suggests a potential utility of MVT-602 to restore reproductive health in anovulatory women with HA.

Interestingly, serum FSH responses to exogenous KP54/MVT-602 or triptorelin were relatively attenuated in women with PCOS. These data seem to disclose that in women with PCOS, conditions of persistently elevated kisspeptin tone might lead to a clear-cut dissociation of gonadotrophin levels. Higher kisspeptin pulse frequencies have been reported in oligomenorrheic PCOS women (Katulski *et al.*, 2018). Therefore, it seems plausible to speculate that conditions of higher endogenous kisspeptin pulse frequencies (Panidis *et al.*, 2006; Katulski *et al.*, 2018) might have prevented a further rise in serum LH following exogenous kisspeptin administration. Consequently, acute administration of kisspeptin/MVT-602 may not be able to significantly alter the patterns of pulsatile release of GnRH, as previously reported in female rats (Roa *et al.*, 2008). It is noteworthy that gonadal hormones such as baseline inhibin and serum AMH levels do not correlate with the changes in serum FSH. Nonetheless, effects of chronic administration of MVT-602, have so far, not been explored and it is possible that the observed phenomenon might alter with prolonged exposure to kisspeptin. Further analyses of serum gonadotrophin profiles following repeated MVT-602 administration might shed light on the underlying mechanisms.

Oestradiol can be an important determinant of LH responses to kisspeptin. Healthy females respond more to kisspeptin in the pre-ovulatory phase in the presence of high oestradiol levels (Dhillo *et al.*, 2007). Thus, I compared basal oestradiol levels to LH response in all the intervention given. Notably,

an association is observed between basal oestradiol and serum gonadotrophin following a subcutaneous bolus MVT-602 0.03nmol/kg in women with HA but not in healthy women or women with PCOS. This suggested that at this dose, the higher the basal oestradiol, the better the response to MVT-602 in women with HA. It is unclear why such a disparity would occur among the groups of women. It is possible that sex steroid feedback mechanisms are entirely different in women with HA. Further work to delineate the sex steroid feedback pathway in women with HA are indicated.

Regardless of whether women had ovulatory cycles or anovulatory cycles, a single bolus of MVT-602 induced a rise in serum LH with a similar amplitude as native KP54 9.6nmol/kg. This indicates the remarkably consistent limited secretion that can be accessed via the kisspeptin receptor, as compared with direct pituitary GnRH stimulation. For example, more than a 4-fold rise in serum gonadotrophins was observed following triptorelin when compared with MVT-602 in healthy women. This would be consistent with the observation that not all GnRH neurons express KISS1R and some GnRH neurons are not contiguous with pituitary GnRH receptors (Han *et al.*, 2005; Irwig *et al.*, 2004). Thus, equimolar doses of GnRH can far exceed the gonadotrophin secretion induced through kisspeptin-based therapeutics (Jayasena *et al.*, 2014). Repeated dosing with GnRH receptor agonists can be associated with a risk of desensitisation (Clarke *et al.*, 1985; Broekmans *et al.*, 1993). As such, activation of the endogenous releasable pool of GnRH via kisspeptin receptors might be a more physiological means to intervene in the HPG axis compared to the excessive pituitary simulation by longer-acting GnRH receptor agonists.

3.5.1 Limitations

As this is the first exploratory study conducted in women with subfertility, it would be helpful to repeat the studies in larger cohorts and in women with other causes of subfertility (e.g. women with hyperprolactinaemia). Nonetheless, as mentioned previously, the recruitment is limited by the prolonged study duration per visit (24 hrs study visit) and the long study period (at least 6 months with one

intervention per month). Further blood sampling between 10 hrs and 24 hrs following triptorelin 0.2 mg would be helpful to delineate the serum gonadotrophin profile. The HPG axis in females is highly variable with feedback interactions, thus measurement of leptin and insulin might shed more light into the potential interaction between serum gonadotrophin responses.

3.6 SUMMARY

In summary, I present novel data on the hormonal profile in women with HA, women with PCOS and healthy women following administration of MVT-602. In this chapter, I have shown that MVT-602 induces an augmented gonadotrophin response in women with HA. More importantly, the exaggerated FSH response increased serum oestradiol in women with HA, therefore highlighting a potential role for MVT-602 to be used as an ovulation induction agent in women with anovulatory HA. Further studies are indicated to evaluate repeated administration of MVT-602 as a novel therapeutic approach to restore fertility in HA.

3.7 FUTURE WORK

Overall, my findings showed an augmented gonadotrophin and oestradiol profile following MVT-602 in women with HA. Nonetheless, further validation of the therapeutic potential of MVT-602 in chronic protocols in women with HA is indicated. Of note, women with PCOS exhibit an attenuated FSH response to KP54/MVT-602 in comparison to women with HA. These findings allude to different feedback pathways in women with different aetiologies of amenorrhoea. Admittedly, the intricate mechanisms causing such differential responses requires further research. More importantly, this observed differential gonadotrophin response presents the possibility of using KP54 as a diagnostic tool in women presenting with anovulatory disorders and could resolve the diagnostic dilemma between HA and PCOS, faced by many clinicians.

CHAPTER 4

THE EFFECT OF KISSPEPTIN ON GONADOTROPHIN RESPONSES IN HEALTHY MEN AND MEN WITH CONGENITAL HYPOGONADOTROPHIC HYPOGONADISM (CHH)

CHAPTER 4: THE EFFECT OF KISSPEPTIN ON GONADOTROPHIN SECRETION IN HEALTHY MEN AND MEN WITH CONGENITAL HYPOGONADOTROPHIC HYPOGONADISM

4.1 INTRODUCTION

Hypogonadotrophic hypogonadism (HH) is characterised by hypogonadism in the context of low/inappropriately normal gonadotrophin levels (Boehm *et al.*, 2015). When assessing patients with HH, a specific clinical test of pituitary function can be conducted i.e. gonadotrophin releasing hormone (GnRH) test. However, GnRH test is often of 'poor diagnostic value' and could not reliably distinguish men with partial HH from healthy controls. At present, a direct test of hypothalamic GnRH neuronal function that would be expected to have greater diagnostic specificity in patients with defects of GnRH secretion, is yet to be available (Boehm *et al.*, 2015). Therefore, establishing a test of hypothalamic GnRH neuronal function would offer the unique ability to delineate the precise defect in patients with hypogonadotrophic hypogonadism and offer greater diagnosis accuracy.

In 2003, seminal reports from two independent research laboratory groups described congenital HH (CHH), with failure to proceed through puberty, due to mutations affecting the kisspeptin receptor. Following that, a wealth of animal studies has confirmed that kisspeptin is a specific stimulator of hypothalamic GnRH function and plays a quintessential role in reproductive physiology (Dhillo *et al.*, 2005, 2007; Abbara *et al.*, 2013). Thereafter, our group and others have explored the physiological effects of kisspeptin in healthy men (Dhillo *et al.*, 2005), healthy women (Dhillo *et al.*, 2007), women with hypothalamic amenorrhoea (Jayasena *et al.*, 2009, 2010; Jayasena *et al.*, 2013), subfertility (Jayasena *et al.*, 2014; Abbara *et al.*, 2017), in patients with chronic hyperprolactinaemia (Millar *et al.*, 2017), or men with diabetes related hypogonadism (George *et al.*, 2013).

The ability of kisspeptin to directly stimulate hypothalamic GnRH release offers the opportunity to specifically interrogate hypothalamic GnRH neuronal function when assessing patients with HH. A subset of patients with HH are those with Congenital Hypogonadotrophic Hypogonadism (CHH).

Typically, such patients have mutations in genes that encode GnRH neuronal migration and or function (Seminara *et al.*, 1998; Boehm *et al.*, 2015). In half of these CHH cases, patients may present with anosmia, which is also termed as "Kallmann syndrome". Other mutations may also be associated with non-reproductive features such cleft lip/palate, dental agenesis, hearing impairment, renal agenesis, bimanual synkinesis or skeletal anomalies (Seminara *et al.*, 1998; Boehm *et al.*, 2015). Though genetic testing has been informative in the diagnosis of CHH, the diverse phenotypes and genetic heterogeneity are formidable obstacles to the accurate determination of the disease (Boehm *et al.*, 2015). In clinical practice, a GnRH test is sometimes used to help diagnose these patients. Whilst a blunted GnRH test may identify men with CHH, a partial GnRH stimulated response could not reliably distinguish men with partial CHH from healthy controls (Boehm *et al.*, 2015; Bang *et al.*, 2017). Hence, patients with CHH represent a suitable cohort to investigate the potential for development of kisspeptin as a specific diagnostic test of hypothalamic GnRH function.

In contrast to patients with functional hypogonadism, patients with CHH may have ultrastructural deficits, such as a failure of GnRH neuronal migration and thus would not be expected to respond to kisspeptin. Thus, in this chapter, I investigated the performance of a KP54 challenge test to that of a GnRH test in identifying men with CHH from healthy men.

4.2 AIMS AND HYPOTHESIS

4.2.1 Aims

(1) To determine and compare the gonadotrophin responses in healthy men and men with CHH following a single intravenous administration of KP54 (6.4nmol/kg) and GnRH (100 micrograms).

4.2.2 Hypothesis

(1) Men with CHH do not exhibit gonadotrophin response to an intravenous bolus of KP54 to the same magnitude as healthy individuals.

(2) The graduation of gonadotrophin responses to KP54 can be used to identify men with CHH from healthy men.

(3) A KP54 challenge test performs better than the currently available GnRH test in the assessment of hypothalamic function in men with CHH.

4.3 MATERIALS AND METHOD

4.3.1 Ethical approval

Ethical approval for this study was granted by the West London Research Ethics Committee, London, UK (reference: 12/LO/0507). All participants provided informed consent and the study was conducted in accordance with the Declaration of Helsinki.

4.3.2 Subjects

Healthy eugonadal men (n=21) and men with CHH (n=21) were recruited through newspaper advertisements or from endocrine clinics. All participants underwent a detailed medical assessment prior to participation in the study including full medical history and clinical physical examination. Eugonadal healthy men fulfilled the following criteria: age 18-35 years, BMI 18-30 kg/m^2, absence of significant systemic disease or co-morbidity, absence of recreational or therapeutic drug use, and a normal clinical and biochemical reproductive function. Men with CHH had HH with a history of incomplete progression through puberty. Further examination in men with CHH included measurement of height, weight, arm span, testicular volume using a Prader orchidometer, signs of hypopituitarism and evaluation for non-reproductive clinical features e.g. cleft palate, synkinesis. Olfactory function in men with CHH was quantified objectively using the University of Pennsylvania 40-items Smell Identification Test (UPSIT) and subjectively by a self-reported inability to smell. All participants had measurement of serum levels of luteinising hormone (LH), follicle stimulating hormone (FSH), testosterone, inhibin B (IHB), anti-Müllerian hormone (AMH), prolactin, sex hormone binding globulin (SHBG) and inhibin B at baseline. Men with CHH also had genetic testing to identify genes implicated in the etiology of CHH.

4.3.2 Study protocol

Recruited participants attended the Clinical Research Unit at Imperial College Healthcare NHS Trust for 2 study visits. Study visits were commenced a 9am and participants were able to eat and drink *ad libitum*. Participants were asked to refrain from strenuous exercise, sexual activity and to abstain from

alcohol, caffeine and tobacco for the 24 hrs period preceding each study visit. On arrival, an intravenous cannula was inserted into the antecubital fossa. After a 30 minutes period of baseline blood sampling, a single intravenous bolus of either KP54 (6.4nmol/kg) or GnRH (100 mcg) was administered, in random order. Serial blood sampling was continued every 15 minutes for 6 hrs. A second study visit was conducted following a washout period of at least one week to receive the other study hormone. A summary of the study protocol is shown in **Figure 28**.

