Imperial College London

The role of Kisspeptin and its analogue in the diagnosis and treatment of reproductive disorders in humans

Thesis submitted for the degree of

Doctor of Philosophy

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August 2020

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Declaration of Originality

I, Maria Phylactou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been acknowledged and referenced. I completed this research project under the guidance of my supervisors Professor Waljit Dhillo and Dr Ali Abbara.

Copyright Declaration

TABLE OF CONTENTS

ABSTRACT **8**

INDEX OF TABLES **10**

INDEX OF FIGURES **11**

DECLARATION OF CONTRIBUTORS **17**

ACKNOWLEDGEMENTS **18**

CHAPTER 1: INTRODUCTION **20**

SECTION 1: The Hypothalamic-Pituitary-Gonadal axis **20**

1.1 The Hypothalamic-Pituitary-Gonadal axis **20**

1.2 The Menstrual cycle **22**

1.3 Ovarian control of gonadotrophins **25**

1.4 GnRH Pulsatility **26**

1.5 KNDy neurons **27**

1.5.1 NKB antagonists for the treatment of reproductive disorders 30

SECTION 2: Kisspeptin **31**

2.1 Kisspeptin and the *KISS1* gene **31**

2.2 The Kisspeptin receptor **33**

2.3 Kisspeptin expression and distribution **33**

2.3.1 Kisspeptin expression in the brain 33

2.3.2 Kisspeptin expression in other tissues 35

2.5 Kisspeptin in Reproductive Endocrinology **35**

2.5.1 Kisspeptin's role in the initiation of puberty 36

2.5.2 Effect of kisspeptin on gonadotrophin release 36

2.5.3 Kisspeptin's role in the sex steroid feedback control of gonadotrophins 37

2.6 Kisspeptin receptor desensitisation 38

2.7 Metabolic regulation of kisspeptin 39

2.8 Kisspeptin administration in humans 41

2.8.1 Kisspeptin administration in healthy men and women 41

2.8.2 Kisspeptin's effects on GnRH pulsatility 42

2.9 Kisspeptin based therapeutics 43

2.9.1 The use of kisspeptin for oocyte maturation in *In Vitro* Fertilisation (IVF) 43

2.9.2 The use of kisspeptin in women with Hypothalamic Amenorrhoea (HA) 45

2.9.3 The use of kisspeptin in men with diabetes related hypogonadism 45

2.10 The case for kisspeptin analogues 46

SECTION 3: The diagnostic challenge of reproductive endocrine disorders 47

3.1 Congenital Hypogonadotrophic Hypogonadism 47

3.2 Anovulatory disorders 47

SECTION 4: Aims and Hypotheses 50

CHAPTER 2: THE EFFECT OF KISSPEPTIN-54 ON GONADOTROPHIN RESPONSES IN HEALTHY MEN AND MEN WITH CONGENITAL HYPOGONADOTROPHIC HYPOGONADISM (CHH) 54

2.1 Introduction 54

2.2 Aims and Hypotheses 56

2.2.1 Aims 56

2.2.2 Hypotheses 56

2.3 Methodology 57

2.3.1 Study Overview 57

2.3.2 Ethical Approval 57
2.3.3 Study Subjects 57
2.3.4 Study Protocol 58
2.3.5 Peptides 60
2.3.6 Hormone Assays 61
2.3.7 Statistics 61
2.3.8 Genetic testing 61
2.4 Results 63
2.4.1 Baseline characteristics 63
2.4.2 Gonadotrophin responses to KP54 and GnRH in healthy men 66
2.4.3 Comparison of gonadotrophin responses between healthy men and men with CHH 68
2.4.4 Other variables affecting gonadotrophin responses 76
2.4.5 Genetic variants in men with CHH 83
2.5 Discussion 86
2.5.1 Limitations 91
2.5.2 Conclusion and future work 92
CHAPTER 3: THE EFFECT OF KISSPEPTIN-54 AND ITS RECEPTOR AGONIST, MVT602, ON GONADOTROPHIN RESPONSES IN HEALTHY WOMEN 94
3.1 Introduction 94
3.2 Aims and Hypotheses 97
3.2.1 Aims 97
3.2.2 Hypotheses 97
3.3 Methodology 98
3.3.1 Study Overview 98
3.3.2 Ethical approval 98
3.3.3 Study Subjects 98
3.3.4 Study Phase Overview 99
3.3.5 Study Design 100

3.3.6 Peptides 106
3.3.7 Hormone Assays 107
3.3.8 Statistical analysis 108
3.4 Results 109
3.4.1 Baseline Characteristics 109
3.4.2 Results of Phase 1 112
3.4.3 Results of Phase 2 120
3.5 Discussion 124
3.5.1 Clinical significance of findings 128
3.5.2 Study limitations 130
3.5.3 Conclusion and future work 131
CHAPTER 4: THE EFFECT OF KISSPEPTIN-54 AND ITS RECEPTOR AGONIST, MVT602, ON GONADOTROPHIN RESPONSES IN WOMEN WITH POLYCYSTIC OVARIAN SYNDROME AND WOMEN WITH HYPOTHALAMIC AMENORRHOEA 134
4.1 Introduction 134
4.1.1 Subfertility 134
4.1.2 Polycystic Ovarian Syndrome 135
4.1.3 Hypothalamic amenorrhoea 137
4.2 Aims and Hypotheses 139
4.2.1 Aims 139
4.2.2 Hypothesis 139
4.3 Methodology 140
4.3.1 Study Overview 140
4.3.2 Ethical approval 140
4.3.3 Subjects 140
4.3.4 Study Phase Overview 141
4.3.5 Study Design 143
4.3.6 Peptides 149

4.3.7 Hormone Assays 149
4.3.8 Statistical analysis 149
4.4 Results 150
4.4.1 Baseline Characteristics 150
4.4.2 Results of Phase 1 152
4.4.3 Results of Phase 2 169
4.5 Discussion 172
4.5.1 Limitations 175
4.5.2 Conclusion and future work 175
CHAPTER 5: DISCUSSION 178
REFERENCES 183

ABSTRACT

Kisspeptin, a hypothalamic neuropeptide encoded by the KISS1 gene, is a key regulator of the hypothalamic-pituitary-gonadal (HPG) axis, by stimulating pulsatile gonadotrophin releasing hormone (GnRH) secretion. Since 2005, there has been a huge body of work on the effects of exogenous kisspeptin administration on gonadotrophin responses in humans. These have demonstrated kisspeptin's potential as a therapeutic agent in reproductive disorders and highlighted its potential as a diagnostic tool to probe hypothalamic GnRH neuronal function. The translational application of kisspeptin as a diagnostic and therapeutic tool in the field of reproductive endocrinology has been the focus of this research project.

Firstly, I investigated kisspeptin's ability to differentiate men with congenital hypogonadotrophic Hypogonadism (CHH) from healthy men compared to the currently available GnRH test.

All circulating isoforms of kisspeptin have relatively short half-lives due to their rapid enzymatic degradation. Furthermore, repeated kisspeptin administration causes kisspeptin receptor desensitisation. Thus, a more stable, longer acting kisspeptin analogue would be an ideal candidate for use in the field of kisspeptin based therapeutics. MVT602 is one such agonist, and whilst it has shown promising results during application in men, its effects in women had not been previously explored. I thus investigated the effects of MVT602 on the gonadotrophin responses of healthy women in the follicular phase, and how these change after oestrogen pre-treatment.

In the final study of this research project I compared the gonadotrophin responses elicited by both KP54 and MVT602 in women with the two commonest anovulatory disorders, namely Polycystic Ovarian Syndrome (PCOS) and Hypothalamic Amenorrhoea (HA).

In summary, the results of this research project highlight the huge potential of KP54 and MVT602 to improve the diagnosis and treatment of patients with reproductive disorders, and also add to the existing body of work in the field of translational research for kisspeptin.

INDEX OF TABLES

Table 2.1: Baseline characteristics of men with congenital hypogonadotrophic hypogonadism (CHH) and healthy men 64

Table 2.2 Baseline Characteristics of CHH men 66

Table 3.1: Comparison between the amino acid sequence of KP10 and MVT602 96

Table 3.2: Baseline characteristics of participants 111

Table 4.1 Baseline characteristics of the three groups 152

INDEX OF FIGURES

Figure 1.1: The hypothalamic-pituitary gonadal (HPG) axis..... 22

Figure 1.2 Hormonal, ovarian and endometrial changes during the menstrual cycle 24

Figure 1.3 The 'KNDy neuron' model for regulation of GnRH secretion and sex steroid feedback control..... 29

Figure 1.4 The amino acid structure of kisspeptins 32

Figure 1.5 The effect of systemic metabolic cues on the HPG axis 41

Figure 2.1 Study Protocol..... 59

Figure 2.2 Preliminary dose-response for KP54 in healthy men. 60

Figure 2.3: Hormone changes after KP54 and GnRH in healthy men..... 67

Figure 2.4 LH levels after GnRH and KP54 in healthy men and CHH men..... 69

Figure 2.5 Maximal LH levels after GnRH and KP54 in healthy men and CHH men..... 70

Figure 2.6 ROC of LH levels after KP54 and GnRH to differentiate healthy men from those with CHH..... 71

Figure 2.7 Change in serum FSH following KP54 and GnRH in healthy men and men with CHH..... 73

Figure 2.8 Maximum change in serum FSH following administration of KP54 and GnRH in healthy men and men with CHH..... 74

Figure 2.9 Change in serum testosterone following administration of KP54 and GnRH in healthy men and men with CHH..... 75

Figure 2.10 Maximum change in serum gonadotrophins following administration of KP54 and GnRH in healthy men and men with CHH 78

Figure 2.11 Relationship between baseline LH levels and maximum change in serum LH following administration of KP54 and GnRH in healthy men and men with CHH 79

Figure 2.12 Relationship between baseline *FS*H levels and maximum change in serum *FS*H following administration of KP54 and GnRH in healthy men and men with CHH 80

Figure 2.13 Relationship between mean testicular volume and maximum change in serum gonadotrophins following KP54 81
Figure 2.14 Relationship between baseline testosterone levels and maximum change in LH following KP54 in healthy men and men with CHH 82
Figure 2.15 Relationship between baseline AMH levels and maximum change in serum LH following KP54 in healthy men and men with CHH 82
Figure 2.16 Maximum change in serum LH according to results of genetic analysis in men with CHH 84
Figure 2.17 Maximum change in serum gonadotrophins following KP54 according to olfactory status in men with CHH 85
Figure 3.1: The chemical structure of MVT602 96
Figure 3.2: Diagram of study protocol for Phase 1 103
Figure 3.3: Diagram of study protocol for Phase 2 105
Figure 3.4 Change in serum gonadotrophin and oestradiol levels following administration of MVT602, KP54 and 0.9% Saline 113
Figure 3.5 Area under the curve of change in gonadotrophins and oestradiol 114
Figure 3.6 Other determinants of gonadotrophin responses to MVT602 116
Figure 3.7 Blood pressure measurements during Phase 1 118
Figure 3.8: Heart rate measurements during Phase 1 119
Figure 3.9 Serum gonadotrophin and oestradiol levels following MVT with and without Oestradiol supplementation 121
Figure 3.10 Change in serum LH and FSH following MVT602 with and without Oestradiol supplementation 122
Figure 4.1: Diagram of the protocol for Phase 1 146
Figure 4.2: Diagram of protocol used for Phase 2 148
Figure 4.3 Change in gonadotrophins and oestradiol following administration of KP54 in the three groups 153

Figure 4.4: Maximal change in serum gonadotrophins and oestradiol following administration of KP54 in the three groups..... 154
Figure 4.5: Change in gonadotrophins and oestradiol levels in the three groups following administration of MVT602 156
Figure 4.6: Area under the curve of change in gonadotrophins and oestradiol in the three groups following MVT602..... 157
Figure 4.7: Time to first peak LH rise in the three groups following MVT602 158
Figure 4.8: Maximal change in serum gonadotrophins following KP54 and MVT602 in the three groups..... 159
Figure 4.9: Change in LH compared to baseline oestradiol following KP54 and MVT602.. 160
Figure 4.10: Change in FSH compared to baseline oestradiol in the three groups following administration of MVT602 and KP54..... 161
Figure 4.11 Blood pressure and heart rate measurements in women with HA..... 164
Figure 4.12 Blood pressure and heart rate measurements in women with PCOS 166
Figure 4.13 Effects of MVT602 on blood pressure and heart rate readings in the three groups 168
Figure 4.14 Change in serum gonadotrophins following administration of MVT602 in the three groups pre-treated with oestrogen..... 170
Figure 4.15 Maximum change in serum LH compared to baseline oestradiol following administration of MVT602 in the three groups with and without oestrogen pre-treatment.. 171

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
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
KISS1R/Kiss1r	Kisspeptin receptor
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 ribonucleuic acid
ME	Median Eminence
MUA	Multi-unit Activity
N	Normosmia
n/a	Not applicable
NKB	Neurokinin B
NMDA	N-methyl-D-L-aspartic acid
NPFFR	Neuropeptide FF Receptor
NT	Not Tested

OHSS	Ovarian Hyperstimulation Syndrome
PCOS	Polycystic Ovarian Syndrome
PLC	Phosopholipase 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
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 Undeconoate (Nebido)
TV	Testicular Volume
UPSIT-40	University of Pennsylvania 40 Item Smelling Test
VEGF	Vascular Endothelial Growth Factor
VUS	Variance of uncertain Significance

DECLARATION OF CONTRIBUTORS

Declaration of originality: I hereby declare that this thesis describes my own original work. All collaborations and assistance I have received are declared below:

Chapter 2: Professor W.S. Dhillo and Dr Ali Abbara designed the study protocol. I carried out participant recruitment and study visits with the help of my colleagues Dr Pei Chia Eng and Dr Sophie Clarke. I also performed the analysis of the blood samples with assistance from Dr Pei Chia Eng.