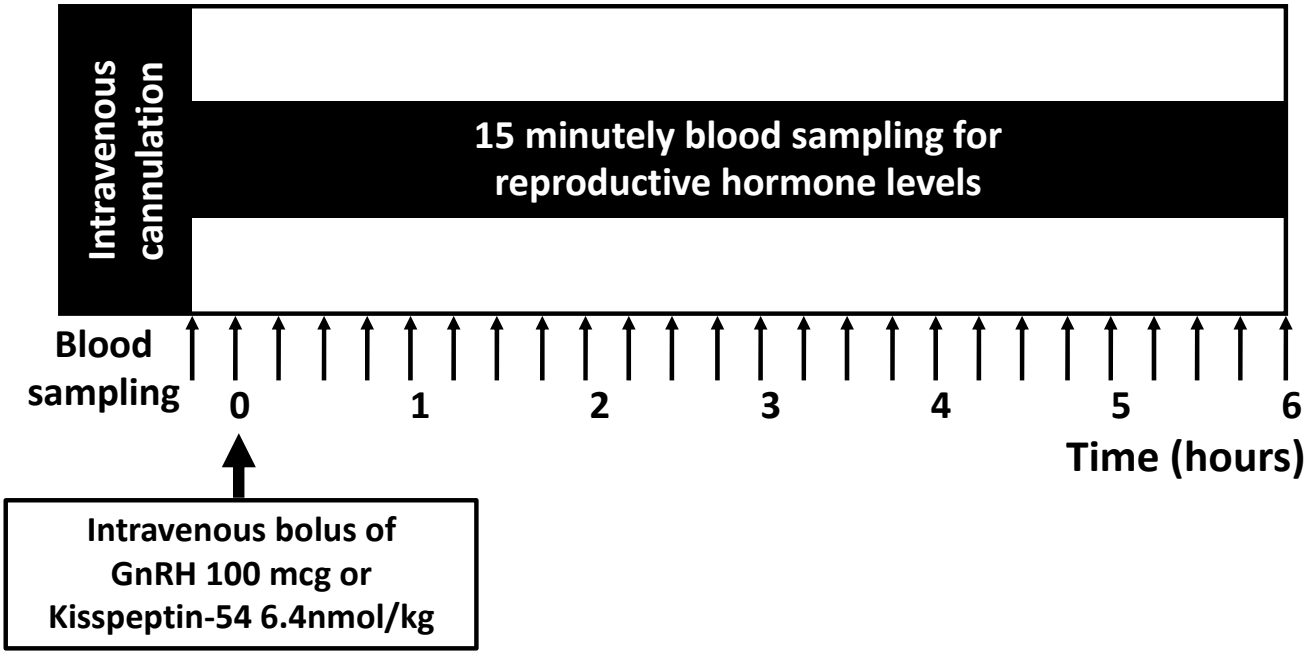


Figure 28 Protocol diagram showing the study visit for healthy males and men with CHH. Reproductive hormone levels were taken every 15 minutes for up to 6 hrs. Reproductive hormones taken include serum LH, serum FSH and serum testosterone.

The dose of KP54 was selected based on a preliminary dose-finding study in five healthy men who received a single intravenous bolus of 6.4, 12.8 and 25.6nmol/kg on subsequent visits at least one week apart **(Figure 29)**. All three doses elicited robust rises in serum LH with no significant effect of dose (P=0.57 by two-way ANOVA), thus we chose 6.4nmol/kg to represent a near-maximal response to KP54 **(Figure 29)**. Lippincott *et al.* have demonstrated the sex-steroid milieu does not influence the response to kisspeptin, however, men with CHH on testosterone gel were asked for at least 1 week prior to

participation in the study. To wash out longer acting intramuscular preparations of testosterone undecanoate would have taken many months to a year to fully washout, and thus study visits were conducted at trough levels prior to the next injection. Men with CHH did not receive gonadotrophin therapy for at least 3 months prior to participation in the study.

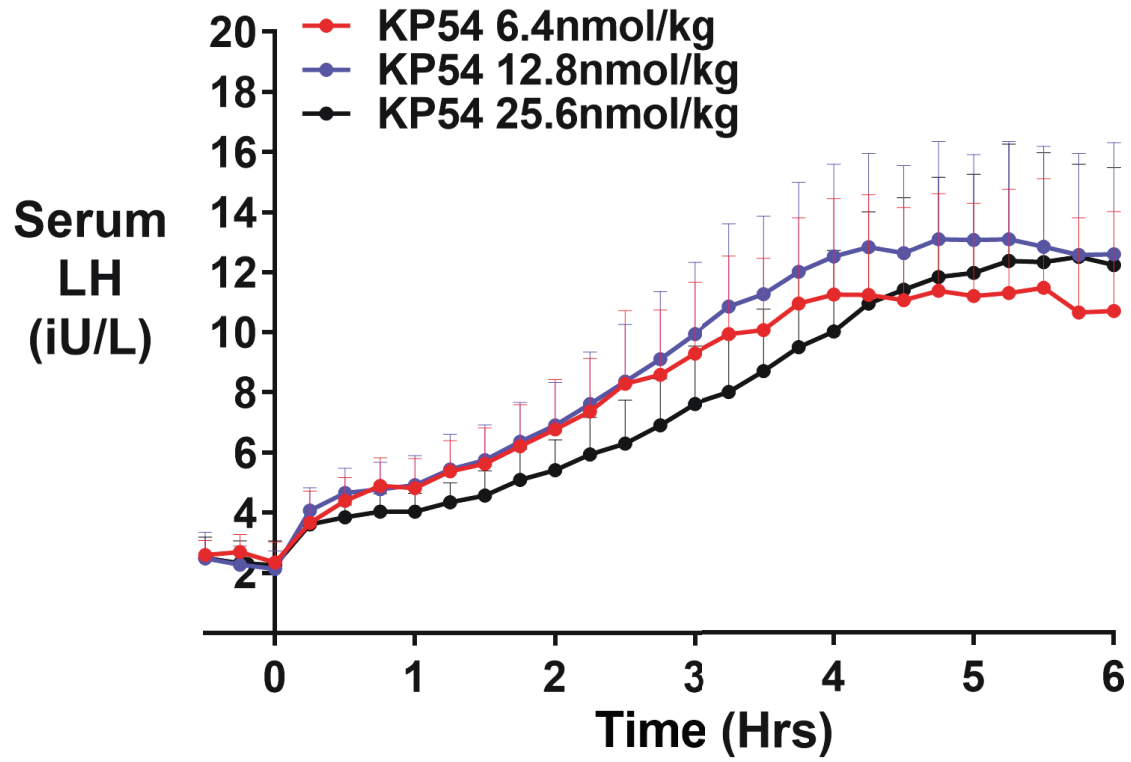


Figure 29 Serum LH (iU/L) levels from baseline over 6 hrs in healthy men (n=5) receiving an intravenous bolus of KP54 at 6.4nmol/kg, 12.8nmol/kg and 25.6nmol/kg. Change in serum LH did not significantly differ between doses when compared by two-way ANOVA (P=0.57).

4.3.3 Peptide

Human KP54 was synthesised by Bachem AG (Liverpool, UK) and further purified and tested as previously described consistent with the standards for physiological research studies. Vials of freeze-dried KP54 were stored at -20º and then reconstituted with 0.9% saline. Gonadorelin 100ug (GnRH) was purchased from Intrapharm Laboratories Ltd (Maidenhead, Berks, UK) and was reconstituted with 1ml of sterile water for injection prior to administration.

4.3.4 Hormone assays

Blood samples were collected in plain serum vacutainer tubes at fifteen minutes intervals throughout the 6 hr study. The samples were allowed to clot prior to centrifugation at 1210 g for 10 minutes and the serum was separated and then frozen at -20ºC until analysis. Frozen samples were defrosted and analyzed for measurement of serum LH, FSH and testosterone at all time points using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Reference range for serum LH for men are 2-12iU/L, serum FSH 1.7-8iU/L, serum testosterone 10-30nmol/L. The respective intra-assay and interassay coefficients of variation, LH 4.1% and 2.7%; FSH 4.1% and 3.0%, and total testosterone 4.2% and 2.8%. Analytical sensitivities were 0.03iU/L (LH), 0.05iU/L (FSH), and 0.05nmol/L (total testosterone).

4.3.5 Statistics

Statistical analyses were conducted using Graphpad Prism version 8.0. Continuous variables that were parametrically distributed were reported by mean ± standard deviation (SD), whereas skewed continuous variables were summarised using median±interquartile range (IQR). Parametrically distributed variables were compared using unpaired student's t-test (two groups) or one-way ANOVA (multiple groups). Non-parametrically distributed variables were compared using Kruskal-Wallis test or Mann Whitney U Test as appropriate. The relationship between two continuous variables was assessed by simple linear regression.

4.3.6 Genetic testing

Exome sequencing in CHH patients was performed by Health 2030 Genomic Center in Geneva and analyzed using previously described methods (Cassatella *et al.*, 2018). Forty-five CHH genes were included in this study: *ANOS1, AMH, AMHR2, CCDC141, CHD7, DCC, DMXL2, FEZF1, FGF17, FGF8, FGFR1, FSHB, GNRH1, GNRHR, HS6ST1, IL17RD, KISS1, KISS1R, KLB, LEP, LEPR, LHB, NDNF, NR0B1, NSMF, NTN1, OUTD4, PCSK1, PLXNA1, PNPLA6, POLR3A, POLR3B, PROK2, PROKR2, RNF216, SEMA3A, SEMA3E, SMCHD1, SOX10, SOX2, STUB1, TAC3, TACR3, TUBB3,*

WDR11. Nonsynonymous rare sequencing variants (RSVs), splicing variants (+/-2bp), with minor allele frequency (MAF) less than 1% in from the Genome Aggregation Database (gnomAD, http://gnomAD.broadinstitute.org/) were selected for further analysis. Variants were interpreted using American College of Medical Genetics and Genomics (ACMG) criteria (Richards *et al.*, 2015), and only variants predicted to be 'pathogenic', 'likely pathogenic' or 'of uncertain significance' are reported in this paper.

4.4 RESULTS

4.4.1 Baseline characteristics

The clinical characteristics of the healthy men (n=21) and men with CHH (n=21) are presented in **Table 6**. Men with CHH were older and had a higher BMI than healthy men. Serum LH, FSH, inhibin B and testosterone were also lower in men with CHH than healthy men in their first study visit. The individual clinical characteristics of twenty-one men with CHH are presented in **Table 7**. Eleven of twenty-one men with CHH (52%) had previously received gonadotrophin treatment to induce spermatogenesis (at least six weeks prior to participation in the study). Eighteen patients (86%) were on testosterone replacement, with the vast majority on testosterone undecanoate (Nebido), whilst two patients were not receiving any treatment at all for at least 3 years at the time of participation (10%). One patient was on gonadotrophin treatment but had stopped for 2months prior to his study visits. Eight of twenty-one (38%) of men with CHH were categorised as anosmic after an objective UPSIT-40 item smelling test; four of twenty-one (19%) were mildly microsmic while the remaining (43%) were normosmic.

Clinical Characteristics	Men with CHH (n =21)	Healthy men (n =21)	P-value
Age (years)	39.1±14.4	23.9±4.6	<0.0001
Weight (Kg)	78.7 (65.8, 92.9)	71.9 (63.8, 79.4)	0.19
Body Mass Index (Kg/m^2)	26.1±5.3	22.9±2.2	0.016
Mean Testicular Volume (mL)	6.0±3.7	n/a	n/a
Serum LH (iU/L)	0.46±0.6	2.9±1.0	<0.0001
Serum FSH (iU/L)	0.4 (0.1, 1.2)	1.9 (1.6, 3.0)	<0.0001
Serum AMH (pmol/L)	26.8 (12.9, 106.6)	49.8* (37.8, 83.1)	0.20
Serum Inhibin B (ng/L)	35.0 (8.0, 79.0)	136 (111, 176)	<0.0001

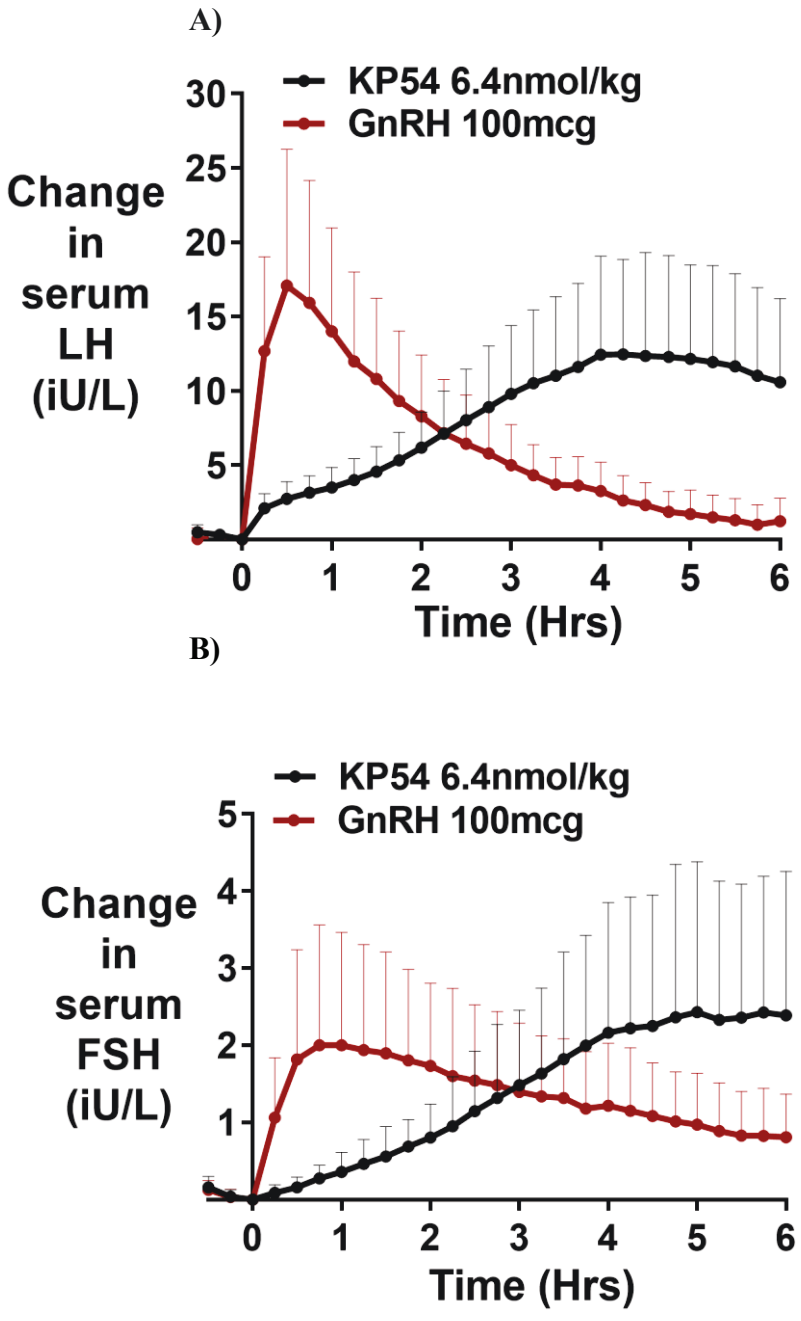
Table 5: Baseline characteristics of men with congenital hypogonadotrophic hypogonadism (CHH) and healthy men. Mean ± SD is presented for parametrically distributed values (age, BMI, testicular volume, LH, AMH) and median (25^{th} centile, 75^{th} centile) is presented for non-parametrically distributed data (weight, FSH, inhibin B). Comparison between the parameters were made using Mann-Whitney U test. Reference range for serum LH for men are 2-12iU/L, serum FSH 1.7-8iU/L, serum testosterone 10-30nmol/L, serum AMH 10.2-82.8pmol/L, serum inhibin B 25-325ng/L.