Chapter 3: Professor W.S. Dhillo and Dr Ali Abbara designed the study protocol. I carried out participant recruitment and study visits with the help of my colleagues Dr Pei Chia Eng and Dr Sophie Clarke. I also performed the analysis of the blood samples with assistance from Dr Pei Chia Eng. Myovant Sciences Ltd provided the kisspeptin analogue MVT602.

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ACKNOWLEDGEMENTS

I am grateful to my academic supervisor, Professor Waljit Dhillo for giving me the opportunity to join the kisspeptin team and for his guidance and encouragement throughout the years.

I am indebted to my supervisor Dr Ali Abbara for investing countless hours in mentoring me, for the many opportunities he has given me and for his constant encouragement and unwavering support. I would especially like to thank my colleague Dr Pei Chia Eng for her hard work, dedication, guidance, and exceptional organisational skills. For the clinical studies, I am grateful to my colleagues Dr Sophie Clarke, Dr Chioma-Izzi Engbeaya, Dr Tia Hunjan, Dr Edouard Mills, Dr Lisa Yang, Dr Layla Thurston, Dr Bryn Owen, Dr Manish Modi, Dr Christos Panayi, Ms Debbie Papadopoulou, Dr Bella Pumptre, Ms Beatrice Muzzi and Ms Ellen Huo for all their help and moral support. Thank you also to Olga, Olu, Sinead, Geoffrey, Jay and all the other staff at the St Mary's Hospital biochemistry lab for accommodating my long hours of analysing there and for always being so supportive.

I would like to thank the study participants, without whom this research work would not have been possible.

This thesis is dedicated to my parents Marios, Eleni and Iphigenia, my siblings Michalis and Margarita, to my fiancé Ricky and of course to Nellie Noodles.

Chapter 1

Introduction

CHAPTER 1: INTRODUCTION

SECTION 1: The Hypothalamic-Pituitary-Gonadal axis

1.1 The Hypothalamic-Pituitary-Gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis is responsible for endocrine reproductive function, thus being critical for fertility.

Anatomically the HPG axis consists of three entities:

1. Hypothalamus: Consisting of the pre-optic area and infundibular nucleus (the human homologue of the arcuate nucleus), where kisspeptin-neurokinin-dynorphin (KNDy) and GnRH-producing neurons are situated (Schally et al. 1971).
2. Pituitary: The anterior pituitary, where pituitary gonadotrophs synthesize and release the two gonadotrophin hormones Follicle-Stimulating Hormone (FSH) and Luteinising Hormone (LH) (Bliss et al. 2010; Stamatiades and Kaiser et al. 2018).
3. Gonads: where sex steroids and gametes are produced under the influence of LH and FSH (Bliss et al., 2010; Brown and Roberson et al. 2017).

Gonadotrophin releasing hormone (GnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2) that is secreted in the preoptic area and the arcuate nucleus of the hypothalamus. GnRH neurons project into the median eminence (ME), where GnRH is released in the hypophyseal-portal circulation to act on the anterior pituitary gland (Marques et al. 2000). There, GnRH 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 (**Figure 1.1**).

GnRH is secreted in a pulsatile manner that controls the pattern of secretion of LH and FSH (Marques et al. 2000). Furthermore, it is now recognised that the stimulatory effects of GnRH on the pituitary gland result in differential LH and FSH secretion (Millar et al. 2004). It has been shown that up to 93% of GnRH pulses are associated with LH pulses, whereas FSH secretion

is less dependent on GnRH pulsatility (Padmanabhan et al. 1997a). The axis is regulated by both positive and negative feedback from sex steroids and gonadal peptides (Tena-Sempere et al. 2005)(J. M. Castellano, Navarro, Fernández-Fernández, et al. 2005).

In males, FSH acts on Sertoli cells to stimulate spermatogenesis, whilst in females it is responsible for ovarian follicular growth (Plant and Marshall 2001). In men, LH acts on Leydig cells to stimulate testosterone synthesis. In females, LH stimulates aromatase to produce oestrogen in granulosa cells (Plant and Marshall 2001). The gonadal steroids, oestrogen and testosterone, exert negative feedback at the level of the hypothalamus and pituitary to inhibit further LH release (Conn et al., 2002). In females, the rising oestradiol levels observed during the mid-ovarian cycle cause a change in the feedback control from negative to positive, which results in the LH surge necessary for ovulation. Following ovulation, the remainder of the follicle forms the corpus luteum, which secretes progesterone to support the endometrium for implantation. Progesterone exerts negative feedback and subsequently reduces gonadotrophin secretion in order to commence the next menstrual cycle if the current one does not result in pregnancy (Conn et al., 2002; Bulun et al. 2011).

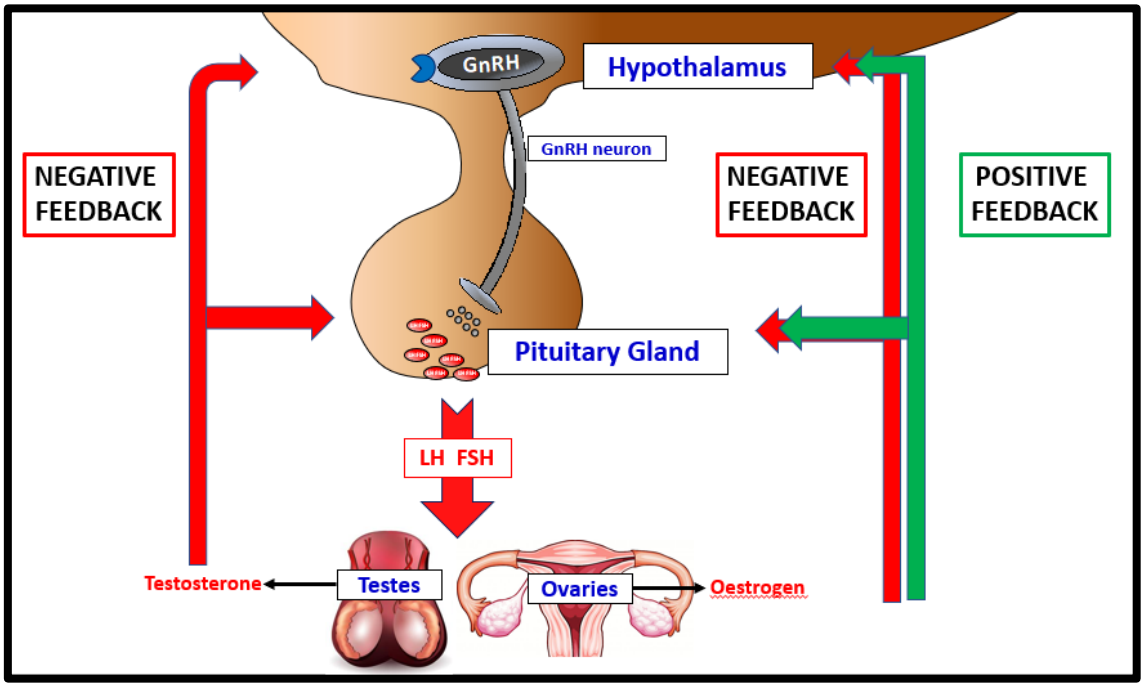


Figure 1.1: The hypothalamic-pituitary gonadal (HPG) axis

Gonadotrophin (GnRH) releasing hormone is secreted in the hypothalamus and is released in the hypophyseal-portal circulation to act on the anterior pituitary gland. There, it stimulates the secretion of Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH). These act on the gonads to stimulate sex steroid secretion and gametogenesis. Whilst testosterone exerts negative feedback control on the gonadotrophins, oestradiol exerts both negative and positive feedback control on the axis (Marques et al. 2000).

1.2 The Menstrual cycle

Menstruation signifies the beginning of a new menstrual cycle, which is divided by ovulation into two parts: the follicular phase and the luteal phase (**Figure 1.2**) (Bulun, 2011; Bates et al. 2013). In healthy women with regular menstrual cycles, the mean (SD) menstrual cycle length is 29.3 ± 5.2 days (Bull et al. 2019) and ranges from 24 days to 38 days (Fraser et al. 2011; Helena J Teede et al. 2018). The average duration of the follicular phase is 16.9 ± 5.3 days and of the luteal phase is 12.4 ± 2.4 days. Cycle length shortens with age after 25 years down to 27.4 ± 4.3 days between the ages of 40 - 45 years, predominantly due to shortening of the follicular phase (Bull et al. 2019; Mihm, Gangooly, and Muttukrishna 2011). The variability in menstrual cycle length is mainly due to variation in the length of the follicular phase and the timing of ovulation (Fehring, Schneider, and Raviele 2006).

By convention, the menstrual cycle begins on the first day of menstruation as a result of luteolysis and a fall in progesterone – i.e. the breakdown of the unfertilised corpus luteum in the previous menstrual cycle. This is associated with falling levels of oestrogen, progesterone, and Inhibin A and due to the reduced negative feedback exerted by these peptides, secretion of the gonadotrophins LH and FSH rises (Mihm et al. 2011). During the follicular phase, the granulosa cells of pre-antral follicles start to proliferate under the influence of FSH. Aromatase enzymes are activated and drive oestrogen production by the granulosa cells. A rise of androgens and Inhibin B is also observed. The FSH drives the selection of a dominant follicle that will eventually undergo ovulation at the end of the follicular phase, whilst the rest will

undergo atresia (Mihm et al. 2011). The dominant follicle drives further increases in oestradiol levels, which will switch feedback on LH from negative to positive once a certain threshold is reached. This drives a surge in LH secretion which typically occurs ~12 hours after peak oestradiol levels are observed (Häggström 2014; Pauerstein et al. 1978)

The dominant follicle rises to the surface of the ovary, and granulosa cells start to express LH receptors, in order to be remodelled under the influence of the LH surge to form the corpus luteum. Ovulation occurs approximately 12 hours following the LH surge (Häggström 2014). Once ovulation has occurred, the luteal phase of the cycle starts, which is characterised by increasing progesterone levels. During the luteal phase, progesterone secretion by the corpus luteum becomes predominant, which exerts negative feedback at the level of the hypothalamus and pituitary to inhibit further gonadotrophin secretion to prevent the growth of a new follicle. In the absence of fertilisation, the corpus luteum degenerates with a subsequent reduction in the levels of oestrogen and progesterone. This leads to loss of the negative feedback and a subsequent rise in FSH levels that leads to a new menstrual cycle (Pauerstein et al. 1978; Bates et al. 2013).