Patient ID	Age at screening (years)	Age at diagnosis (years)	Sense of smell (UPSIT -40)	TV at screening (mL)	Mean TV (mL)	Serum LH (iU/L)	Serum FSH (iU/L)	Duration of gonadotrophin therapy (years)	Time since stopping gonadotrophin (years)	Current treatment	Genetic Mutation	ACMG criteria
1	43	27	N	R-5, L-4	4.5	0.97	1.3	5	1	TU	PLXNA1 p.D429E	VUS
2	44	37	M	R-12, L-15	13.5	<0.03	0.1	2	3	TU	KLBp.G34E	VUS
3	58	17	M	R-1, L-1	1.0	0.04	0.1	Nil	n/a	Nil	-	Nil
4	60	16	N	R-4, L-4	4.0	1.44	2.0	1	19	TU	-	Nil
5	50	16	A	R-2, L-5	3.5	0.03	0.1	1	0.5	hCG	SEMA3Ap.R 734Q	VUS
6	53	16	A	R-4, L-0	2.0	<0.03	0.1	1	35	TU	-	Nil
7	48	16	N	R-12, L-12	4.0	0.22	0.8	1	1	TU	-	Nil
8	28	18	N	R-4, L-5	4.5	0.91	1.1	Nil	n/a	TU	CHD7p.S1604T	VUS
9	65	23	A	R-8, L-5	6.5	1.01	1.9	2	34	Nil	-	Nil
10	23	17	N	R-4, L-4	4.0	0.36	0.8	Nil	n/a	TG	WDR11p.A768V	VUS
11	19	16	A	R-3, L-4	3.5	0.04	0.1	Nil	n/a	TU	ANOS1 deletion	LP
12	17	16	N	R-7, L-7	7.0	1.42	1.1	Nil	n/a	TU	-	Nil
13	27	17	A	R-8, L-6	7.0	0.05	0.1	0.5	9	TU	-	Nil
14	31	17	N	R-12, L-10	11.0	1.47	3.0	2	1	TU	-	Nil
15	26	16	N	R-5, L-3	4.0	0.05	2.5	Nil	n/a	TU	FGFR1p.N724K(LP), PLXNA1p.A14797T	LP
16	31	18	N	R-6, L-10	8.0	0.04	0.4	Nil	n/a	TP	-	Nil
17	31	16	A	R-2, L-3	2.5	0.04	0.1	Nil	n/a	TU	PROKR2p.H20Lfs*24	LP
18	46	18	N	R-8, L-8	8.0	1.44	0.3	Nil	n/a	TU		NT
19	43	19	N	R-15. L-8	11.5	0.10	2.5	Nil	n/a	TP	-	Nil
20	37	16	A	R-12, L-15	13.5	0.13	0.5	Nil	n/a	TU		NT
21	39	16	A	R-3, L-5	4.0	<0.03	0.6	1	6	TU	SEMA3A	LP

Table 6 Clinical and genetic characteristics of men with congenital hypogonadotrophic hypogonadism (CHH). Serum LH, FSH at the first study visit are presented. UPSIT-40 university of Pennsylvania 40 items smelling (N: Normosmia, M: Microsmia, A: Anosmia), TV, Testicular volume at screening, TU, Testosterone undecanoate (Nebido); hCG: human chorionic gonadotrophin; TP, Testosterone proprionate (Sustanon); TG, Testosterone Gel. American College of Medical Genetics, ACMG (VUS: Variant Uncertain Significance; Nil: No mutation identified; LP: Likely Pathogenic mutation identified; NT: Not Tested). n/a Not applicable.

4.4.2 Gonadotrophin responses to KP54 and GnRH in healthy men

Serum LH reached a maximum of 18.2iU/L in all healthy men at 30 minutes following an intravenous bolus of GnRH, whereas the maximal rise in serum LH following KP54 occurred later at 4.4 hrs to 13.2iU/L. Maximal rise in serum FSH levels occurred much later at 45-50minutes after the peak rise in LH following both GnRH (2.3iU/L) and KP54 (2.7iU/L) **(Figure 30A, B)**. Testosterone was increased similarly following both hormones to ~4nmol/L at 3 hrs following administration **(Figure 30C).**



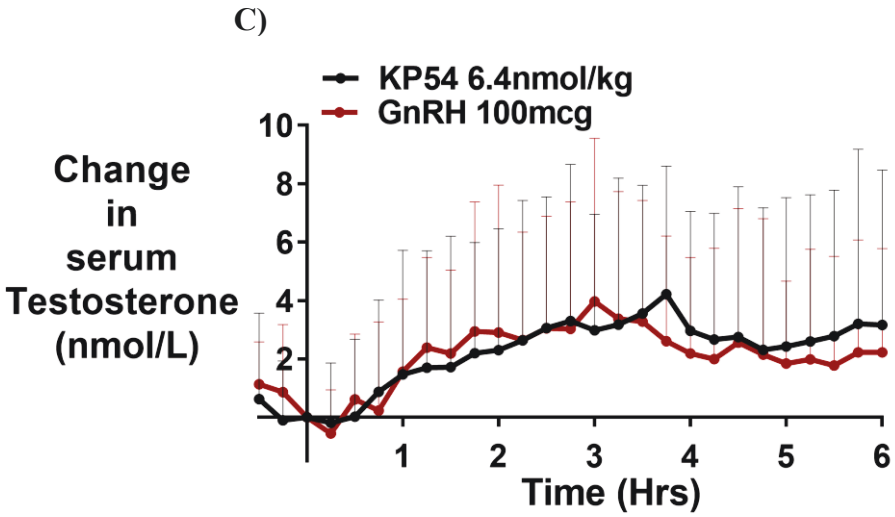


Figure 30: Mean±SD of change from baseline levels of (A) serum LH (iU/L) (B) serum FSH (iU/L) (C) serum testosterone (nmol/L) in healthy men (n=21) over 6 hrs following a single intravenous bolus of KP54 6.4nmol/kg (in black) and GnRH 100 micrograms (in maroon). Groups were compared by two-way ANOVA. Changes in serum LH (P-value=0.054), FSH (P-value=0.69) and testosterone (P-value=0.92) did not significantly differ between the two peptides.

4.4.3 Gonadotrophin responses in healthy men and men with CHH

4.4.3.1 Serum LH

LH levels remained undetectable following KP54 in men with CHH in comparison to healthy men (P<0.0001) **(Figure 31A)**. LH-rises after GnRH were ~3-fold lower in men with CHH than in healthy men (P<0.0001) **(Figure 31B**).

The median maximal change in serum LH from baseline following KP54 was 12.5iU/L in healthy men and 0.4iU/L in men with CHH (P-value<0.0001). The minimum rise of serum LH following KP54 in healthy men was 4.1iU/L, whereas the maximal rise in men with CHH was 2.0iU/L **(Figure 32A)**. Therefore, the LH rise following KP54 could accurately differentiate all healthy men from those with CHH (Area under ROC 1.0) (**Figure 33A**). The median maximal LH-rise following GnRH was 18.2iU/L for healthy men and 2.0iU/L for men with CHH (P-value<0.0001) (**Figure 32B**). However, there was an overlap of LH responses between the groups, with maximal rises in LH as high as 40iU/L in men with CHH. Therefore, a GnRH test less accurately differentiated healthy men from those with CHH (AuROC 0.88, 95% CI 0.76 to 0.99) **(Figure 33B).**

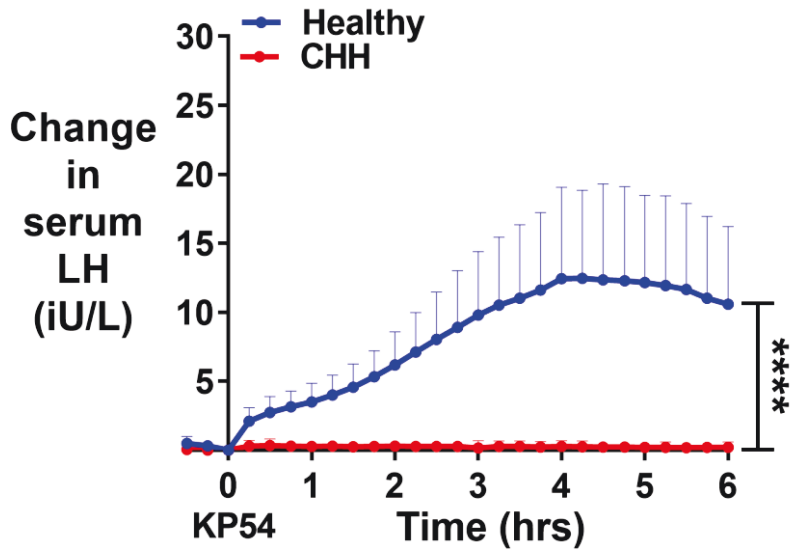
4.4.3.2 Serum FSH levels

Change in serum FSH levels from baseline were also markedly reduced in men with CHH compared to healthy men after a KP54 challenge test (P-value<0.0001). Median FSH-rise after KP54 was also significantly lower in men with CHH (0.3iU/L) than in healthy men (2.2iU/L) (P-value<0.0001) **(Figure 34A and 35A)**. However, the FSH-rise after GnRH did not significantly differ between men with CHH and healthy men (1.1iU/L vs 1.8iU/L; P-value=0.079) (**Figure 34B and 35B)**.

4.4.3.3 Serum Testosterone levels

Changes in testosterone levels did not significantly differ between KP54 (**Figure 36A**) and GnRH (**Figure 36B**) in both healthy men and those with CHH (P-value>0.12).

A)



B)

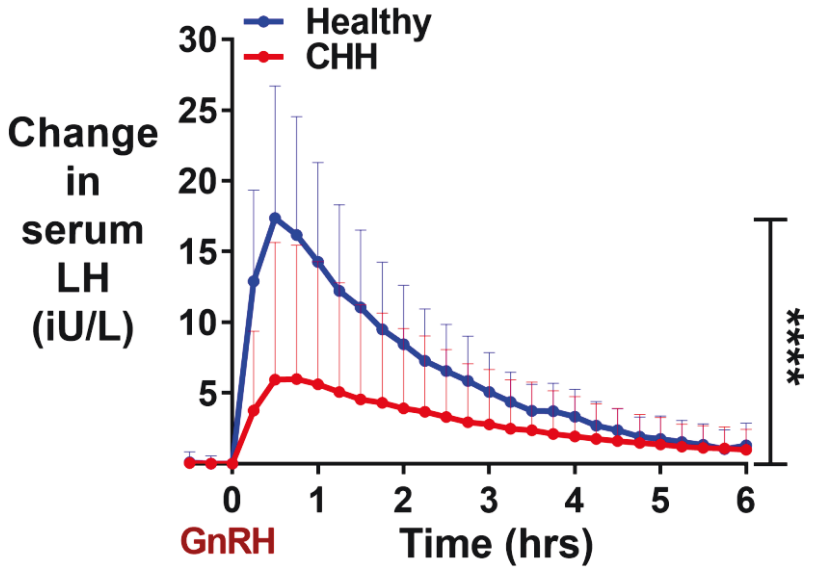
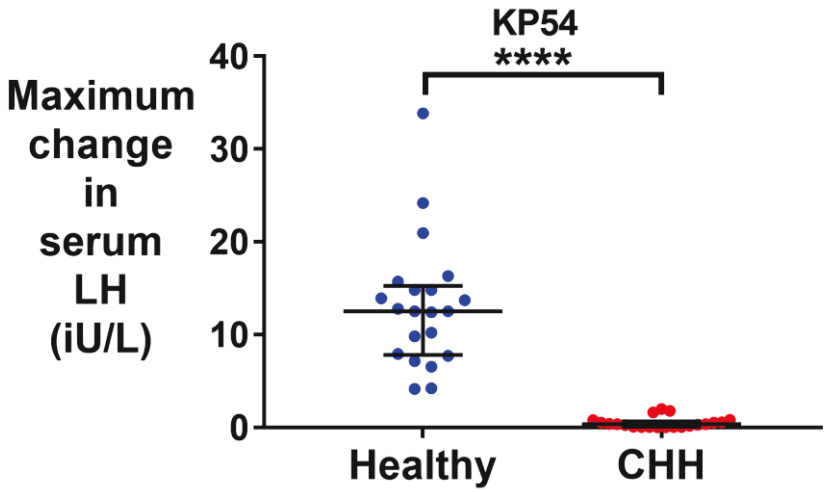


Figure 31 Mean±SD of change from baseline levels of serum LH (iU/L) after (A) an intravenous bolus of KP54 6.4nmol/kg (B) an intravenous bolus of GnRH over 6 hrs in healthy men (blue) and in men with CHH (red). Groups were compared by two-way ANOVA (**** P-value<0.0001).

A)



B)

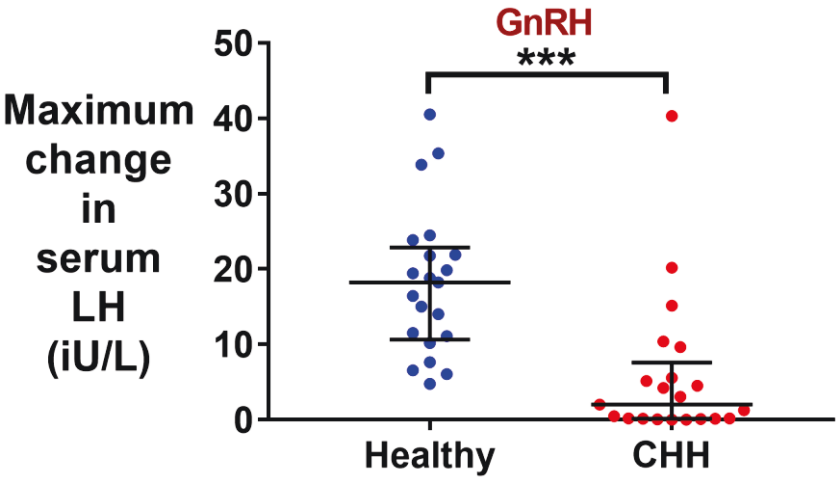


Figure 32 Scattergram of Median±IQR of maximum change in serum LH (iU/L) in healthy men (blue)and men with CHH (red) following (C) an intravenous bolus KP54 6.4nmol/kg (D) an intravenous bolus of GnRH 100 micrograms. Groups were compared by Mann-Whitney U Test. (**** denotes P-value<0.0001, *** denotes P-value< 0.001).

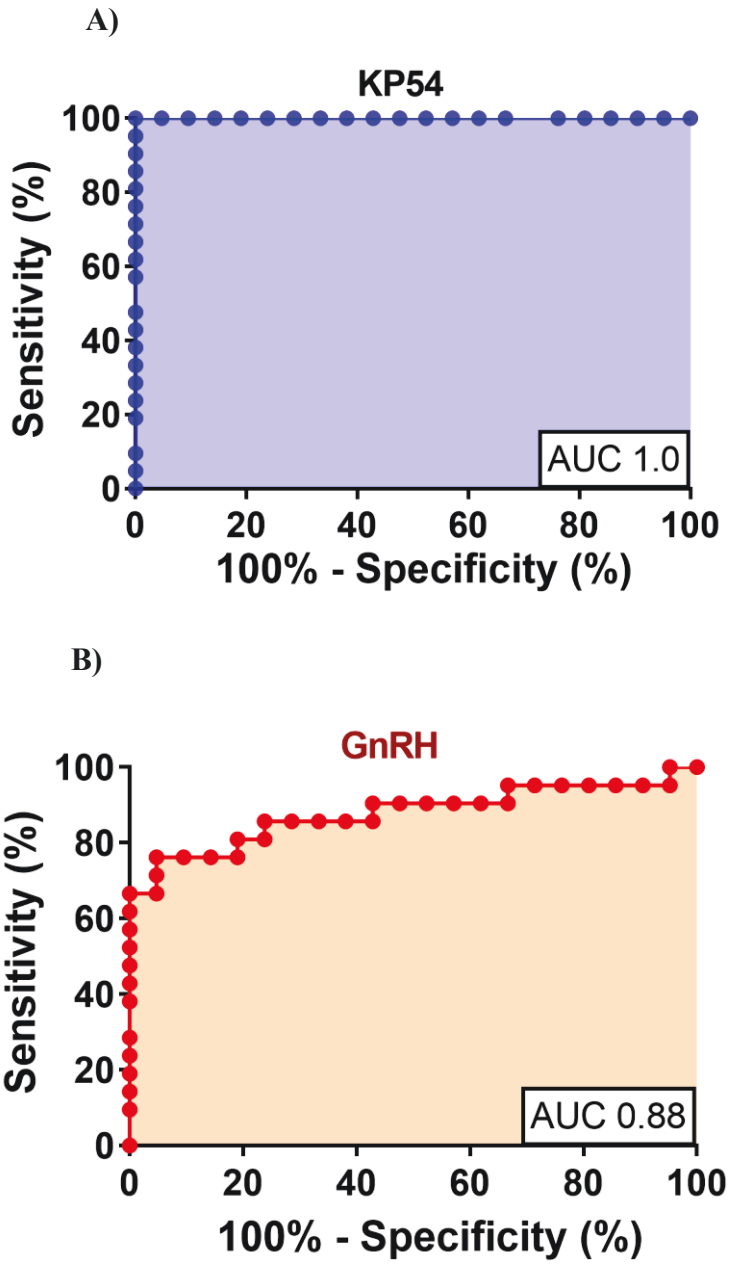
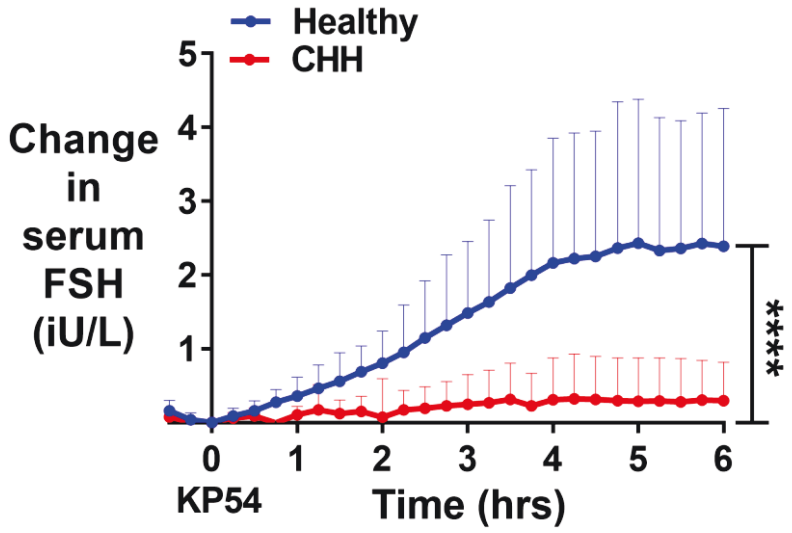


Figure 33 Area under receiving operating characteristics (AuROC) for (A) maximum change in serum LH (iU/L) following an intravenous bolus of KP54 6.4nmol/kg to differentiate men with CHH (B) maximum change in serum LH following an intravenous bolus of GnRH 100 micrograms to differentiate men with CHH.

A)



B)

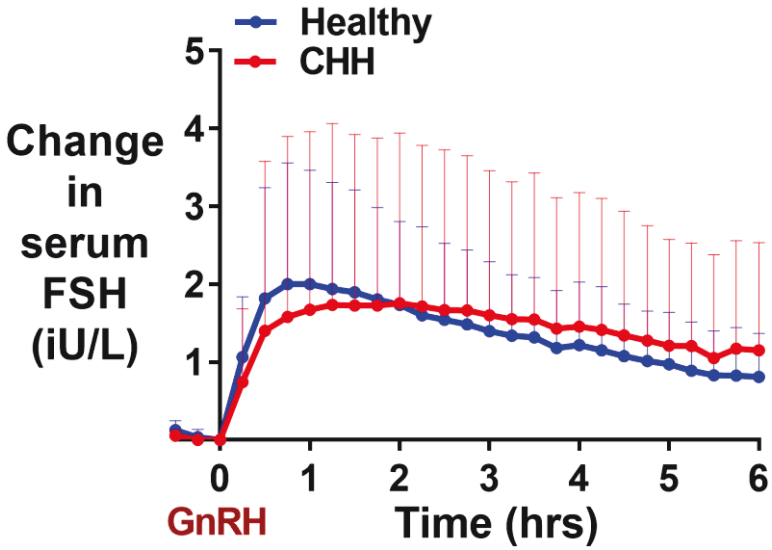


Figure 34 Mean±SD of change from baseline of serum FSH in healthy men (blue) and men with CHH (red) following (A) an intravenous bolus of KP54 6.4nmol/kg (B) an intravenous bolus of GnRH 100 micrograms over 6 hrs. Groups were compared by two-way ANOVA. (****P-value<0.0001).

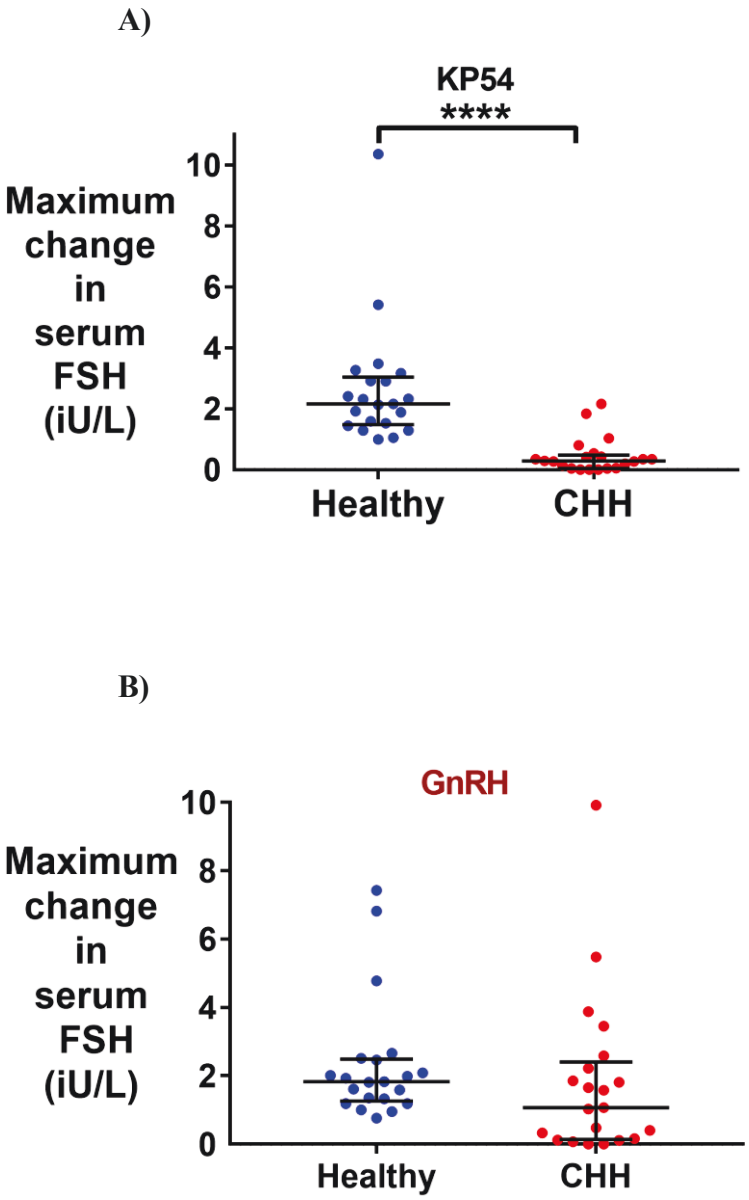
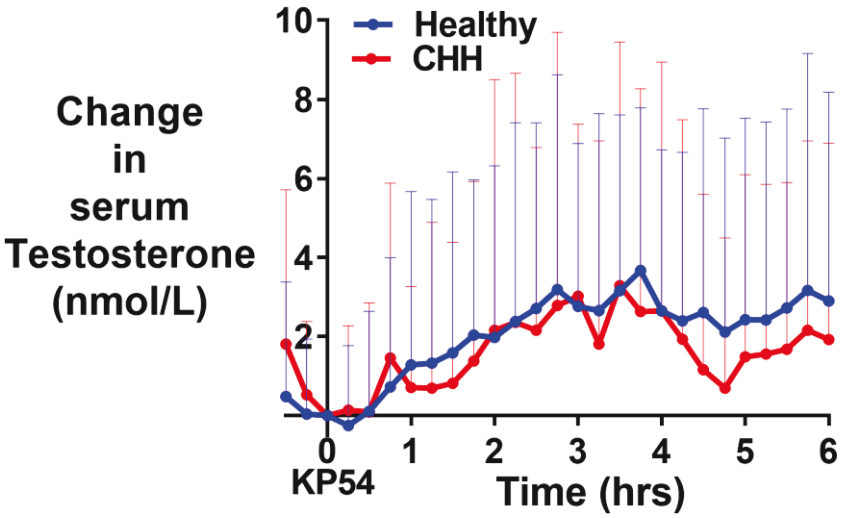


Figure 35 Scattergram (Median±IQR) of maximum change in serum FSH (iU/L) from baseline in healthy men (blue) and men with CHH (red) following (C) an intravenous bolus of KP54 6.4nmol/kg (D) an intravenous bolus of GnRH 100 micrograms. Groups were compared by the Mann-Whitney U test (****P-value<0.0001).