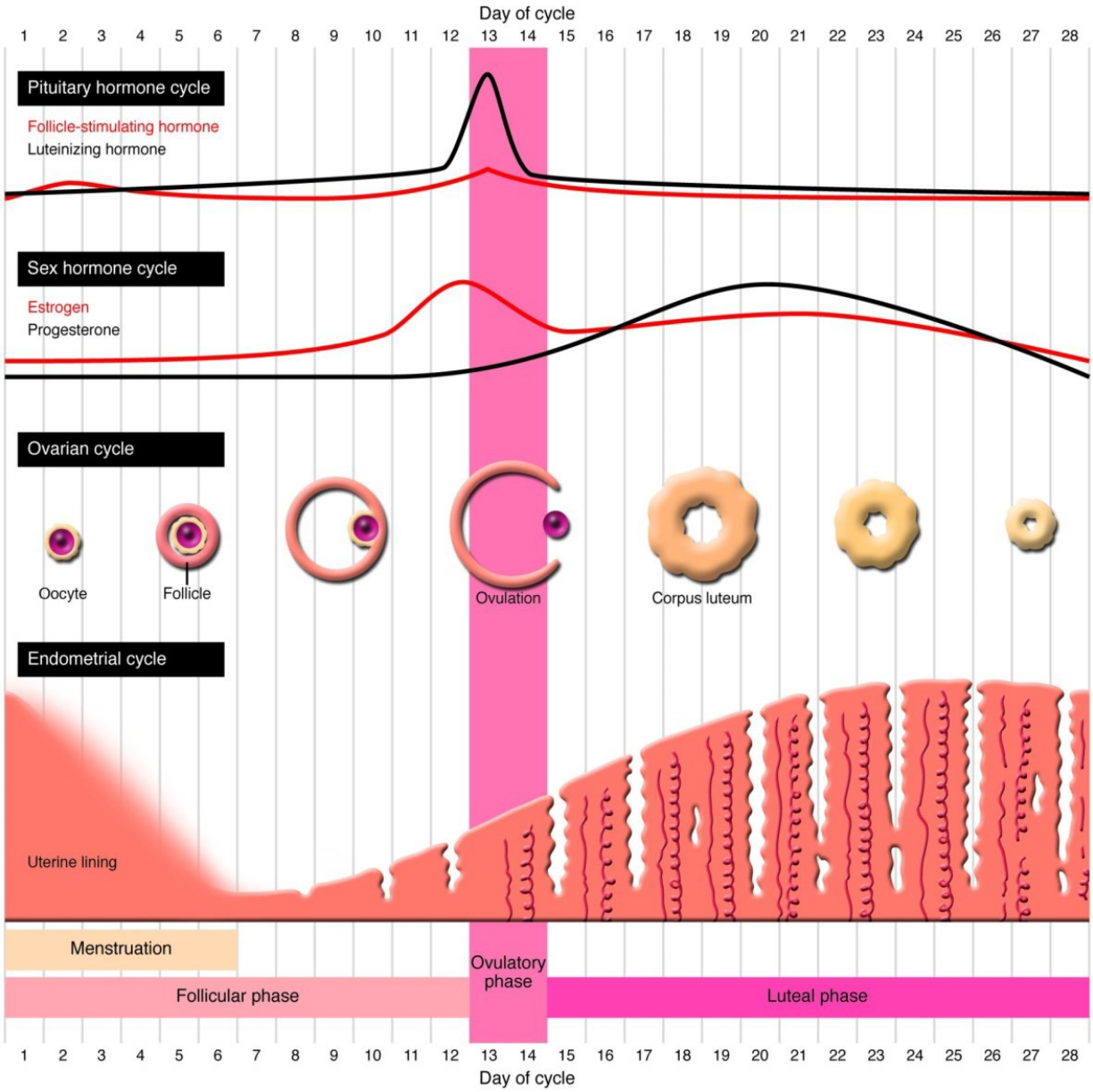


Figure 1.2 Hormonal, ovarian and endometrial changes during the menstrual cycle (Aitken et al. 2008). Menstruation signifies the beginning of a new menstrual cycle, which is a consequence of luteolysis and a fall in oestrogen and progesterone levels. This leads to a rise in FSH and subsequent proliferation of the granulosa cells of the pre-antral follicles and the selection of a dominant follicle for ovulation at the end of the follicular phase. The dominant follicle drives an increase in circulating oestradiol levels and one a certain threshold is reached, the feedback control exerted by oestradiol is switched from negative to positive. This is responsible for the LH surge required for ovulation. Ovulation signifies the start of the luteal phase that is characterised by the rupture of the dominant follicle to form the corpus luteum,

which secretes progesterone levels. If fertilization does not occur, the corpus luteum degenerates with a resultant fall in oestrogen and progesterone levels that lead to the rise of gonadotrophins and the start of a new menstrual cycle.

1.3 Ovarian control of gonadotrophins

Whilst GnRH secretion is primarily responsible for the synthesis of LH and FSH, other endocrine or paracrine signals are also involved in the regulation of gonadotrophin secretion. Among these, are peptides belonging to the Transforming Growth Factor β (TGF β) superfamily (such as Inhibins, activins and bone morphogenic proteins 2 and 4) that are thought to exert influence on *fshb* expression and FSH production and thus also contributing to the differential release of gonadotrophins (Attardi et al. 1989; Lee et al. 2007). Inhibins A and B inhibit further FSH secretion, to enable selection of a dominant follicle (Luisi et al. 2005). Activin, synthesized by the granulosa cells but also the pituitary gland, has the opposite effects to inhibin and thus stimulates FSH release and sensitises the ovary to the effects of FSH (Kitaoka et al. 1988). Follistatin is an activin-binding glycoprotein that reduces FSH secretion (Besecke et al. 1997). It decreases activin-stimulated FSH secretion but has no effect on GnRH-stimulated FSH secretion (Meriggiola et al. 1994). Moreover, anti-Müllerian hormone (AMH) is a homodimeric glycoprotein also belonging to the TGF β superfamily, with a high affinity for its receptor AMH type II receptor (AMHR2) (Josso, Di Clemente, and Gouédard 2001). In women, AMH is produced by the granulosa cells of growing antral follicles in the ovary (Dewailly et al. 2014). Its actions include aiding the emergence of a dominant follicle, reducing the sensitivity of individual follicles to FSH and inhibiting aromatase activity in the ovary, which can lead to increased androgen levels (Pellatt et al. 2011; Teede et al. 2019).

1.4 GnRH Pulsatility

It is now established that GnRH is secreted in two distinct manners: a pulsatile mode and a surge mode (Maeda et al. 2010). Pulsatile GnRH secretion involves the episodic release of GnRH in distinct bursts called pulses (Moenter et al. 2003). On the other hand, the GnRH surge occurs during the pre-ovulatory phase in females which is characterised by persistent release of GnRH into the portal circulation (Maeda et al. 2010).

GnRH pulsatility is a prerequisite for the normal functioning of the HPG axis. Its importance was first recognised when gonadotrophin secretion in rhesus monkeys with hypothalamic lesions was restored following intermittent administration of synthetic GnRH, but not following a constant infusion of the decapeptide (Belchetz et al. 1978). The pulsatile pattern of GnRH secretion was later also confirmed in humans, through frequent blood collected from the pituitary gland during transsphenoidal surgery (Antunes et al. 1978). LH secretion is also pulsatile due to GnRH pulsatility (Caraty, Martin, and Montgomery et al. 1984). In humans LH pulse frequency is now used as a surrogate of GnRH pulsatility (Reame et al. 1985).

GnRH pulsatility is frequently described in terms of the frequency and the amplitude of pulses. The pulse and amplitude of GnRH pulses vary during the menstrual cycle, but also during a human's lifecycle (Marques et al. 2000). *In utero*, GnRH secretion is active during gestation and during early neonatal life. This early activation of the reproductive axis is a phenomenon called 'mini-puberty', that lasts for six months in boys and two years in girls (Tsutsumi and Webster 2009). After years of inactivity during infancy, the axis is activated during puberty. This transition is characterised by pulses that initially occur only nocturnally, prior to then also persisting during daytime (Plant 2015).

LH pulsatility varies during the menstrual cycle, with the frequency of LH pulses changing from 1 to 2 hours during the early follicular phase, to continuous mid-cycle secretion (during the LH surge) and subsequent pulse frequency reduction to four hourly pulses that characterise the luteal phase (Tsutsumi et al. 2009; Tony M. Plant et al. 2015). Low GnRH pulsatility favours

FSH predominant secretion, whereas increased frequency and amplitude of GnRH pulses favours LH secretion (Karsch et al.1987; Marshall et al. 1991).

Circulating sex steroids also impact the GnRH pulsatility, with higher oestradiol levels during the follicular phase of the menstrual cycle switching its feedback from negative to positive and thus increasing GnRH pulsatility (Kirk et al. 1994; Marshall et al. 1991). The increased levels of progesterone produced by the corpus luteum during the luteal phase exert negative feedback on GnRH pulse frequency, that favours FSH predominant secretion, necessary for the commencement of a new menstrual cycle (Petersen, Ottem, and Carpenter 2003). Remarkably, the oestrogen receptor alpha (ERα), which is required to mediate sex-steroid control of gonadotrophin secretion, is not expressed in GnRH neurons. Kisspeptin neurons express ERa and are thus considered important regulators of the effects of oestrogen on GnRH secretion (Yeo et al. 2014; Dubois et al. 2015).

GnRH secretion is also influenced by metabolic, nutritional and psychological cues (Marques et al. 2000). Low body weight is associated with reduced GnRH pulsatility and thus low activity of the HPG axis which is considered an energy saving evolutionary adaptation (Bala et al. 2020).

1.5 KNDy neurons

A hypothalamic neuropeptide intimately involved in GnRH regulation is Neurokinin B (NKB). It shares a common C-terminal amino acid motif with the tachykinin family (Goodman et al. 2007). Kisspeptin, NKB and dynorphin (DYN) (collectively called the KNDy neurons) were found to be collectively situated within the hypothalamic ARC neurons of sheep (Goodman et al. 2007). KNDY neurons have since been reported in the arcuate nucleus of mice, rats, goats as well as in the human infundibular nucleus (Hrabovszky et al. 2010; Navarro et al. 2009; Rance et al. 2009; Skrapits et al. 2014; Wakabayashi et al. 2010).

Neurons expressing these peptides form a network whose role is to regulate GnRH secretion **(Figure 1.3)**. Infusion of NKB receptor blockers in ewes suppresses pulsatile LH release, whereas administration of DYN receptor blockers has the opposite effect (Goodman et al. 2013). Furthermore, progesterone receptors were found on Dynorphin neurons in the POA and ARC of ewes, suggesting that these neurons may have a role in mediating the inhibitory effect of progesterone on GnRH pulsatility (Foradori et al. 2002). Furthermore, in humans inactivating mutations of the NKB (TAC3) genes or its receptor, NK3R (TACR3), result in congenital hypogonadotrophic hypogonadism (Topaloglu et al. 2009; Young et al. 2010). Moreover, administration of pulsatile exogenous GnRH in these patients results in a rise in gonadotrophin levels, thus suggesting that NKB acts upstream of the GnRH neurons to control GnRH secretion (Young et al. 2010).

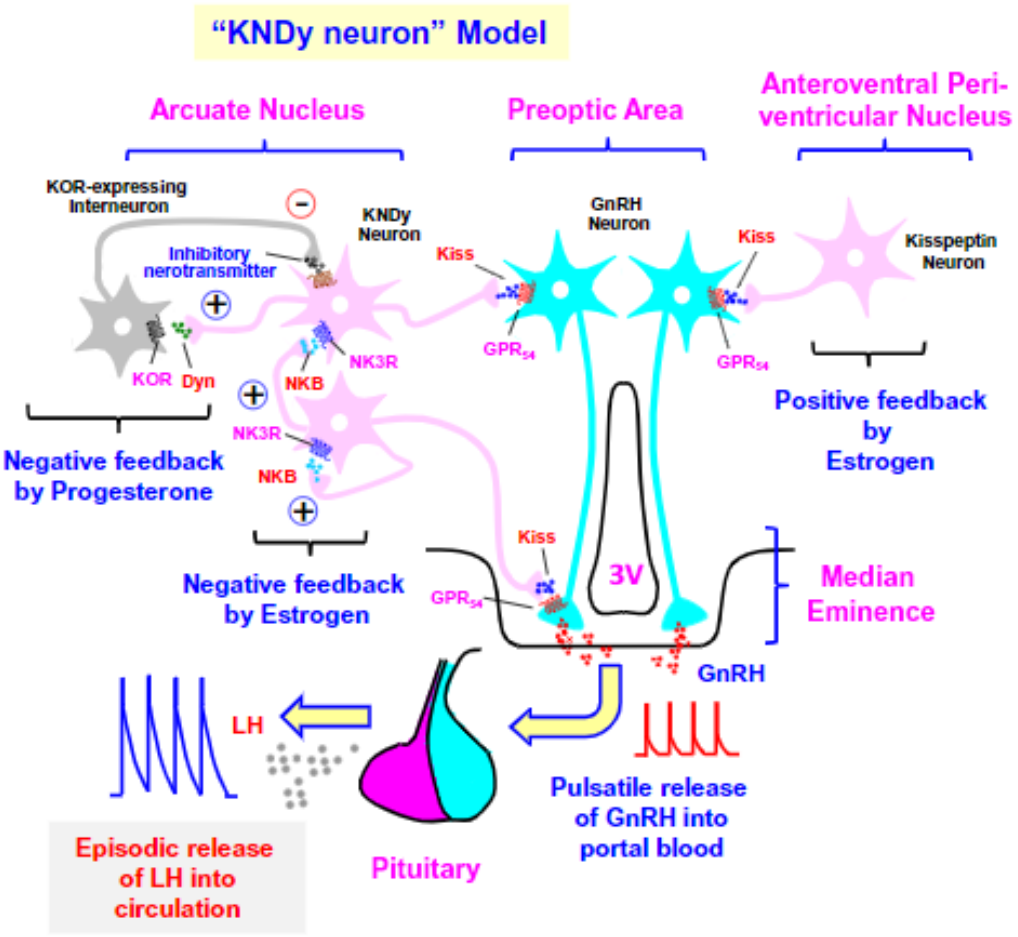


Figure 1.3 The 'KNDy neuron' model for regulation of GnRH secretion and sex steroid feedback control

(Hu et al. 2014). KNDy neurons located in the arcuate nucleus release Neurokinin B (NKB) that activates the type 3 Neurokinin receptor (NK3R) to trigger kisspeptin release. Kisspeptin then stimulates GnRH secretion by acting on both GnRH cell bodies and GnRH nerve terminals. KNDy neurons also secrete Dynorphin A (Dyn) that exerts negative feedback control on kisspeptin release. The KNDy neuron is also a target for negative sex steroid feedback such as oestrogen and progesterone.