A)



B)

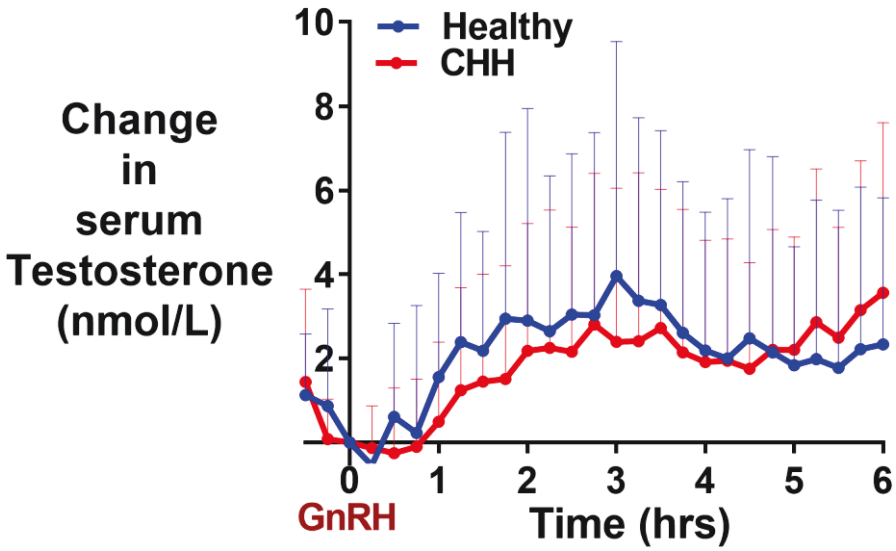


Figure 36 Mean±SD of change from baseline of serum testosterone (nmol/L) in healthy men (blue) and men with CHH (red) following (A) an intravenous bolus of KP54 6.4nmol/kg. Groups were compared by two-way ANOVA (P-value=0.16 (B) an intravenous bolus of GnRH 100 micrograms over 6 hrs Groups were compared by two-way ANOVA and did not show a significant difference between them (P-value=0.12).

4.4.4 Baseline variables affecting gonadotrophin responses following KP54 and GnRH test

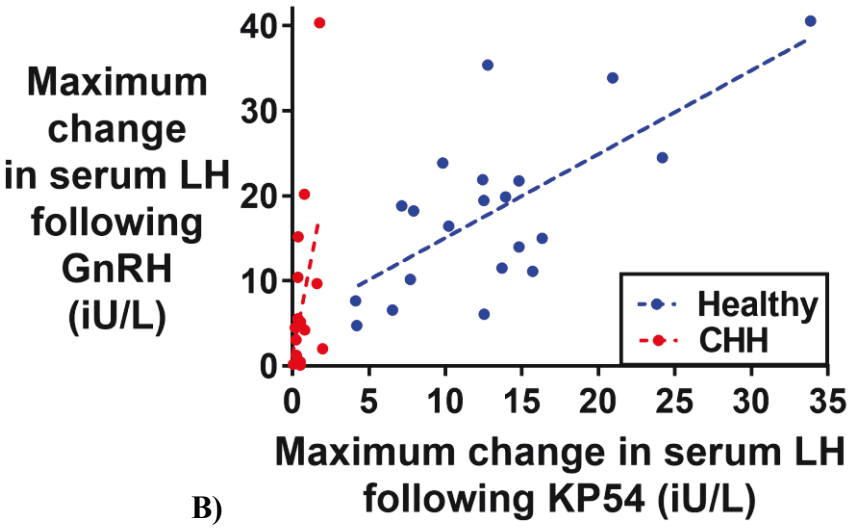
The LH-rise following KP54 was associated with the LH-rise following GnRH in healthy men (P-value=0.0005, r^2=0.48). However, in men with CHH, serum LH response was attenuated following KP54 even though LH response to GnRH is preserved (P-value=0.007, r^2=0.33). Thus, the ratio of LH-rise following GnRH to that following KP54 was 1.5 in healthy men and 10.8 in men with CHH (P-value=0.0005 by two-way ANOVA) **(Figure 37)**.

The LH levels at baseline before administration predicted the subsequent stimulated LH response following KP54 test in healthy men, but this was attenuated below the projected level due to a lower baseline LH in men with CHH. By comparison, with the GnRH test, the LH levels at baseline did not predict the stimulated LH response in healthy men (P-value=0.17, r^2=0.09) but this relationship was preserved in men with CHH (P-value<0.001, r^2=0.71) **(Figure 38).**

FSH has a longer half-life than LH. FSH-rise following KP54 was associated with the FSH-rise following GnRH in healthy men (P-value<0.001, r^2=0.76). However, this association is lost in men with CHH (P-value=0.70, r^2 =0.008). The FSH levels at baseline predicted the subsequent stimulated FSH response following KP54 test (P-value<0.0001, r^2=0.59) and GnRH test (P-value<0.0001, r^2=0.66) in healthy men. However, in men with CHH, FSH response is preserved following GnRH test P-value<0.0001, r^2=0.76) but not after KP54 test (P-value=0.99, r^2=0.001) **(Figure 39)**.

There was also a trend towards greater gonadotrophin responses following GnRH and KP54 in men with CHH with larger testicular volume **(Figure 40**). All healthy men had a serum AMH level of less than 140pmol/L. Five men with CHH on testosterone treatment had high levels of serum AMH of at least more than 140pmol/L, likely reflecting the absence of pubertal maturation of Sertoli cells despite achieving a physiological replacement testosterone level of 14.5±5.6nmol/L. Baseline serum AMH and baseline testosterone levels do not interfere with the maximum LH or FSH-rise following KP54 or GnRH **(Figure 41 and Figure 42).**

A)



B)

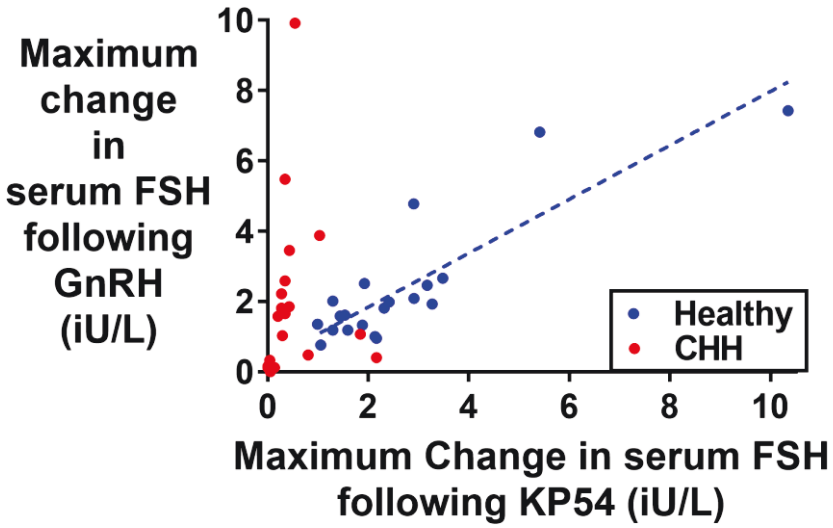
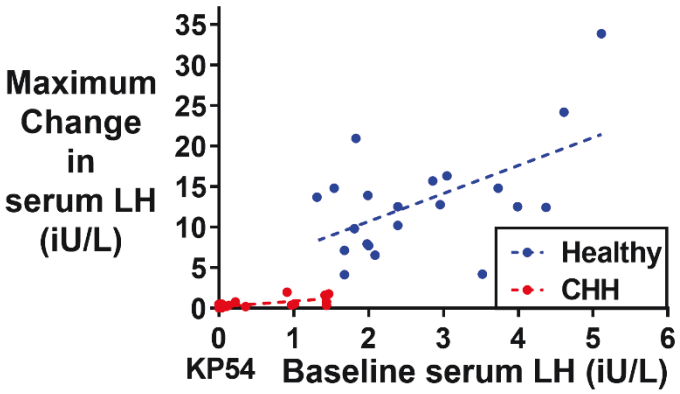


Figure 37 Simple linear regression of (A) maximum change in serum LH (iU/L) following an intravenous bolus of 100 micrograms of GnRH and an intravenous bolus of KP54 6.4nmol/kg in healthy men (n=21) in blue (P-value=0.0005, r^2=0.48) and men with CHH (n=21) in red (P-value=0.007, r^2=0.33). (B) maximum change in serum FSH (iU/L) following an intravenous bolus of 100 micrograms of GnRH and an intravenous bolus of 6.4nmol/kg of KP54 in healthy men (n=21) in blue (P-value<0.001, r^2=0.76). and men with CHH (n=21) in red (P-value=0.70, r^2=0.008).

A)



B)

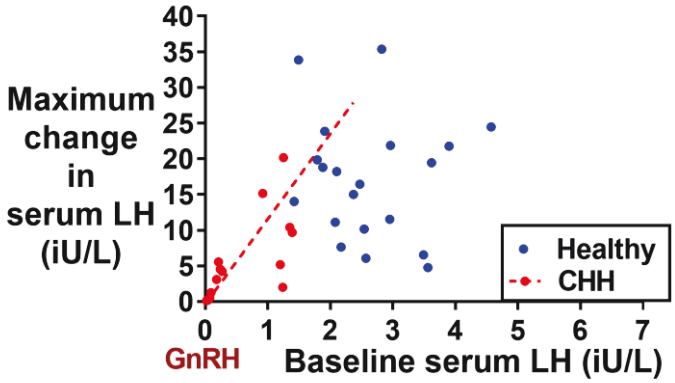
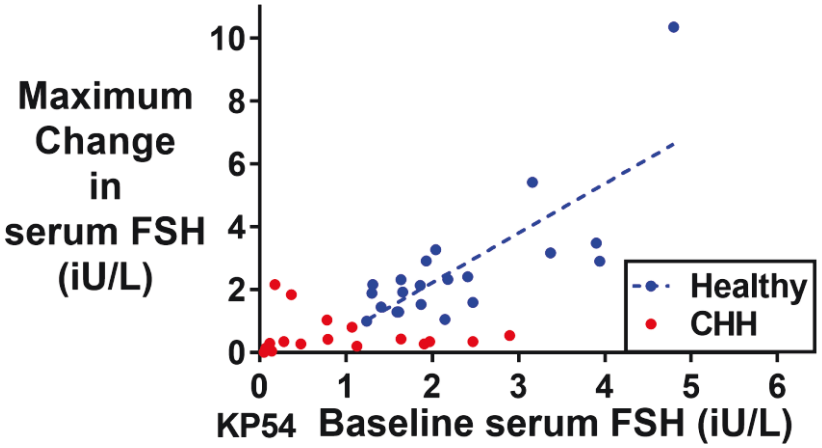


Figure 38 Simple linear regression between maximum change from baseline in serum LH (iU/L) and baseline serum LH (iU/L) following: (A) an intravenous bolus of KP54 6.4nmol/kg in healthy men (n=21) in blue (P-value=0.009, r^2=0.31) and men with CHH (n=21) in red (P-value=0.001, r^2=0.47). (B) following an intravenous bolus of GnRH (100 micrograms) in healthy men (n=21) (P-value=0.17, r^2=0.09) and men with CHH (n=21) in blue (P-value<0.001, r^2=0.71).

A)



B)

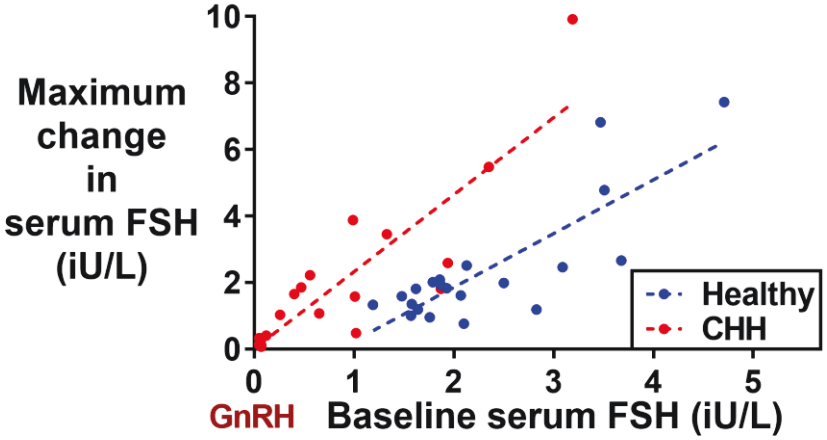


Figure 39 Simple linear regression between maximum change in serum FSH (iU/L) from baseline and baseline serum FSH (iU/L) following: (A) an intravenous bolus of KP54 6.4nmol/kg in healthy men (n=21) in blue (P-value<0.001, r^2=0.59) and men with CHH (n=21) in red (P-value=0.88, r^2=0.0001). (B) following an intravenous bolus of GnRH (100 micrograms) in healthy men (n=21) (P-value<0.0001, r^2=0.66) and men with CHH (n=21) in blue (P-value<0.001, r^2=0.76).