1.5.1 NKB antagonists for the treatment of reproductive disorders

Whilst NKB administration to healthy men and women did not affect reproductive hormone levels or GnRH pulsatility, it can cause flushing and heat intolerance in some study participants, especially when given at higher doses (Jayasena et al. 2014). Indeed a 30-minute infusion of NKB induced flushing, whilst this was not reported when the same participants received placebo (Jayasena et al. 2015). This led to the hypothesis that KNDY upregulation could be involved in the pathophysiology of menopausal flushes. Indeed in a ground breaking study conducted by our team, administration of a NK3R antagonist in women suffering from menopausal hot flushes induced a 45% reduction in the weekly number of hot flushes (Prague et al. 2017).

Polycystic ovary syndrome (PCOS) is one of the commonest causes of infertility in women of reproductive age and can affect up to 21% of women (Helena J Teede et al. 2018). It is characterised by a spectrum of signs and symptoms which include anovulation, androgen excess and polycystic ovarian morphology on ultrasound (Teede et al. 2014). Increased GnRH pulsatility is commonly found in women with PCOS, which in turn results in elevated LH levels, whilst FSH levels are relatively preserved (Diamanti-Kandarakis, Kandarakis, & Legro, 2006). NK3R blockade has been trialed in women with PCOS and resulted in a 52% reduction in the area under the curve of LH exposure, a significant reduction in the number of eight hourly LH pulses, as well as a 28.7% reduction in total testosterone levels (George et al. 2016).

SECTION 2: Kisspeptin

2.1 Kisspeptin and the *KISS1* gene

Kisspeptin is a hypothalamic neuropeptide encoded by the *KISS1* gene (Ohtaki et al. 2001). *KISS1* was first discovered in 1996 to be overexpressed in melanoma cell lines with reduced metastatic capacity (Lee et al. 1996). There are four end products of the *KISS1* gene in humans, cleaved from the 145-amino acid polypeptide precursor: Kisspeptin-54 (KP54), Kisspeptin-14 (KP14), Kisspeptin-13 (KP13) and Kisspeptin-10 (KP10), with the suffix signifying the number of amino acids (M Kotani et al. 2001). They all share a common C terminal ten amino acid sequence, including an Arg-Phe-NH_2 motif that is required for activation of the kisspeptin receptor (**Figure 1.4**) (M Kotani et al. 2001; Lee et al. 1996; Ohtaki et al. 2001; West et al. 1998). In fact, this distinctive arginine-phenylalanine residue is what distinguishes kisspeptins from other members of the RF amide family of peptides (Tsutsui et al. 2010). KP54 is the predominant circulating isoform in humans (M Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). In rodents the end product of the *kiss1* gene consists of 52 amino acids, and the terminal RF amide motif is substituted by an Arg-Tyr-NH2 motif (Terao et al. 2004).

Whilst initially discovered as a metastasis suppression gene in melanoma and breast cancer cell lines (Lee and Welch 1997), *KISS1* later emerged as an important regulator of the HPG axis. Patients with inactivating mutations of the gene encoding the kisspeptin receptor were found to have congenital hypogonadotrophic hypogonadism (CHH) (de Roux et al. 2003; Seminara et al. 2003), whereas activating mutations of the same gene were found to cause central precocious puberty (CPP) (Silveira et al. 2010). *KISS1* was discovered in Hershey, Pennsylvania, USA and was named after the famous chocolate produced there, called ‘kisses’. The *KISS1* gene is located on the long arm of chromosome 1, in the region of 1q32-q41. Although it consists of four exons, the first two are not translated (West et al. 1998).

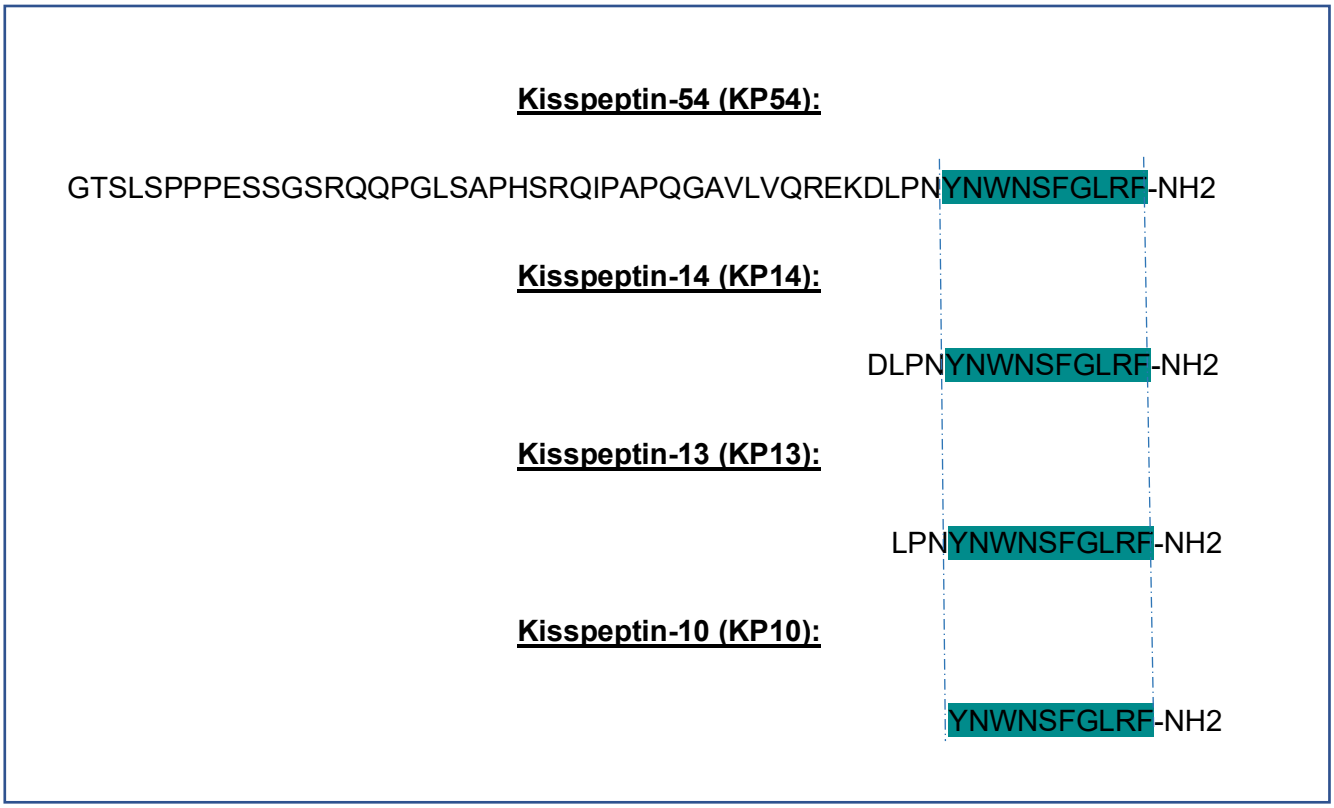


Figure 1.4 The amino acid structure of kisspeptins

All four isoforms of kisspeptin (KP54, KP14, KP13, KP10) share a common carboxyl-terminal decapeptide sequence, which is required for receptor activation (M Kotani et al. 2001)

2.2 The Kisspeptin receptor

Kisspeptin acts on the kisspeptin receptor, previously known as G-protein coupled receptor 54 (GPR54), which is a member of the rhodopsin-gamma family (Ohtaki et al. 2001). It was first discovered in the rat in 1999 as an orphan receptor and was found to have >40% homology with the transmembrane regions of galanin receptors (Lee et al. 1999). A few years later, the human orthologue of KISS1R was cloned and called AXOR12 or hOT7T175 (Muir et al. 2001; Ohtaki et al. 2001). Binding of the ligand to the receptor stimulates a cascade of intracellular signalling pathways including hydrolysis of phospholipase C (PLC) and the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (M Kotani et al. 2001). These result in an increase in calcium flux, the release of arachidonic acid and the phosphorylation of protein kinases including p38 MAP kinases and ERK1/2. The final consequence of receptor activation involves the opening of potassium and non-selective cation channels (M Kotani et al. 2001). Work by Ursula Kaiser's lab has revealed that continuous activation of KISS1R results in a biphasic increase in intracellular calcium signaling, characterised by an initial short first-phase response lasting 5 minutes, and a more sustained 'second-phase' response that lasts for at least 30 minutes (Min et al. 2013). The kisspeptin receptor is continuously internalised and recycled during the second phase, to avoid receptor desensitisation (Min et al. 2013).

2.3 Kisspeptin expression and distribution

2.3.1 Kisspeptin expression in the brain

There is considerable variation in the hypothalamic distribution of *Kiss1* across different species. The sites with the highest density of kisspeptin-expressing neurons are the rostral periventricular area of the third ventricle (RP3V) of rodents or the preoptic area (POA) of other mammals and the arcuate nucleus of the hypothalamus (ARC) (Lehman, Hileman, and Goodman 2013).

Furthermore, *Kiss1* mRNA is also found in the ventromedial hypothalamus and paraventricular nucleus, which are areas involved in reproductive behaviour (Clarkson et al. 2009). In mammals kisspeptin neurons are situated within the infundibular nucleus and preoptic area (POA), which is the equivalent to the arcuate nucleus in rodents. Interestingly the anatomical area equivalent to the rodent AVPV kisspeptin neurons has not yet been discovered in humans, however kisspeptin neurons facilitating oestradiol related positive feedback have been detected in the rostral periventricular zone (RP3V) in female monkeys (Rometo et al. 2007).

The majority of these neurons express receptors vital for sex steroid feedback control of gonadotrophins: the oestrogen receptor α (ERα), the progesterone receptor, and the androgen receptor (Franceschini et al. 2006; Smith, Cunningham, et al. 2005a; Smith, Dungan, et al. 2005). Interestingly, oestradiol increases kisspeptin levels in neurons located within the RP3V, but decreases *Kiss1* in the ARC, thus suggesting the existence of different kisspeptin subpopulations with divergent roles in the control of steroid hormone feedback (Smith, Cunningham, et al. 2005b).

The distribution of kisspeptin neurons in the hypothalamus also exhibits sexual dimorphism. Female rodents have a larger population of *Kiss1* neurons in their AVPV than male rodents, (Clarkson et al. 2009; Kauffman et al. 2007). Furthermore kisspeptin neurons that facilitate positive sex steroid feedback have not been identified in male monkeys or men, demonstrating their significance in mediating oestradiol positive feedback necessary for the LH surge (Hrabovszky et al. 2010; Ramaswamy et al. 2008).

Whilst the density and distribution of kisspeptin neurons in the ARC of rodents appears to be the same between the two sexes (Clarkson et al. 2006; Kauffman et al. 2007; Clarkson et al. 2009), in sheep, the distribution is sexually dimorphic with half the number of kisspeptin neurons found in rams than in ewes (Cheng et al. 2010).

2.3.2 Kisspeptin expression in other tissues

KISS1 is also expressed in the theca cells of growing follicles, the corpus luteum, testes, carotid bodies, pancreas, liver and small intestine (Cejudo Roman et al. 2012; M Kotani et al. 2001; Ohtaki et al. 2001; Owens et al. 2018a; Terao et al. 2004). The kisspeptin receptor (KISS1R) was also found in smooth muscle of vessels with the same developmental origins: aorta, coronary artery, and umbilical vein (Mead et al. 2007).

KISS1 is also highly expressed in the syncytiotrophoblast cells of the placenta, and indeed kisspeptin has recently emerged as a putative marker of placentation (Hu et al. 2019). Human maternal kisspeptin levels rise linearly throughout pregnancy, with first trimester plasma kisspeptin levels found to be 900-fold higher than in non-pregnant women, and the levels increase further to 7000-fold higher during the third trimester (Horikoshi et al. 2003). Whilst the physiological role of circulating kisspeptin in pregnancy is unknown, it has been hypothesised to be involved in the processes of implantation, placentation and uterine decidualisation (Reynolds et al. 2009; Jayasena et al. 2015).

2.5 Kisspeptin in Reproductive Endocrinology

It was not until 2003 when the crucial role of kisspeptin as a potent regulator of reproductive hormone secretion was recognised when two seminal papers revealed that absence of kisspeptin signaling resulted in hypogonadotrophic hypogonadism (HH) (de Roux et al. 2003; Seminara et al. 2003). Subsequently, various animal models of kisspeptin receptor inactivation explored this further and confirmed the same phenotypic presentation, namely the presence of secondary hypogonadism, with low serum gonadotrophins despite low sex steroid levels and failure to initiate puberty (Funes et al. 2003; Lapatto et al. 2007; Seminara et al. 2003). Administration of GnRH in *Kiss1r* knock out mice results in potent secretion of LH and FSH, suggesting that the lack of kisspeptin signaling does not affect the responses of pituitary gonadotrophs (Seminara et al. 2003). On the other hand, people with activating mutations in kisspeptin signalling develop central precocious puberty (Teles et al. 2008).

2.5.1 Kisspeptin's role in the initiation of puberty

It is now recognised that kisspeptin signalling is prerequisite for pubertal initiation (Seminara et al. 2003). In animal models, exogenous kisspeptin enhances vaginal opening in prepubertal rats by approximately four days (Navarro et al. 2004). Prepubertal male monkeys primed with GnRH exhibit LH responses following administration of kisspeptin similar to those seen in post-pubertal monkeys (Shahab et al. 2005; Plant et al. 2006). Moreover, administration of a kisspeptin receptor antagonist (p234) delayed vaginal opening in juvenile female rats (Sahin et al. 2015).

Kiss1 mRNA expression is increased in the hypothalamus of peripubertal rats of both sexes (Navarro et al. 2004). The same is observed in female and male rhesus monkeys (Shahab et al. 2005), even in the absence of sex steroids, thus suggesting that this process is independent of gonadal steroid feedback (Shahab et al. 2005). Furthermore, increased *Kiss1r* mRNA expression in GnRH neurons is also reported in mice at the time of pubertal transition (Herbison et al. 2010).

2.5.2 Effect of kisspeptin on gonadotrophin release

These findings uncovered many new possibilities for kisspeptin and its use in the neuroendocrine control of reproduction. A multitude of studies have since investigated the effects of kisspeptin on gonadotrophin secretion. Central administration of kisspeptin elicited significant LH responses, even at doses as low as 1fmol (Gottsch et al. 2004; Matsui et al. 2004). FSH secretion, whilst also responsive to the stimulatory effect of kisspeptin, was 100-fold less sensitive than LH (Gottsch et al. 2004). The gonadotrophin effects of exogenous kisspeptin were preserved with different routes of administration, such as intravenous, intraperitoneal and subcutaneous, but also with the use of different isoforms of kisspeptin (KP10 and KP54) (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Messager et al. 2005; Navarro, Castellano, et al. 2004; Shahab et al. 2005). Furthermore it was found that KP10 acts on GnRH nerve terminals to stimulate potent GnRH secretion *in situ*, even without

the presence of GnRH neuronal cell bodies (d'Anglemont de Tassigny et al. 2008; Han et al. 2005). KP54, the isoform with the longest amino-acid sequence, crosses the blood brain barrier (BBB) to reach GnRH cell bodies (De Tassigny et al. 2017). Moreover, the stimulatory effect of KP on gonadotrophins was lost after administration of GnRH receptor antagonists, thus suggesting that its effects are mediated via GnRH neurons (Gottsch et al. 2004; Irwig et al. 2004; Shahab et al. 2005).

Because kisspeptin receptors are also expressed in the pituitary, it is plausible that some of kisspeptin's effects on gonadotrophin release could be as a result of direct action there (M Kotani et al. 2001; Muir et al. 2001). However, data from *in vitro* experiments on rat pituitary explants do not support this. For example, even though rat pituitary cultures incubated in KP10 (in doses ranging from 1nM to 10,000nM) resulted in a dose-dependent increase in LH after a few hours of exposure, the impact of kisspeptin's direct pituitary effect *in vivo* is thought to be small (Navarro et al. 2005). This is because *in vitro* doses of kisspeptin required to generate LH release were much higher than those used *in vivo* (Navarro et al. 2005). Moreover, work from other teams found no effect on gonadotrophin secretion when rat pituitary cells were incubated with either KP10 (Thomson et al. 2004) or KP54 (Matsui et al. 2004).

2.5.3 Kisspeptin's role in the sex steroid feedback control of gonadotrophins

GnRH neurons lack the oestrogen receptor, ERα, which suggests that other neurons are required to act as mediators of peripheral signals for GnRH regulation. Kisspeptin neurons, which possess the ERα, have been proposed as the mediators of sex steroid feedback control of gonadotrophins. Indeed, gonadectomy (and thus a lack of oestradiol) increases kisspeptin mRNA expression in the ARC in female rodents, but downregulates kisspeptin expression in the anteroventral periventricular nucleus (AVPV) (Navarro, Castellano, et al. 2004). These changes are reversed following administration of exogenous oestradiol, suggesting differential regulation of kisspeptin in different anatomical areas of the hypothalamus (Smith, Dungan, et al. 2005). When oestradiol is administered to mice that have undergone kisspeptin cell-specific ERα ablation, the kisspeptin expression in the ARC is not affected, whereas that in the AVPV

is significantly reduced (Dubois et al. 2015). Furthermore, the distribution of kisspeptin neurons in the AVPV is sexually dimorphic, with females possessing a higher number than males (Smith, Cunningham, et al. 2005b). Moreover, *Kiss1* mRNA expression in the AVPV is enhanced at the time of ovulation signifying that this positive feedback loop is necessary for the facilitation of the pre-ovulatory LH surge (Smith, Popa, et al. 2006). In summary, there is conclusive evidence that kisspeptin neurons in the AVPV facilitate positive feedback control of GnRH secretion, whereas negative feedback regulation of kisspeptin neurons occurs in the ARC.

2.6 Kisspeptin receptor desensitisation

The hallmark of HPG integrity and function is the episodic nature of GnRH release. GnRH pulsatility is paramount for the integrity of the axis, which appears to be sensitive to continuous GnRH receptor activation. Receptor desensitisation is a phenomenon seen in many G-protein coupled receptors (GPCR) (Kaiser, Conn, and Chin 1997). Prolonged stimulation of these results in phosphorylation of the carboxyl terminal tail, the site of high affinity binding of the arrestin family of proteins. This affects the interaction between GPCR and its ligand and eventually leads to clathrin-mediated endocytosis (Luttrell and Lefkowitz 2002). Other downstream signalling pathways (such as the IP3 and MAPK cascade) are also affected, and the sustained influx of extracellular calcium necessary for receptor activation cannot be maintained (Bianco et al. 2011; Babwah AV et al. 2012). Overall continuous activation of the receptor leads to the loss of dynamic trafficking and cell surface recycling (Min et al. 2013).

Indeed, continuous intravenous infusion of KP10 at a dose of 100 µg/hour in prepubertal rhesus monkeys led to a transient increase in LH levels, prior to a subsequent reduction (Seminara et al. 2006). This is thought to be occurring at the level of the kisspeptin receptor rather than the pituitary gland, as a single bolus of kisspeptin, N-methyl-D-L-aspartic acid (NMDA) and GnRH given at the end of the KP10 infusion, resulted in LH rises (Seminara et al. 2006). Furthermore, the same group repeated the experiment in eugonadal animals with

similar results (Ramaswamy et al. 2007). In summary, chronic stimulation of the kisspeptin receptor leads to its desensitisation, which results in downregulation of the reproductive axis.

2.7 Metabolic regulation of kisspeptin

Energy availability and metabolic cues greatly influence the attainment and maintenance of reproductive capability. For example conditions associated with energy depletion and negative energy balance, such as anorexia nervosa, can lead to hypogonadotrophic hypogonadism (Welt et al. 2004; Gordon et al. 2017a; Thurston et al. 2019). Equally, excess metabolic states such as morbid obesity are associated with precocious puberty in females (Castellano et al. 2011) or hypogonadism in males (Tajar et al. 2010).

Kisspeptin is considered an important conduit integrating metabolic cues to the reproductive system (Tena-Sempere, 2007; Sanchez-Garrido et al. 2013) and there is evidence that it is directly affected by changes in metabolic state (**Figure 1.5**). Male and female pubertal rats exhibited decreased hypothalamic *Kiss1* mRNA expression following a 72 hour fast (J. M. Castellano, Navarro, Fernandez-Fernandez, et al. 2005). This was also replicated in other animal models involving adult rats in the setting of acute food deprivation (Luque et al. 2007; Brown et al. 2008) and in female rats following a sustained state of negative energy balance (True et al. 2011). Moreover, exogenous KP10 administration in prepubertal female rats undergoing chronic undernutrition resulted in potent rises in gonadotrophin and sex steroid levels (J. M. Castellano, Navarro, Fernandez-Fernandez, et al. 2005). Further, there is evidence of enhanced sensitivity to exogenous kisspeptin in experimental models of food restriction and reduced energy availability (J. M. Castellano, Navarro, Fernandez-Fernandez, et al. 2005). Interestingly, there seems to be a correlation between the magnitude of LH responses to KP10 and the duration of undernutrition, suggesting the presence of a hypersensitised endocrine axis, likely resulting from a compensatory increase in kisspeptin receptors (Roa and Tena-Sempere 2007).

It is known that approximately 40% of the kisspeptin neurons in the ARC express leptin receptors (Smith et al. 2006), the adipocyte hormone secreted by white adipose tissue. In fact,

GnRH neurons do not express leptin receptors (Quennell et al. 2009). Thus, leptin might be able to exert its effects as a metabolic modulator on the reproductive system via the hypothalamic kisspeptin system. As such, there are a number of studies demonstrating the effects of leptin on kisspeptin. Ob/Ob mice, an animal model of leptin deficiency, have reduced *Kiss1* mRNA expression in the ARC and RP3V (Smith et al. 2006; Quennell et al. 2011) which is reversed following administration of leptin (Smith et al. 2006). Furthermore, leptin can depolarise up to 82% of the ARC Kiss1 neurons in the guinea pig (Qiu et al. 2011).

In humans, mutations of genes encoding leptin and its receptor are associated with obesity syndromes and hypogonadotropic hypogonadism. Furthermore, Farooqi and colleagues have demonstrated that administration of recombinant leptin in patients with congenital leptin deficiency restores the onset of puberty (Farooqi et al. 2002). Furthermore women with hypothalamic amenorrhoea have been found to have lower leptin levels compared to controls (Welt et al. 2004). In conclusion, kisspeptin neurons can receive and relay signals of metabolic stress to the reproductive system. Kisspeptin's role as a conduit between metabolic cues and the HPG axis renders it a promising therapeutic target for the treatment of reproductive disorders associated with metabolic conditions.

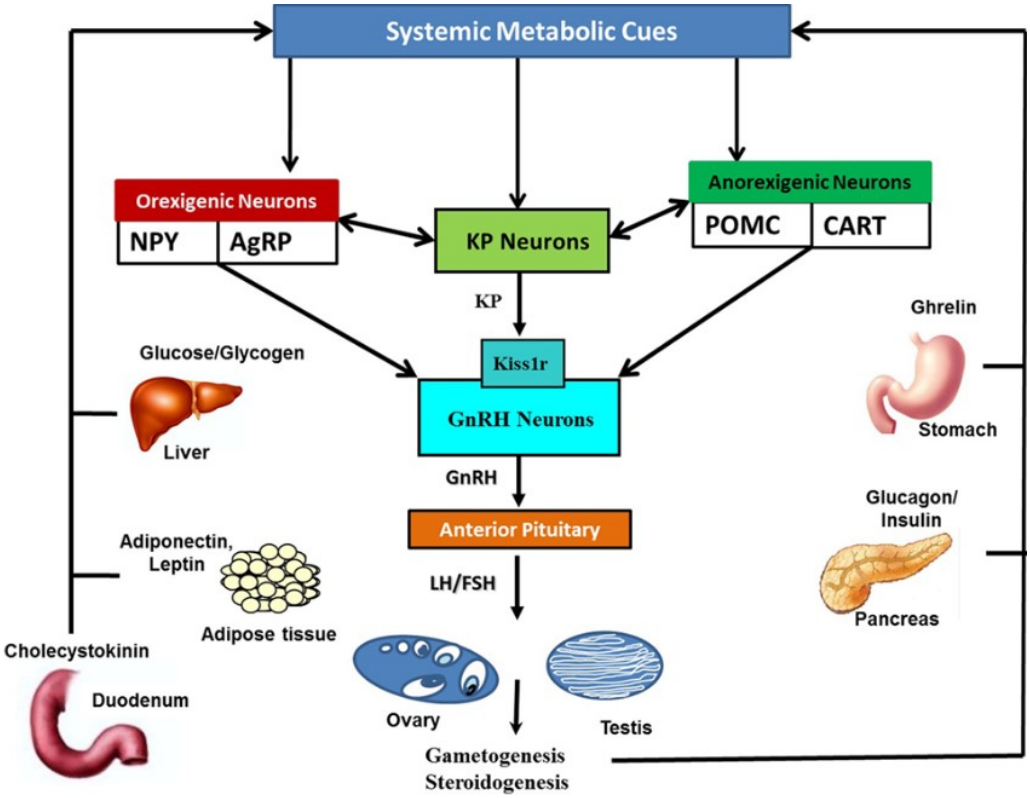


Figure 1.5 The effect of systemic metabolic cues on the HPG axis

(Wahab et al. 2018) Perturbations in the metabolic status result in the secretion of metabolic cues such as leptin, adiponectin and ghrelin, that modulate kisspeptin neuronal activity via orexigenic and anorexigenic neurons.