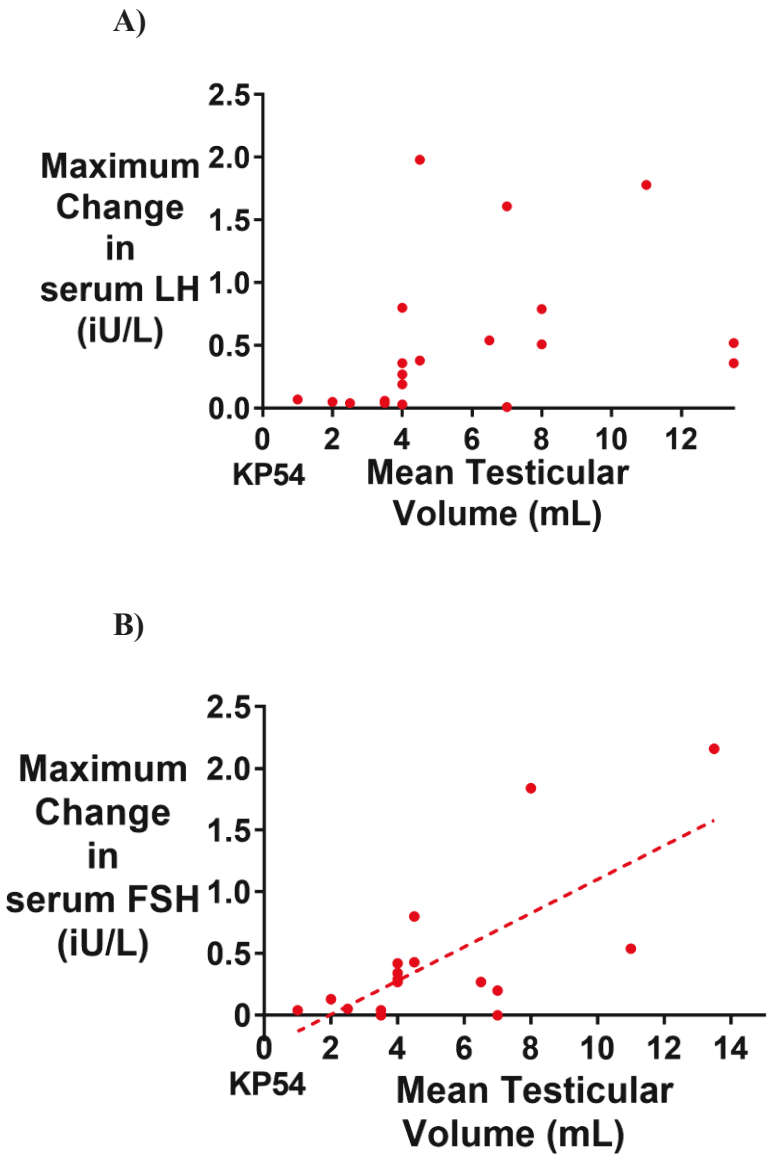


Figure 40 Simple linear regression between mean testicular volume and maximum change in (A) serum LH (iU/L) from baseline and (B) serum FSH from baseline following an intravenous bolus of KP54 6.4nmol/kg in men with CHH (n=21) (P-value=0.001, r^2=0.51).

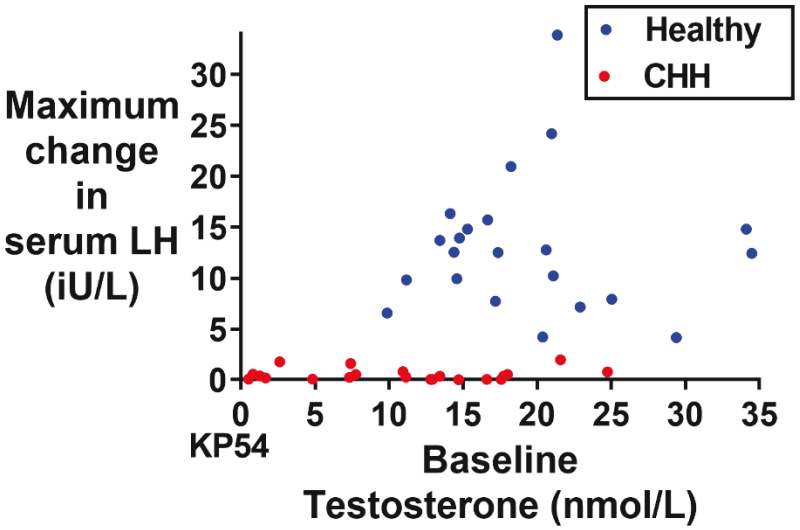


Figure 41 Simple linear regression between the maximum change in serum LH (iU/L) from baseline and testosterone level (nmol/L) at time 0hrs in healthy men (n=21) in blue and men with CHH (n=21) in red (P-value>0.86).

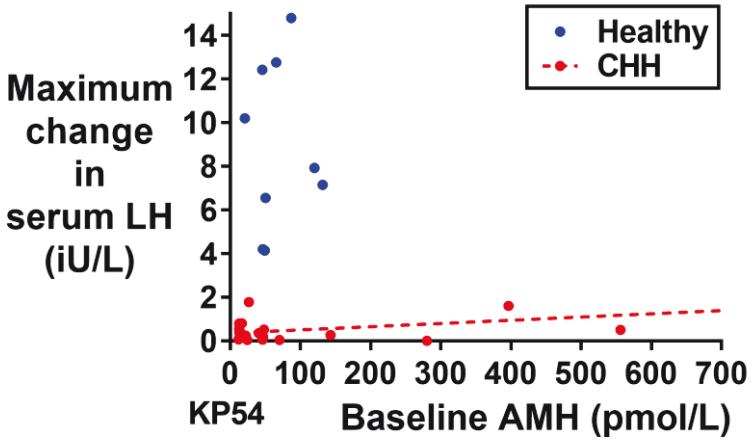


Figure 42 Simple linear regression between maximum change in serum LH (iU/L) following an intravenous bolus of KP54 6.4nmol/kg and that of baseline serum AMH (pmol/L) (P-value=0.02, r^2=0.02).

4.4.5 Genetic mutations in men with CHH

Four men with CHH had mutations identified that were likely to be pathogenic, five men had variants of uncertain significance (VUS) and ten men had no abnormality identified on genetic testing (**Table 7**). The four men with pathogenic mutations identified had even lower LH-rises after KP54 administration than other men with CHH who had either no mutation identified or VUS (**Figure 43**; P-value=0.04). Notably, the maximal LH-rise after KP54 in men with CHH also differed by olfactory status (normosmia 0.79iU/L; microsmia 0.22iU/L; anosmia 0.05iU/L) (**Figure 43**). Anosmic men with CHH (Kallmann syndrome) had significantly lower LH-rises than normosmic men with CHH (P-value=0.003). Serum FSH levels were also higher in men with CHH who were normosmic, but one man with CHH with microsmia had a much higher rise in serum FSH than normosmic men with CHH (Normosmia: 0.43iU/L; microsmia: 0.38iU/L; anosmia: 0.045iU/L) **(Figure 44).**

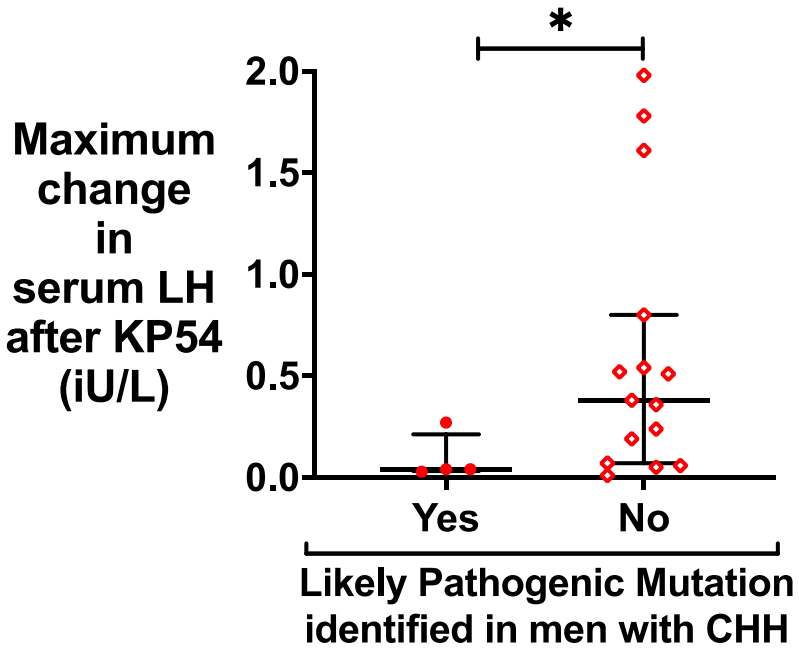


Figure 43 Scattergram Median (±IQR) of maximum change in serum LH after KP54 in men with CHH grouped by those with likely pathogenic mutations (n=4) in closed circles, and those with variants of uncertain significance (VUS) or no abnormality detected on genetic testing (open circles) (P-value=0.04).

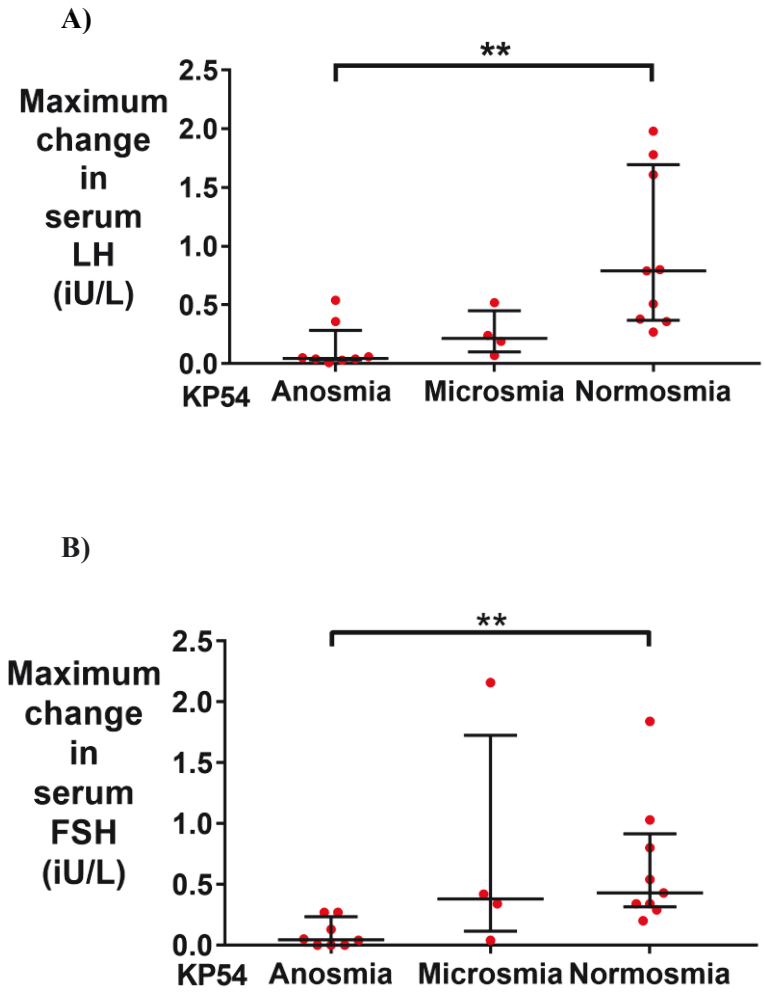


Figure 44 Scattergram (Median±IQR) of maximum change in (A) serum LH (iU/L) (B) serum FSH (iU/L) in men with CHH with anosmia, microsmia and normosmia determined by UPSIT-40 item Smelling test. Groups were compared by Kruskal-Wallis test with post hoc Dunn's multiple comparison test (** P-value< 0.01).

4.5 DISCUSSION

In this part of my thesis, I investigated the potential for KP54 to be used as a specific test of hypothalamic function in the investigation of men with hypogonadotrophic hypogonadism. All men with CHH, irrespective of their specific genetic mutation, had an attenuated response to KP54 consistent with impaired hypothalamic function, even if the pituitary responsiveness to GnRH was preserved. This highlighted the value of a KP54 test in interrogating the hypothalamic function in men with CHH. The KP54 test more accurately distinguished men with CHH from healthy men than a GnRH test. Indeed, all men with CHH had an LH rise after KP54 of <2iU/L, whereas all healthy men had an LH rise of >4iU/L. Whilst there was no overlap in LH-rise following KP54 in healthy and CHH men (healthy >4iU/L vs CHH <2iU/L), LH-rises as high as 40iU/L were observed after GnRH in men with CHH. Thus, a KP54 test (auROC 1.0) markedly surpassed GnRH (auROC 0.88) in the differentiation of men with CHH from healthy men.