2.8 Kisspeptin administration in humans

2.8.1 Kisspeptin administration in healthy men and women

In 2005, Dhillo and colleagues were the first to administer exogenous kisspeptin to humans. A 90-minute infusion of KP54 (4 pmol/kg/min) resulted in a dose-dependent rise in gonadotrophin secretion in healthy eugonadal men (Dhillo et al. 2005). Whilst there was a rise in the plasma levels of both LH and FSH, the FSH response was less pronounced compared to LH, consistent with previous data from animal studies. Other isoforms of kisspeptin were also studied; intravenous boluses of KP10 resulted in stimulation of LH secretion as well as

increased LH pulse frequency and pulse amplitude (George et al. 2011). Bolus administration of KP10 (dose range of 0.01-3ug/kg) also resulted in acute LH rises (George et al. 2011). KP10 has a very short half-life of 4 minutes (Dhillo et al. 2005; Jayasena, Abbara, et al. 2015a), which renders it less suitable for subcutaneous bolus administration. By contrast, KP54 has a longer half-life of 27.6 minutes and is thus more biologically stable and could be used to persistently stimulate gonadotrophin secretion by intermittent bolus administration (Dhillo et al. 2005).

In women, kisspeptin also potently stimulates gonadotrophin secretion, but its effects vary according to the phase of the menstrual cycle (Dhillo et al. 2007). Kisspeptin is most potent during the pre-ovulatory phase with LH rises that are five times greater than those observed during the follicular phase (Dhillo et al. 2007). KP10 results in LH stimulation in women during the luteal and preovulatory phase, but only half of women in the early follicular phase were responsive (Y.-M. Chan et al. 2012). This is in part due to background circulating sex steroid milieu during the different phases of the menstrual cycle (George, Anderson, and Millar 2012). Furthermore, kisspeptin also stimulates LH pulsatility in women with hypothalamic amenorrhoea (Channa N. Jayasena, Abbara, Veldhuis, et al. 2014). Indeed, it has been shown that GnRH neuronal cell lines demonstrate an enhanced response to kisspeptin in the presence of oestradiol (Tonsfeldt et al. 2011).

2.8.2 Kisspeptin's effects on GnRH pulsatility

GnRH pulsatility is a prerequisite for the integrity of the HPG axis. Reproductive disorders such as Hypothalamic Amenorrhoea (HA) and Polycystic Ovarian Syndrome (PCOS) are also characterised by abnormal GnRH pulsatility. A bolus of KP54 can temporarily increase LH pulsatility when administered in the follicular phase of healthy women (Jayasena, Comninos, Veldhuis, et al. 2013). A bolus of KP10 in healthy men results in an increase in LH amplitude and resets the GnRH pulse generator (Y M Chan et al. 2011). Furthermore a prolonged

continuous infusion of KP10 increased LH pulse frequency and secretory burst mass in healthy men (J T George et al. 2011).

2.9 Kisspeptin based therapeutics

Kisspeptin's action on GnRH neurons results in a more physiological stimulation of pituitary secretion when compared to gonadotrophin responses elicited by GnRH or GnRH receptor analogues (C N Jayasena, Abbara, et al. 2015). Furthermore, the preferential effects of kisspeptin on LH secretion in women enables its utilisation as a therapeutic agent to target anovulatory conditions by providing the LH-surge (H Matsui and Asami 2014). Moreover, kisspeptin receptor desensitisation can also be exploited when gonadal axis downregulation is desired – for example in sex steroid responsive cancers (e.g. prostate cancer) or gynaecological disorders such as fibroids (MacLean et al. 2014).

2.9.1 The use of kisspeptin for oocyte maturation in *In Vitro* Fertilisation (IVF)

The physiological midcycle LH surge is prerequisite for oocyte maturation and subsequent ovulation (Wallach et al. 1995). During IVF treatment, this LH surge is achieved through the administration of exogenous human Chorionic Gonadotrophin (hCG), which is structurally and biochemically similar to LH (Castillo, Humaidan, and Bernabéu 2014). The half-lives of LH and hCG however are quite distinct ($t_{1/2}$ LH 60 minutes vs hCG ~48 hours) (Yen et al. 1968). The longer half-life of hCG increases its luteotrophic effect and causes elevation of oestrogen and progesterone for at least six days (Itskovitz et al. 1991). Thus, the excessive stimulation by hCG when compared to the endogenous mid-cycle LH surge can lead to the occurrence of the complication 'Ovarian Hyperstimulation Syndrome' (OHSS). Our group conducted the first study of kisspeptin as an ovulation trigger in women undergoing IVF treatment. A single subcutaneous bolus of KP54 (1.6-12.8nmol/kg) induced an LH-surge, with peak LH levels of approximately 40 iU/L, that lasted for 12 to 14 hours (Jayasena *et al.*, 2014). Egg maturation was observed following KP54 at all doses used, whilst the mean number of mature eggs in each patient increased in a dose-dependent fashion. Overall, 49 out of 53 (92%) subfertile women had successful fertilization and embryo transfers. Further, 40% of the women had

biochemical confirmation of pregnancy (21 from 53 women), whilst 12 women (23%) also had clinical confirmation of pregnancy on ultrasound (Jayasena *et al.*, 2014).

This duration of gonadotrophin exposure was shown to be sufficient for oocyte maturation to occur, but crucially without also causing the most significant complication of IVF treatment, namely OHSS (Abbara et al. 2015). Furthermore, extending the duration of LH exposure by administering a second dose of kisspeptin 10hrs following the first, further improved oocyte maturation but without causing OHSS (Abbara et al. 2017a). Whilst the improvement in the risk of OHSS has predominantly been ascribed to the shorter duration of action of KP54, emerging data has suggested that kisspeptin may have an additional direct action at the ovary to reduce vascular endothelial growth factor (VEGF) production, which is the predominant instigator of OHSS (Zhai et al. 2017). OHSS is a serious and potentially life-threatening iatrogenic complication, characterised by hyper-enlarged ovaries and increased capillary permeability leading to 'third space' oedema (Whelan and Vlahos 2000). At least 16% of IVF cycles are complicated by moderate to severe OHSS (Toftager et al. 2016), which is predominantly caused by the use of human chorionic gonadotrophin (hCG) and its non-physiological stimulation. This has triggered a search for more physiologic therapies to induce oocyte maturation during IVF treatment, associated with a safer side-effect profile.

Thus, the evidence to date suggests that a longer acting kisspeptin analogue could be ideal for use during IVF treatment to optimise oocyte maturation, but without causing OHSS Abbara, Clarke, and Dhillo 2018). The increased sensitivity to kisspeptin in healthy women during their pre-ovulatory phase could be utilised as a therapeutic target for ovulation induction. Moreover, it has been recently demonstrated that KP54 enhances differential gene expression of steroidogenenic pathways in granulosa cells when compared to traditional ovulation trigger agents (Owens et al. 2018a). It has been suggested that this can instigate a more favourable ovarian environment for luteal phase implantation (Owens et al. 2018a).

2.9.2 The use of kisspeptin in women with Hypothalamic Amenorrhoea (HA)

Hypothalamic amenorrhea (HA) is an anovulatory disorder associated with reduced GnRH pulsatility, low circulating sex-steroid levels and low leptin levels (Jayasena, Comninos, Nijher, et al. 2013). The 2017 Endocrine Society guidelines on the diagnosis of HA suggest that it can be diagnosed in women with: menstrual cycle length persistently more than 45 days, or amenorrhoea for at least 3 months, history of weight loss, and/or vigorous exercise, and/or stress and the presence of hypogonadotrophic hypo-oestrogenism, after excluding anatomic or organic causes of amenorrhea (Gordon et al. 2017b). In a rodent model of HA, hypothalamic *Kiss1* expression was reduced, whilst kisspeptin receptor mRNA expression was increased (J M Castellano et al. 2005). It has since been demonstrated that a continuous intravenous infusion of kisspeptin in women with HA resulted in gonadotrophin secretion (Jayasena et al., 2014). However, twice daily subcutaneous administration resulted in tachyphylaxis and loss of stimulation (Jayasena et al. 2009), whereas gonadotrophin stimulation is maintained with twice weekly administration (C N Jayasena et al. 2009). As tachyphylaxis in response to kisspeptin has been observed most frequently following high dose and frequent/continuous administration (Abbara et al. 2013), it is possible that a longer acting kisspeptin analogue, administered less frequently could facilitate the restoration of physiological gonadotrophin secretion in this patient cohort (Abbara et al. 2017b).

2.9.3 The use of kisspeptin in men with diabetes related hypogonadism

Type II diabetes mellitus (T2DM) has been associated with hypogonadotrophic hypogonadism and it is estimated that almost half of men with diabetes have this condition (Dandona and Dhindsa 2011). Previous work has demonstrated that men with diabetes related hypogonadism have normal responses to GnRH stimulation testing, consistent with the underlying cause being at the level of the hypothalamus (J T George et al. 2011). Intravenous infusion of KP10 in men with T2DM and secondary hypogonadism resulted in LH and total testosterone rises and enhanced LH pulse frequency (J T George et al. 2011). Thus,

kisspeptin based therapeutics could be developed for the treatment of age, obesity or diabetes related hypogonadism and could potentially restore testosterone levels to a more physiological range as opposed to the levels achieved following exogenous testosterone therapy (Swerdloff et al. 2000).

2.10 The case for kisspeptin analogues

Frequent administration of KP54 in a chronic stimulation protocol can lead to tachyphylaxis, thus limiting its potential to restore reproductive health in women with anovulation. Thus, longer acting kisspeptin analogues enabling less frequent administration could be of value in the treatment of reproductive disorders.

Longer acting kisspeptin analogues have been developed through modification of KP10 to increase water solubility, receptor potency and stability (Hisanori Matsui and Asami 2014; Nishizawa et al. 2016; Orsini et al. 2007). MVT602 (formerly known as TAK448) is one such analogue with a longer half-life than KP54 (1.5-2.2hrs vs 27 minutes) (MacLean et al. 2014). Subcutaneous administration of MVT602 in rodent models caused an initial rise in gonadotrophin secretion, prior to rapid and sustained reduction in testosterone levels, observed with chronic high-dose exposure (Matsui et al. 2012). MVT602 was therefore studied in patients with prostate cancer, where high doses were administered in order to induce tachyphylaxis (MacLean et al. 2014). Indeed McLean et al. demonstrated that high doses of MVT602 induce chemical castration in this cohort (MacLean et al. 2014). In healthy men, a single dose of MVT602 caused sustained LH-rises with peak levels observed between 8-12hrs before returning to baseline levels by 72hrs (MacLean et al. 2014). A longer acting kisspeptin analogue could have huge potential in the treatment of women with ovulation disorders if used at lower doses, in order to induce stimulation of gonadotrophin secretion.

SECTION 3: The diagnostic challenge of reproductive endocrine disorders

3.1 Congenital Hypogonadotrophic Hypogonadism

Hypogonadotrophic hypogonadism (HH) is characterized by hypogonadism accompanied by low or inappropriately normal gonadotrophins. It can arise from disorders affecting either the pituitary or the hypothalamus. Whilst we are able to assess pituitary function during routine assessment of patients with HH by the use of a gonadotrophin releasing hormone (GnRH) test, we do not have an equivalent test for investigating hypothalamic function (Boehm et al. 2015). Furthermore, the discriminatory potential of a GnRH test is rather poor (Adulwahid et al. 1985). A test of hypothalamic function would enable us to precisely interrogate GnRH neuronal activity and describe the specific defect in patients with hypogonadotrophic hypogonadism.

3.2 Anovulatory disorders

Menstrual disturbance is a frequent indication for referral to the endocrine clinic, often indicative of oligo / anovulation (Burgers et al. 2010). Secondary amenorrhoea occurs in 3-5% of women of reproductive age (Meczekalski et al. 2014), and two of the commonest causes are Polycystic Ovarian Syndrome (PCOS) and Hypothalamic Amenorrhoea (HA) (Golden and Carlson 2008). PCOS is reported to occur in 8-13% of women of reproductive age (Teede et al. 2018), whereas HA is the second commonest cause of secondary amenorrhea.

PCOS is diagnosed by the presence of at least two of the following features: hyperandrogenism (clinical or biochemical), oligomenorrhoea (cycle length > 35 days) and polycystic ovarian morphology on ultrasound (PCOM) (Anon 2004; Teede et al. 2018). The diagnosis of PCOS has recently been updated from the 2003 Rotterdam criteria in the 2018 international guidance (Teede et al. 2018), predominantly differing by an increase in the number of follicles per ovary required to define PCOM (20 vs 12 per ovary) reflecting improvements in ultrasonographic resolution (Teede et al. 2018). Diagnosis of both conditions is qualified by the need to ‘exclude other causes of menstrual disturbance’.