Our studies complemented previous data demonstrating that KP10 elicited an attenuated response in men with CHH. Chan *et al.* administered KP10 at 0.24nmol/kg (0.313µg/kg) to probe the hypothalamic function in 11 patients with CHH. Whilst this dose had previously elicited a rise in LH of 5iU/L at 30 minutes in healthy men (Chan *et al.*, 2011), LH did not rise in any person with CHH, regardless of genotype. This was the case even with repeated dosing of KP10 or using doses up to 2.4nmol/kg, or after GnRH priming (Chan *et al.*, 2014). Evidence suggests that KP54 may cross the blood brain barrier whereas KP10 may not (Comninos *et al.*, 2017; De Tassigny *et al.*, 2017), thus KP54 could theoretically provide more specific information on the functionality of GnRH neuronal cell bodies as opposed to stimulation of GnRH neuronal terminals at the median eminence (De Tassigny *et al.*, 2017). Furthermore, due to the longer half-life of KP54 ($t_{1/2}$ KP54:28 minutes, KP10:3 minutes), it could provide preferable pharmacokinetic properties for intravenous bolus administration during a 'kisspeptin test' (C N Jayasena, Abbara, *et al.*, 2015). Indeed, the median LH-rise was 13.2iU/L after KP54 in the present study, whereas the highest reported mean LH-rise following KP10 is 8.3iU/L after a dose of 0.77nmol/kg (George *et*

al., 2011). Thus, KP54 could facilitate better differentiation between healthy and abnormal responses, which could be of relevance for partial responders/milder phenotypes, or potentially helping to differentiate CHH from functional pubertal delay in teenage boys.

There is considerable heterogeneity in the genetic mutations that cause CHH. Whilst some genetic mutations causing CHH impair GnRH migration, others, such as those in genes affecting neurokinin B signalling, impair GnRH secretion and can present in a partial or milder clinical phenotype (Boehm *et al.*, 2015). Young *et al.* investigated the gonadotrophin response to a 12 hrs infusion of KP10 (dose 1.5µg/kg/hr) in 2 patients with TAC3 mutations and 2 patients with TAC3R mutations (Young *et al.*, 2013). KP10 infusion in these patients increased both mean LH (Saline 0.4iU/L, KP10 1.0iU/L) and FSH (Saline 3.1iU/L, KP10 5.0iU/L) (Young *et al.*, 2013). However, the rise in gonadotrophins was markedly lower than in healthy men treated with the same protocol (*LH in healthy men*: Saline 5.2iU/L, KP10 14.1iU/L; *FSH in healthy men*: Saline 2.7iU/L, KP10 5.0iU/L) (George *et al.*, 2011). Thus, although patients with deficits in NKB signalling can respond to KP10, the response is not as great as in healthy individuals. Therefore, the greater magnitude of gonadotrophin response following KP54 administration is advantageous for a more precise differentiation of patients with CHH from healthy individuals.

Indeed, there was no over-lap in LH-responses between men with CHH and healthy men irrespective of the specific causative mutation. However, pathogenic mutations that were identified in four men with CHH exhibited lower LH-rises following KP54 administration than men with CHH with either VUS or no abnormality identified on genetic testing. Additionally, men with CHH with anosmia had even more attenuated responses after KP54 administration than normosmic men with CHH. Conceivably, this is due to patients with anosmia being more likely to have a failure of GnRH neuronal migration (rather than secretion) and thus GnRH neurons are not in the appropriate anatomical location to respond to a KP54 challenge test. In line with this, anosmic men with CHH are also reported to have a more severe and complex phenotype than normosmic men with CHH (Quinton *et al.*, 2001; Bonomi *et al.*, 2018).

Although men with CHH regardless of genotype had an attenuated response to KP54, those with larger testicular volumes indicative of partial puberty had relatively greater gonadotrophin responses. This was consistent with previous studies demonstrating that response to KP10 increases with increasing Tanner stage in children (Nabi *et al.*, 2018). Men with CHH also exhibited an FSH-predominant response to GnRH but not to KP54. Though the alterations in GnRH pulsatility can underlie differential FSH and LH responses in physiological pulsatile secretion, this is unlikely to explain this observation to a bolus stimulus. A possibility is that men with CHH have lower inhibin B levels, thus reducing feedback on FSH levels and leading to FSH predominant gonadotrophin secretion. This is in keeping with data suggesting that an FSH predominant response to GnRH were observed in absent puberty, whereas men with partial puberty and higher inhibin B, basal LH and testosterone levels, had more adult-like LH predominant response (Pitteloud *et al.*, 2009).

It was noteworthy that serum LH rises following KP54 could be predicted based on baseline LH in healthy men (correlation coefficient, r=3.5). However, the LH rise following a KP54 test were attenuated beyond baseline LH levels in men with CHH (correlation coefficient, r^2=1.7). This suggests that a KP54 test, unlike a GnRH test, is able to provide additional information over and beyond the knowledge of the baseline LH levels. Additionally, stimulated LH response to GnRH in healthy men exhibited a great degree of variation, suggesting a high degree of inaccuracy with this test. Moreover, post GnRH LH levels in men with CHH were closely associated with baseline LH levels and therefore did not offer any additional information than the knowledge of baseline levels itself.

The gonadotrophin response to an intravenous bolus of KP54 is characterised by a brief initial LH rise, followed by plateau between 30 and 75 minutes and a second rise peaking between 4-6hrs. The timings of this profile were remarkably preserved across a range of circumstances including healthy men and women, hypothalamic amenorrhoea and IVF treatment (Jayasena *et al.*, 2010; 2014; 2015b Abbara *et al.*, 2014, 2017). Recent *in vitro* work on the KISS1R has suggested that kisspeptin stimulates a biphasic intracellular response, with an acute rapid release in intracellular calcium (lasting 5 minutes) followed

by a more sustained second-phase calcium response (lasting 30 minutes) (Min *et al.*, 2013). Serum FSH peaks about an hour later than LH due the longer half-life of FSH compared to LH ($t_{1/2}$ 3.9hrs vs 20minutes). Though the half-life of GnRH is much shorter than KP54 ($t_{1/2}$ 3minutes vs 28minutes), the amplitude of peak LH was similar following GnRH and KP54. Interestingly, the FSH response following KP54 seemed to exceed that induced by GnRH, perhaps due to a longer duration of action of KP54 and a potential delay to allow for transport of KP54 across the blood brain barrier whereas the pituitary resides outside the blood brain barrier.

A fifth of patients with CHH can undergo spontaneous reversal of their hypogonadism that is associated with restoration of their responsiveness to kisspeptin (Lippincott *et al.*, 2016a). Some men with CHH may be on treatment with testosterone replacement, however the response to kisspeptin has not been shown to be influenced by sex-steroid replacement (Lippincott *et al.*, 2018). In my data, it is notable that baseline testosterone does not seem to interfere the response to KP54, therefore our data complements previous researcher's findings, by suggesting that a 'KP54 test' could be used to expedite the assessment of hypothalamic GnRH function in patients with CHH with potential reversal without interfering their treatment.

In summary, I demonstrated that KP54 offers the unique opportunity to interrogate hypothalamic GnRH function in patients presenting with hypogonadism. As KP54 has a longer half-life than KP10 ($t_{1/2}$ 28min vs 3min), it offers a favourable pharmacokinetic profile compared to KP10. The gradation of response after KP54 is more useful than shorter acting peptides (KP10) in identifying patients with CHH from healthy individuals. Thus, KP54 provides important diagnostic value in the accurate investigation of men with hypogonadotrophic hypogonadism in comparison to currently available investigations.

4.5.1 Limitations

The response to kisspeptin is through the hypothalamic pituitary axis, predicated on a responsive pituitary gland. Consequently, the lack of GnRH priming could impair the response to GnRH and contributed to an attenuated kisspeptin response. However, GnRH priming is not readily available and recent evidence suggested that GnRH priming did not alter the categorisation of LH response to KP54 (YM *et al.*, 2018).

In the earlier chapters, I described the ability of sex steroids to alter the responsiveness of GnRH neurons to kisspeptin. In healthy women, kisspeptin responsiveness changes throughout the menstrual cycle with an augmented response observed in preovulatory phase when oestradiol levels were at their highest level (Dhillo *et al.*, 2007; Chan *et al.*, 2012). In rhesus monkeys, kisspeptin responsiveness changes as sex steroid milieu changes across pubertal development (Seminara *et al.*, 2006). Endocrinologists treat patients with CHH with sex steroids to correct their hypogonadotrophic hypogonadism. Therefore, it is theoretically possible that the presence of sex steroids may modulate the gonadotrophin response to GnRH or KP54. However, to fully washout the effects of sex-steroid replacement would take many months and I did not wish to impede our participant's management. As a result, the hormonal studies were undertaken at trough levels while patients were on testosterone undecanoate or 1 week without transdermal testosterone gels. Despite this, patients with CHH, on repletion of sex steroid to normal physiological levels, did not show a response to KP54. This is consistent with existing data suggesting that sex steroid replacement does not interfere with interpretation of gonadotrophin response to kisspeptin in patients with CHH (Lippincott *et al.*, 2018).

4.5.2 Conclusion and future work

In summary, my data has important diagnostic implications; a KP54 challenge test may have utility in evaluating the hypothalamic function of men with HH who could be directed towards genetic testing. In addition, it may provide value in identifying men with CHH who have undergone spontaneous reversal

of reproductive function. The magnitude of response may allow greater differentiation between healthy and abnormal responses which would be of relevance to individuals presenting with milder or partial phenotypes. Future work would involve looking into the wider application of a KP54 test of hypothalamic GnRH neuronal function in the investigation of other reproductive disorders where hypothalamic function could be impaired such as in individuals with acquired hypogonadotrophic hypogonadism. It would also be of interest to investigate the response to KP54 in children presenting with delayed puberty to see if a KP54 test would better differentiate young adults with CHH and constitutional delay of growth and puberty (CDGP)

Further work encompassing a larger cohort of healthy male individuals would also be of value to establish a normative range of serum gonadotrophin responses following a KP54 challenge test in healthy subjects.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5 General Discussion

Kisspeptin has emerged as a pivotal hormone in the HPG axis. The potent stimulatory action of kisspeptin on serum gonadotrophins has offered a novel option in the treatment of patients with reproductive disorders. To date, human studies have used different isoforms of kisspeptin (KP54 or KP10) via different administration routes (intravenous or subcutaneous; single boluses or infusions). The longer peptide KP54 decays less rapidly compared to KP10 and subcutaneous administration results in a more sustained level of kisspeptin in comparison to KP10. However, the 54 amino acid peptides, are more expensive to produce. Consequently, there is an unmet need to determine the pharmacological profile of kisspeptin receptor agonists with shorter peptide length. Knowledge of the hormonal profile of kisspeptin receptor agonists would be instrumental in the development of kisspeptin-based therapies.

MVT-602 is a novel kisspeptin receptor agonist that has been recently trialled in healthy men and men with prostate cancer (MacLean *et al.*, 2014). However, the hormonal profile of MVT-602 in women was until now, unknown. In this thesis, I have demonstrated for the first time that MVT-602 administration to healthy women in follicular phase elicits an LH surge with a comparable amplitude to KP54 but with a peak LH that occurred much later (at ~22hrs), thereby extending the duration of gonadotrophin-surge. As the physiological effects of MVT-602 in women were not previously known, I selected a broad dose range (~300 fold difference) that would facilitate comparison with the dose of KP54 that has been established to be effective for triggering oocyte maturation in IVF setting (9.6nmol/kg (Abbara *et al.*, 2015). MVT-602 is known to be more potent than KP54 (pEC50 10.75 vs pEC50 8.05), therefore I would expect the dose used for MVT-602 (0.03nmol/kg) to be ~500 fold lower than KP54 (9.6nmol/kg). Based on the initial dose-response study, MVT-602 at 0.03nmol/kg appeared to offer the greatest change in serum LH and serum oestradiol at 24 hrs. Higher doses of MVT-602 (0.1, 0.3 and 1nmol/kg) did not further increase the gonadotrophin response. This implied that I had identified the dose-range that induces maximal serum LH stimulation and doses beyond 0.03nmol/kg would not induce greater stimulation. As a result, this dose (0.03nmol/kg) was taken forward to the second part of the study and

more detailed sampling over the first 24 hrs was carried out to delineate the gonadotrophin profile following MVT-602.

As described in the previous sections, serum LH profile following MVT-602 was different from the response observed in healthy men. Following administration of a single dose of MVT-602, serum LH rise was delayed up to 6 hrs before gradually rising to a peak at ~22 to 24 hrs in healthy women in the follicular phase. This finding appeared to be reproducible even with doses beyond 0.03nmol/kg. As we had purposefully undertook the study in the follicular phase in order to minimise interference from circulating endogenous steroids, this may partly explain the attenuated LH response encountered, as it has previously been established that the response to kisspeptin varies depending on the phase of menstrual cycle with greatest resistance in the follicular phase (Dhillo *et al.*, 2007; Chan *et al.*, 2012). An important focus in future studies will be determining the effect of MVT-602 on gonadotrophin response under different dosing range of oestradiol.

However, differences in basal oestradiol level may not fully explain the suppressive profile of serum LH, as women with HA with a similar basal oestradiol level to healthy women in follicular phase exhibited an exaggerated rise in serum gonadotrophin. On the contrary, women with PCOS, showed a serum gonadotrophin profile that was similar to that of healthy women in follicular phase. Women with HA have been reported to be more sensitive to effects of kisspeptin and exhibited a 4-fold greater response in gonadotrophin stimulation compared to healthy women (Jayasena *et al.*, 2013). However, it remains unknown if women with HA have a different sex steroid feedback regulatory system. Notably, ovarian-derived hormones (e.g. inhibin B or serum AMH) and leptin levels could interfere with gonadotrophin responses in women (Castellano *et al.*, 2009; Robertson *et al.*, 2009). Further studies are required to understand the feedback regulatory system in women with anovulatory disorders.

More recently, *in vitro* studies have provided evidence that kisspeptin can bind and activate other receptors within the same family such as the NPFF1R and NPFFR2 expressed in cell lines (Oishi *et al.*,

2011; Elhabazi *et al.*, 2013) and lower LH levels (Murakami *et al.*., 2008; Clarke *et al.*., 2009; Kriegsfeld *et al.*., 2010). Given that MVT-602 is a novel nano-peptide that has never been trialled in females, it is not unreasonable to speculate a possible ligand-receptor interaction of MVT-602 to different neuropeptide receptors such as gonadotrophin inhibiting hormone (GnIH) receptors leading to variable LH release. The characterisation of GnIH receptors on human GnRH neurons is yet to be established. However, preliminary work by George *et al.* revealed that a simultaneous infusion of GnIH (up to 150µg/kg/hour) up to 3 hrs did not seem to inhibit the kisspeptin-induced LH response in men but caused a reduced serum LH response in post-menopausal women (George *et al.*, 2017). Additional cellular work to understand the mechanism of ligand-receptor binding and GnRH signalling are indicated to validate the findings.

Serum FSH responses following administration MVT-602 occurred in a far less robust manner than the LH rises. These observations were consistent with results seen following endogenous kisspeptin isoforms (KP54, KP10) which are more efficacious on LH release when compared with FSH release, as observed in animal studies (Navarro, Castellano, *et al.*, 2004), healthy women (Dhillo *et al.*, 2007; Narayanaswamy *et al*., 2016), and healthy men (Dhillo *et al.*, 2005; George *et al*., 2011).

Overall, the findings in this study offer valuable insights to the application of kisspeptin-based therapies for reproductive disorders. Previously, our group has shown that a single subcutaneous bolus of KP54 (9.6nmol/kg) in women undergoing IVF treatment can effectively induce an LH-surge and oocyte maturation without causing OHSS. This was the case even when the protocol is repeated in women at increased *a priori* risk of OHSS (Jayasena *et al.*, 2014; Abbara *et al.*, 2015). However, as the LH-surge after a single dose of KP54 only lasts 10 to 12 hrs, an additional dose of KP54 (9.6nmol/kg) was given at 10 hrs (Abbara *et al*., 2017) to replicate the endogenous LH surge of 48 hrs (Damewood *et al.*, 1989). The outcome of the study was promising and no further increase in the rates of OHSS or intermediate markers of OHSS were observed (Abbara *et al*., 2017). MVT-602 evokes a serum LH rise equivalent in amplitude to a dose of KP54 (9.6nmol/kg) but duration of LH-surge was increased. The greater AUC of

LH rise mirrored the endogenous physiological LH surge (~48hrs) and would favour oocyte maturation when used as an ovulation trigger in IVF studies.

More importantly, kisspeptin acts on the hypothalamus to stimulate endogenous GnRH release, which is individually determined and is therefore less likely to produce excessive gonadotrophin response compared to a pharmacological dose of GnRH. Nevertheless, well-designed clinical trials are needed to validate the utility of MVT-602 in IVF studies. Aside from its role in IVF studies, MVT-602 also enables an augmented gonadotrophin (especially the enhanced serum FSH) response observed in women with HA. This exaggerated gonadotrophin rise resulted in an increased serum oestradiol response in women with HA, thus highlighting a potential role for MVT-602 to be used as an ovulation induction agent in women with anovulatory HA. Further studies are indicated to evaluate repeated administration of MVT-602 as a novel therapeutic approach to restore fertility in HA.

Kisspeptin stimulates GnRH-induced gonadotrophin secretion. Therefore, it can be used as a probe to interrogate the functional integrity of hypothalamic GnRH neuronal function in the hypothalamus. Chan *et al.* had previously administered KP10 at 0.24nmol/kg (0.313μg/kg) to determine the hypothalamic function in 11 patients with CHH. Whilst this dose had previously elicited a rise in LH of 5iU/L at 30minutes in healthy men (Chan *et al.*, 2011), LH did not rise in any person with CHH, regardless of genotype. This was the case even with repeated dosing of KP10 or using doses up to 2.4nmol/kg, or after GnRH priming (Chan *et al.*, 2014), suggesting that kisspeptin can be used as a potential tool to assess hypothalamic neuronal function. However, the protocol used in these studies utilised a short-acting peptide (KP10) that often requires frequent dosing or long hospital stays. As KP10 is shorter acting and more easily subjected to degradation, it is noteworthy that the gradation of response in these studies were not as robust and may have practical implications for the implementation of kisspeptin stimulation testing in the clinical setting. Therefore, in the last part of my thesis, I explored the potential for a longer acting peptide (KP54) to be used as a specific test of hypothalamic function in the investigation of men with hypogonadotrophic hypogonadism.

My study provided very encouraging results in that all men with CHH, irrespective of their specific genetic mutation, had an attenuated response to KP54 consistent with impaired hypothalamic function, even if the pituitary responsiveness to GnRH was preserved. Indeed, all men with CHH had an LH rise after KP54 of <2iU/L, whereas all healthy men had an LH rise of >4iU/L. In contrast, LH-rises can go up to as high as 40iU/L after GnRH in men with CHH and overlap of LH responses were observed between healthy men and CHH men suggesting the poor diagnostic specificity of a GnRH test. A KP54 test gives an auROC of 1.0 and markedly surpassed GnRH test which has an auROC of 0.88 in the differentiation of men with CHH from healthy men.

In this study, I have used KP54 instead of KP10 as a probe of the hypothalamic GnRH neuronal network because recent evidence has suggested that KP54 may cross the blood brain barrier and thus could theoretically provide more specific information on the functionality of GnRH neuronal cell bodies (Comninos *et al.*, 2017; De Tassigny *et al.*, 2017) as opposed to stimulation of GnRH neuronal terminals at the median eminence (De Tassigny *et al.*, 2017). Furthermore, due to the longer half-life of KP54 ($t_{1/2}$ KP54: 28 minutes, KP10: 3 minutes), it could provide preferable pharmacokinetic properties for intravenous bolus administration during a 'kisspeptin test' (C N Jayasena, Abbara, *et al.*, 2015). Indeed, the median LH-rise was 13.2iU/L after KP54 in the present study, whereas the highest reported mean LH-rise following KP10 is 8.3iU/L after a dose of 0.77nmol/kg in healthy men (George *et al.*, 2011). Thus, the greater magnitude of response induced by KP54 could facilitate better differentiation between healthy and abnormal responses, which could be of relevance for partial responders/milder phenotypes, or potentially helping to differentiate CHH from a self-limiting condition called constitutional delay of growth and puberty (CDGP) in teenage boys.

Men with CHH due to pathogenic mutations exhibited lower LH-rises following KP54 administration than men with CHH with either VUS or no abnormality identified on genetic testing. Likewise, men with CHH with anosmia displayed even more attenuated responses after KP54 administration than normosmic men with CHH, indicating that patients with failure of GnRH neuronal migration are less

likely to respond to KP54 due to the inappropriate location of GnRH neurons. In line with this, anosmic men with CHH are also reported to have a more severe and complex phenotype than normosmic men with CHH (Quinton *et al.*, 2001; Bonomi *et al.*, 2018). Overall, this implied that the magnitude of gonadotrophin response following KP54 administration can provide information on the functionality of the hypothalamic neuronal network and the magnitude of response can be used to differentiate patients with CHH from healthy individuals.

In addition to being used as a potential tool to accurately differentiate CHH from CDGP in teenage boys, KP54 could also serve as a probe to identify CHH patients who have experienced reversal of their condition. Up to 20% of patients with CHH experience reversal of their condition and normalisation of their reproductive hormones without the use of medication (Quinton *et al.*, 2001; Boehm *et al.*, 2015). Current literature indicates that reversal can occur in any patients with CHH, irrespective of the severity of the phenotype and is not restricted to mutations in any one CHH gene (Boehm *et al.*, 2015). However, the process of reversal could theoretically represent an awakening of the HPG axis analogous to the awakening of the reproductive endocrine cascade at puberty, a process that requires kisspeptin. Indeed, Lippincott and colleagues have shown subjects with CHH with reversal demonstrated an LH-rise to KP10 bolus (0.24nmol/kg) whereas individuals with relapses or persistent CHH did not show any response (Lippincott *et al.*, 2016b). In line with the previous study (Lippincott *et al.*, 2016b), our study confirmed that response to KP54 is not interfered by baseline testosterone level, suggesting that a 'KP54 test' could be used to expedite the assessment of hypothalamic GnRH function in patients with CHH with potential reversal without interfering their treatment.

In summary, I have investigated the physiological profile of kisspeptin receptor agonist, MVT-602 in healthy women, women with HA and women with PCOS. I provide novel evidence that administration of MVT-602 induces a gonadotrophin response that is comparable to KP54 in healthy women, but the duration of gonadotrophin response is prolonged resulting in an increased AUC of gonadotrophin stimulation. In women with HA, a single subcutaneous dose of MVT-602 resulted in augmented

gonadotrophin response and higher levels of oestradiol, suggesting a potential use as an ovulation induction agent in this group of women. Lastly, I demonstrate that the longer peptide KP54 could be used to interrogate the hypothalamic GnRH function in men with CHH. The longer half-life of KP54 also provided a more favourable pharmacokinetic profile compared to KP10 and the gradation of response is far more helpful in identifying patients with CHH from healthy individuals. Therefore, KP54 offered important diagnostic value in the accurate investigation of men with HH compared to currently available investigations.

5.1 Future studies

I have finished each chapter with a separate section on future work, however I would like to just briefly outline the future directions for the study of kisspeptin based on my work.

Over the years, translational studies in humans have not only helped researchers garner phenomenal insight into the physiological effects of kisspeptin in humans, but also presented new possibilities in kisspeptin-based therapeutics. However, human studies are challenging to conduct as they bring with them a range of safety, ethical and regulatory issues. Very often, researchers develop different dosing regimens administered via various routes to guarantee an effective yet safe clinical outcome in patients. Yet, the different isoforms used in different research protocols can sometimes pose challenges to interpretation of results. Therefore, evidence-based studies using kisspeptin/kisspeptin receptor agonists would allow clinicians to better decode the role of kisspeptin in clinical practice.

Kisspeptin stimulates (depending on dose) a more physiological magnitude of downstream hormones by stimulating the release of an endogenous pool of GnRH. This approach has several advantages compared to treatments lower down the HPG axis (GnRH injection, LH/FSH injections, oestradiol/testosterone treatments). These other treatments typically result in supraphysiological levels of downstream hormones. For example, the use of hCG to trigger the mid cycle LH surge in IVF studies has resulted in a potent action on the LH receptors in the ovary. The longer half-life of hCG (24 hrs) results in prolonged

activation of ovarian LH receptor and predispose a woman to the potentially life-threatening condition of OHSS. By contrast, kisspeptin acts at the top of axis and release of GnRH is tailored to an individual's own axis, thus reducing its potential for complications. Given that MVT-602 is longer acting than kisspeptin, it enables a gonadotrophin response that is comparable to KP54 in healthy women, but with prolonged duration of gonadotrophin response, thus greatly mimicking the endogenous physiological LH surge. It would be worthwhile to investigate the utility of MVT-602 as an ovulation trigger in IVF studies or as an ovulation induction agent in chronic protocols in women with HA. As alluded to in my findings, the profile of MVT-602 in healthy women is unexpectedly different from that observed in men. Therefore, further work to explore the signaling mechanisms following receptor-ligand activation would be beneficial. Even more crucially, further experiments in *KISS1R* knock out mice or using specific NPFFR antagonists (Kim *et al.*, 2015) would perhaps, shed light on the possible interaction between kisspeptin and RF amide receptors in the brain.

Given that kisspeptin sits at the apex of the reproductive axis, it may be used to interrogate the hypothalamic GnRH neuronal network in patients with disordered GnRH secretion. Traditionally, GnRH is used as a probe to identify these patients. However, the lack of specificity of GnRH test has led researchers to search for an alternative diagnostic tool that could provide better accuracy and specificity. My findings on the performance of KP54 as a diagnostic test in men with CHH has provided encouraging results. Besides repeating the study in a larger cohort of patients, expanding the patient group to include men and women with acquired HH would be of great value. From a clinical perspective, it would be of benefit to explore the ability of KP54 in accurately differentiating children with CHH and CDGP or in differentiating women with HA from women with CHH or PCOS.

Evidently, there remains much work to be done, fueled by the exciting results observed to date.

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