The 2017 Endocrine Society guidelines on HA suggest that it can be diagnosed in women with: menstrual cycle length persistently more than 45 days, or amenorrhoea for at least 3 months, history of weight loss, and/or vigorous exercise, and/or stress and the presence of hypogonadotrophic hypo-oestrogenism, after excluding anatomic or organic causes of amenorrhea (Gordon et al. 2017a). They recommend that patients have a progestogen challenge test and an MRI pituitary to exclude other causes.

Although, the prevalence of secondary amenorrhea is 2-5% in the general population, it can be as high as 69% in athletes (Nazem and Ackerman 2012). The 'female athlete triad' is a related condition to HA comprised of menstrual disturbance, insufficient energy availability, and reduced bone mineral density (Z score of < -1.0) (Joy et al. 2014; Nattiv et al. 2007).

Both PCOS and HA are common diagnoses and thus it is eminently feasible for both diagnoses to coexist. Early reports suggested that the ovaries of women with HA may be multifollicular, but without the increased stroma consistent with a diagnosis of PCOS (Zhu, Wong, and Yong 2016). A study of nineteen women with weight loss related HA found that 89% of them had multifollicular ovaries, whilst 42.1% of them had increased ovarian volume ≥ $10cm^3$ (Jonard *et al.*, 2005). Recently Alemyar et al. found that 36% of women with HA also fulfilled the Rotterdam criteria, with 24.5% having PCOM and oligo/amenorrhoea, 6.9% having oligo/amenorrhoea and hyperandrogenism and 5% having all three (Alemyar, van der Kooi, and Laven 2020). In a smaller cohort of forty women with HA, 10% were found to have high AMH levels (a putative marker for PCOS) and high ovarian volume (Carmina, Fruzzetti, and Lobo 2016). However, PCOS is a diagnosis of exclusion that cannot be made in the presence of HA (Helena J Teede et al. 2018), and thus such findings are typically discounted. Yet, emerging evidence suggests that they may have clinical relevance and that while both may coexist, HA predominates in patients. Wang and Lobo retrospectively compared the endocrine response to controlled ovarian stimulation (COS) in 6 women with HA/PCOM to 10 women with PCOS and 20 controls seeking fertility treatment for other causes of infertility (Wang and Lobo 2008). They observed that although baseline endocrine profiles in the women with HA/PCOM and normo-ovulatory controls did not differ, women with HA/PCOM demonstrated

increased androgen production following administration of similar doses of gonadotrophins to controls (Δ androstenedione per dominant follicle 0.30 vs 0.10ng/ml respectively, P<0.005) with levels similar to those in women with PCOS. Furthermore, a further 5 women with HA/PCOM, recruited at the same time, but who did not wish to undergo fertility treatment, were followed up prospectively. All of these women experienced an increase in body weight (ranging from 5-18%) with subsequent development of symptoms of PCOS, including oligomenorrhoea and symptoms of hyperandrogenism (Wang and Lobo 2008).

These studies demonstrate that whilst both conditions have distinct pathophysiology and their diagnosis is supported by clinical guidance (Gordon et al. 2017; Teede et al. 2018), in practice, differentiating these two common causes of menstrual disturbance can be challenging.

SECTION 4: Aims and Hypotheses

The kisspeptin system is crucial for normal functioning of the HPG axis. Whilst there is a wealth of data on kisspeptin's prominent role on GnRH regulation, kisspeptin's potential as a diagnostic and therapeutic agent has not yet been fully elucidated. I have thus endeavoured to address some outstanding issues that are fundamental in our journey of translating kisspeptin into clinical practice:

1. **To investigate the use of KP54 as a novel test of hypothalamic GnRH function in the diagnosis of congenital hypogonadotrophic hypogonadism (CHH)**

Hypogonadotrophic hypogonadism (HH) can be either congenital or acquired, and can arise from conditions affecting either the hypothalamus, or the pituitary gland (Boehm et al. 2015). A number of patients presenting with HH have a diagnosis of Congenital Hypogonadotrophic Hypogonadism (CHH). This is a genetic syndrome caused by various mutations in genes regulating GnRH neuronal migration or secretion. These patients present with delayed puberty, primary amenorrhoea (if female) and infertility (Seminara et al. 1998; Boehm et al. 2015). The only readily available test for patients presenting with HH is the GnRH test. However, since the pathophysiological basis of this condition is hypothalamic, the GnRH test can offer limited diagnostic value (Adulwahid et al. 1985). Furthermore, as kisspeptin directly stimulates hypothalamic GnRH neurons, it would be logical to study kisspeptin's ability to discriminate men with CHH from healthy men. Additionally, it would be of great interest to investigate how kisspeptin performs as a diagnostic test of hypothalamic GnRH function compared to the currently available test GnRH, which only assesses pituitary function.

HYPOTHESIS:

Healthy men will have similar responses following aKP54 challenge test and GnRH stimulation test. Patients with CHH should respond to GnRH stimulation but should have attenuated responses to the KP54 challenge test.

2. **To probe the effects of the kisspeptin receptor agonist, MVT602, in healthy women**

MVT602, a kisspeptin receptor agonist, has a longer half-life than native KP54 (1.5-3.5hr vs 27 minutes) (MacLean et al. 2014). It was previously administered in men, whereby a single dose resulted in a potent rise of LH that peaked 8-12hrs and sustained LH elevation before returning to baseline levels at 72hrs (MacLean et al. 2014). This prolonged rise in serum LH levels resembles the pattern of LH secretion observed during the LH surge. As such, this kisspeptin analogue could potentially be used as an oocyte maturation trigger in the setting of *In Vitro* Fertilisation treatment. However, the effects of MVT602 in women are unknown and I therefore investigated MVT602 in women for the first time.

HYPOTHESIS:

Based on the data from men, I hypothesise that MVT602 will induce an LH-surge of comparable amplitude, but more prolonged duration of action compared to native KP54.

3. To compare the effects of KP54 and MVT602 in women with hypothalamic amenorrhoea, women with polycystic ovarian syndrome and healthy women

The two commonest causes of anovulation are polycystic ovarian syndrome (PCOS) and hypothalamic amenorrhoea (HA) (National Institute for Health and Care Excellence, 2013; Thurston et al. 2019). It is becoming increasingly evident that differentiating PCOS from HA can be challenging. Furthermore, no clear discriminatory marker exists that can aid in the resolution of this challenging diagnostic dilemma. Our group has previously demonstrated that women with HA exhibit augmented responses to KP54 compared to eumenorrhoeic women (Dhillo *et al.*, 2005; Jayasena *et al.*, 2010; Jayasena *et al.*, 2014). The effects of KP54 in

women with PCOS are unknown. Furthermore, the effects of MVT602 have not been previously explored in the settings of PCOS or HA in humans. I thus investigated the effects of both KP54 and MVT602 on the gonadotrophin secretion of women with these two common ovulation disorders and compared them to those of healthy women.

HYPOTHESIS:

The gonadotrophin responses of women with PCOS to MVT602 will be comparable to those seen in healthy women, whilst those of women with HA will be augmented.

Thus, these studies will elucidate the ability of KP54 as a diagnostic test of hypothalamic function and determine for the first time the endocrine profile of MVT602 in healthy women and in women with anovulatory disorders such as PCOS and HA.

Chapter 2

The effect of Kisspeptin-54 on gonadotrophin responses in healthy men and men with Congenital Hypogonadotrophic Hypogonadism (CHH)

CHAPTER 2: THE EFFECT OF KISSPEPTIN-54 ON GONADOTROPHIN RESPONSES IN HEALTHY MEN AND MEN WITH CONGENITAL HYPOGONADOTROPHIC HYPOGONADISM (CHH)

2.1 Introduction

Hypogonadotrophic hypogonadism (HH), or secondary hypogonadism, describes the presence of low or inappropriately normal gonadotrophin levels despite low circulating sex steroids (Boehm et al. 2015). A subset of patients with HH are those with Congenital Hypogonadotrophic Hypogonadism (CHH). Typically, these patients have mutations in genes encoding GnRH neuronal migration and/or GnRH neuronal function (Seminara et al. 1998; Boehm et al. 2015). GnRH neurons co-migrate with olfactory pathways. Up to half of patients with CHH also suffer from anosmia and are therefore diagnosed as having "Kallmann syndrome". Other mutations may also be associated with non-reproductive features such as cleft lip, cleft palate, dental agenesis, hearing impairment, renal agenesis, or bimanual synkinesis (Seminara et al. 1998; Boehm et al. 2015). Though genetic testing has been illuminating in the diagnosis of CHH, the diverse phenotypes and variation in the genotypes of patients with this condition pose significant limitations to the accurate and timely diagnosis of this condition (Boehm et al. 2015). In the clinic, when assessing patients with evidence of HH, pituitary function can be readily assessed using a gonadotrophin releasing hormone (GnRH) test. However, as pituitary function is normal in CHH, a GnRH test is often of 'poor diagnostic value' and does not reliably distinguish men with CHH from healthy controls. At present, a direct test of hypothalamic GnRH neuronal function with greater diagnostic accuracy in patients with impaired hypothalamic function is currently unavailable (Boehm et al. 2015).

In 2003, two ground breaking papers from two independent research groups described the presence of CHH, and the resulting failure to proceed through puberty, as a consequence of inactivating mutations of the kisspeptin receptor (de Roux et al. 2003; Seminara et al. 2003). Following those, an array of studies have confirmed kisspeptin's essential role in reproductive physiology, by acting as a specific stimulator of hypothalamic GnRH secretion (Abbara et al.

2013; Waljit S. Dhillo et al. 2005, 2007).Our group and others have investigated the effects of kisspeptin administration on gonadotrophin and sex steroid hormone secretion in healthy men (Waljit S. Dhillo et al. 2005), healthy women (Waljit S. Dhillo et al. 2007), as well as in patients with conditions such as hypothalamic amenorrhoea (Jayasena *et al.*, 2009, 2010; Jayasena *et al.*, 2013), subfertility (Jayasena *et al.*, 2014; Abbara *et al.*, 2017), hyperprolactinaemia (Millar et al. 2017) and diabetes-related hypogonadism (George et al. 2013).

Kisspeptin directly stimulates hypothalamic GnRH release and thus can specifically probe hypothalamic GnRH neuronal function in patients with HH. In clinical practice, a GnRH test is sometimes used to help diagnose these patients. Whilst minimal responses to a GnRH test may be able to distinguish men with CHH from healthy men, a partial response to GnRH might not be able to reliably exclude the diagnosis (Bang et al. 2017; Boehm et al. 2015).

During this study, I explored the gonadotrophin responses of men with CHH to a KP54 challenge test and compared the diagnostic performance of both KP54 and GnRH in distinguishing men with CHH from healthy men.

2.2 Aims and Hypotheses

2.2.1 Aims

(1) To ascertain the gonadotrophin and testosterone responses of men with CHH following administration of an intravenous bolus of KP54 (6.4 nmol/kg) and GnRH (100 micrograms).

(2) To compare the gonadotrophin responses with those of healthy men receiving the same doses of KP54 and GnRH.

2.2.2 Hypotheses

(1) Men with CHH do not respond to intravenous KP54 to the same degree as healthy individuals.

(2) The differences in gonadotrophin responses to KP54 between the two groups can be used to distinguish men with CHH from healthy men.

(3) A KP54 challenge test exhibits higher diagnostic performance compared to the currently available GnRH test in the assessment of men with CHH.

2.3 Methodology

2.3.1 Study Overview

I conducted a prospective, randomised, cross-over physiological study to determine the effects of KP54 in men with CHH and investigate how their gonadotrophin responses compared to those elicited after GnRH.

2.3.2 Ethical Approval

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

2.3.3 Study Subjects

Healthy eugonadal men (n=21) and men with CHH (n=21) were recruited via advertisements in the local press or from endocrine clinics. Prior to participation in the study, all participants underwent a detailed medical assessment including full medical history and physical examination. The inclusion and exclusion criteria for the eugonadal group were as follows: age 18-35 years, BMI 18-30 kg/m^2, absence of symptoms of hypogonadism, absence of significant systemic disease or co-morbidity, absence of recreational or therapeutic drug use, and the presence of normal biochemical reproductive parameters (serum LH, FSH, Total Testosterone). For recruitment of the CHH cohort, patients required an established diagnosis of CHH with a history of incomplete progression through puberty by the age of 18 years and evidence of biochemical secondary hypogonadism. Participants from both groups had their height and weight measured, whilst men with CHH had additional assessments of their arm span (eunuchoid proportions), testicular volume with the use of a Prader orchidometer and evaluation for the presence of any non-reproductive clinical features of CHH such cleft palate and synkinesis. Men with CHH also underwent subjective and objective assessment of their

olfactory function: they were initially asked to subjectively grade their sense of smell (completely absent, present but reduced, or normal) before also having an objective assessment using the University of Pennsylvania 40-item Smell Identification Test (UPSIT). The test consists of 40 questions, with a "scratch and sniff" strip that contains a microencapsulated odorant, which the individual is asked to identify after being given a choice of four answers. The answers are then scored, and the scores are compared to the answers in a normative database from 4000 healthy individuals. All participants had measurements of serum luteinising hormone (LH), follicle stimulating hormone (FSH), total testosterone, inhibin B, anti-Müllerian hormone (AMH), sex hormone binding globulin (SHBG) and the rest of anterior pituitary panel at baseline. Men with CHH were also offered genetic testing to identify the specific genes responsible for their condition.

2.3.4 Study Protocol

I conducted a randomised, single-blinded, cross-over study. Study visits took place in the Clinical Research Unit at Imperial College Healthcare NHS Trust. Study participants were advised to refrain from strenuous exercise, sexual activity and to abstain from alcohol, caffeine and tobacco for 24 hours prior to each study visit. The studies started at 9am and lasted for 6.5 hours. On arrival, an intravenous cannula was inserted into the antecubital fossa and the participant's weight was recorded. After a 30-minute period of baseline blood sampling, a single intravenous bolus of either KP54 at a dose of 6.4nmol/kg or of GnRH at a dose of 100 mcg was administered, in random order. The randomisation of the order of peptide administration was determined by an online randomisation tool (random.org). Serial blood sampling occurred every 15 minutes for 6 hours. The second study visit was conducted following a washout period of at least one week. A summary of the study protocol is shown in **Figure 2.1**.

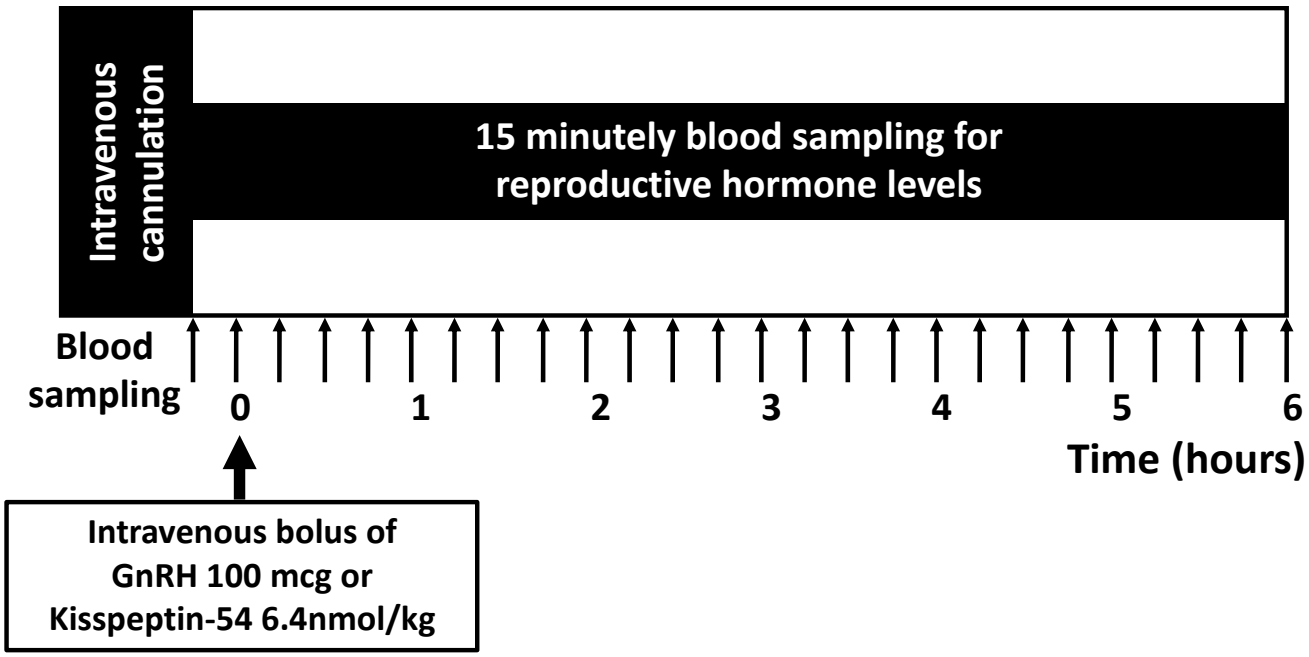


Figure 2.1 Study Protocol.

Diagram outlining the study protocol of this randomised, blinded, cross-over study of healthy men and men with CHH. Reproductive hormone levels were taken every 15 minutes for 6 hours. Reproductive hormones measured include serum LH, serum FSH and serum total testosterone.

The dose of KP54 was chosen following the results of a preliminary dose-finding study involving five healthy men who received three intravenous doses of KP54: 6.4 nmol/kg, 12.8 nmol/kg and 25.6 nmol/kg **(Figure 2.2)**. There was no difference in the LH rises following all three doses (P=0.57 by two-way ANOVA). Thus, 6.4 nmol/kg was determined to be the lowest effective dose that elicits a near maximal rise in LH and was selected for use in the main study. Men with CHH who were taking topical testosterone supplements were asked to abstain from them for at least 1 week prior to the study. Men with CHH on longer acting intramuscular testosterone preparations (such as testosterone undecanoate) had their studies performed just before their next injection, to coincide with trough levels of sex steroids. Men with CHH

on gonadotrophin therapy had this discontinued for at least 3 months prior to commencement of the study visits.

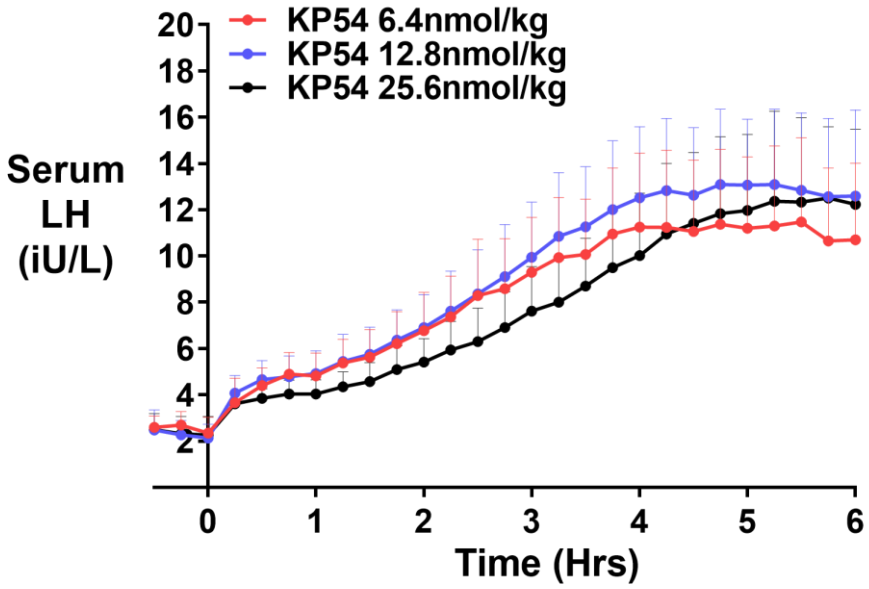


Figure 2.2 Preliminary dose-response for KP54 in healthy men.

Mean ±SD of serum LH (iU/L) levels over six hours, during a preliminary dose finding study in healthy men (n=5) receiving an intravenous bolus of KP54 at 6.4nmol/kg, 12.8nmol/kg and 25.6nmol/kg. Max change in serum LH did not significantly differ between the three doses when compared by two-way repeated measures ANOVA (P=0.57).

2.3.5 Peptides

KP54 was synthesised by Bachem AG (Liverpool, UK) and further purified and tested as previously described (Waljit S. Dhillo et al. 2005). Vials of freeze-dried KP54 (600nmol per vial), stored at -20ºC, were reconstituted at the start of each study in 600µL of 0.9% sodium chloride. Gonadorelin (GnRH) was purchased from Intrapharm Laboratories Ltd (Maidenhead, Berks, UK) and 100mcg were reconstituted in 1ml of sterile water for injection prior to administration.

2.3.6 Hormone Assays

Blood samples were collected in plain serum vacutainer tubes and left to clot for at least one hour, prior to centrifugation at 3000rpm for 10 minutes. The serum was separated and frozen at -20ºC until analysis. Serum samples were analysed for LH, FSH and total testosterone using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). The reference range for serum LH for men was 2-12 iU/L, serum FSH 1.7-8 iU/L, serum total testosterone 10-30 nmol/L. The intra-assay and interassay coefficients of variation were 4.1% and 2.7% for LH; 4.1% and 3.0% for FSH, and 4.2% and 2.8% for total testosterone, respectively. The analytical sensitivities of the three assays were 0.03 iU/L for LH, 0.05 iU/L for FSH and 0.05 nmol/L for total testosterone.

2.3.7 Statistics

Graphpad Prism version 8.0 was used for the statistical analysis of our results. Continuous variables that were parametrically distributed were reported as mean ± standard deviation (SD), whereas non-parametrically distributed continuous variables were represent by 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.

2.3.8 Genetic testing

We collaborated with the Health 2030 Genomic Centre (Geneva, Switzerland), that performed whole exome sequencing in CHH patients, using previously described methods (Cassatella et al. 2018). Blood samples were analysed for the following 45 CHH genes: *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 and WDR11. Non-synonymous rare sequencing variants (RSVs), splicing variants (+/-2bp), with minor allele frequency (MAF) <1% from the Genome Aggregation Database (gnomAD, http://gnomAD.broadinstitute.org/) were analysed further. The American College of Medical Genetics and Genomics (ACMG) criteria were used to interpret the variants identified (Richards et al. 2015). I shall report variants predicted to be 'pathogenic', 'likely pathogenic' or 'of uncertain significance'.

2.4 Results

2.4.1 Baseline characteristics

The baseline characteristics of the healthy men (n=21) and men with CHH (n=21) are presented in **Table 2.1**. Participant age at the time of recruitment and baseline body mass index (BMI) were significantly higher in men with CHH than in healthy men ($p < 0.0001$ for age, $p=0.016$ for BMI). Men with CHH had lower serum LH, FSH, inhibin B and total testosterone levels compared to healthy men **(Table 2.1)**. The individual clinical and biochemical characteristics of the twenty-one men with CHH are presented in **Table 2.2**. Of those with CHH, 52% (11 out of 21 men) had previously received gonadotrophin treatment for induction of spermatogenesis . Of the 21 patients with CHH, two were not on any testosterone replacement at the time of the study, whilst the rest (86%) were on either sex steroid treatment (17/21), or gonadotrophin treatment (1/21). The vast majority of participants were on testosterone undecanoate (Nebido), whilst two were on Testosterone proprionate (Sustanon) and one on Testosterone gel. Two patients were not on any treatment at the time of participation (10%). Eight of twenty-one (38%) of men with CHH were anosmic as identified by the UPSIT40-item smell test; four of twenty-one (19%) were mildly microsmic (reduced sense of smell) whilst the remainder of patients (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 2.1: Baseline characteristics of men with congenital hypogonadotrophic hypogonadism (CHH) and healthy men

Parametrically distributed values (e.g. age, BMI, testicular volume, serum LH, AMH) are presented as Mean ± SD, whilst non-parametrically distributed data (e.g. weight, serum FSH, inhibin B) are presented as median (interquartile range). Comparison between the parameters was done with the Mann-Whitney U test. Reference range for serum LH (in men): 2 -12 iU/L, serum FSH (in men): 1.7 -8 iU/L, serum total testosterone: 10 -30 nmol/L, serum AMH: 10.2 -82.8 pmol/L, serum inhibin B: 25 -325 ng/L.

ID	Age at screening (years)	Age at diagnosis (years)	Sense of smell (UPSIT-40)	TV at screening (ml)	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.R734Q	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 2.2 Baseline Characteristics of CHH men

Baseline clinical, biochemical and genetic parameters of men with congenital hypogonadotrophic hypogonadism (CHH) (n=21). Sense of smell was measured using the University of Pennsylvania UPSIT-40 smell test (N: Normosmia, M: Microsmia, A: Anosmia), TV (Testicular volume) at screening was measured using a Prader Orchidometer, TU, Testosterone undecanoate (Nebido); hCG: human chorionic gonadotrophin; TP, Testosterone propionate (Sustanon); TG, Testosterone Gel, American College of Medical Genetics (ACMG) classification : VUS (Variant of Uncertain Significance); Nil (No mutation identified); LP (Likely Pathogenic mutation identified); NT (Not Tested); n/a (not applicable).

2.4.2 Gonadotrophin responses to KP54 and GnRH in healthy men

Following administration of GnRH in healthy men, the maximal serum LH was observed 30 minutes after administration, to a mean serum LH level of 18.2 iU/L. In contract, the rise in LH following an intravenous bolus of KP54 occurred much later at around 4.5 hours to a mean serum LH level of 13.2 iU/L **(Figure 2.3A)**. The FSH response to both GnRH and KP54 mirrored the respective LH responses, however the peak rise in serum FSH levels occurred approximately 45-50 minutes after the peak rise in LH. The maximal FSH following GnRH (2.3 iU/L) was similar to the peak serum FSH following KP54 (2.7 iU/L) **(Figure 2.3B)**. Testosterone increased similarly following both interventions, to a mean peak of 4nmol/L 3 hours following administration **(Figure 2.3C)**.

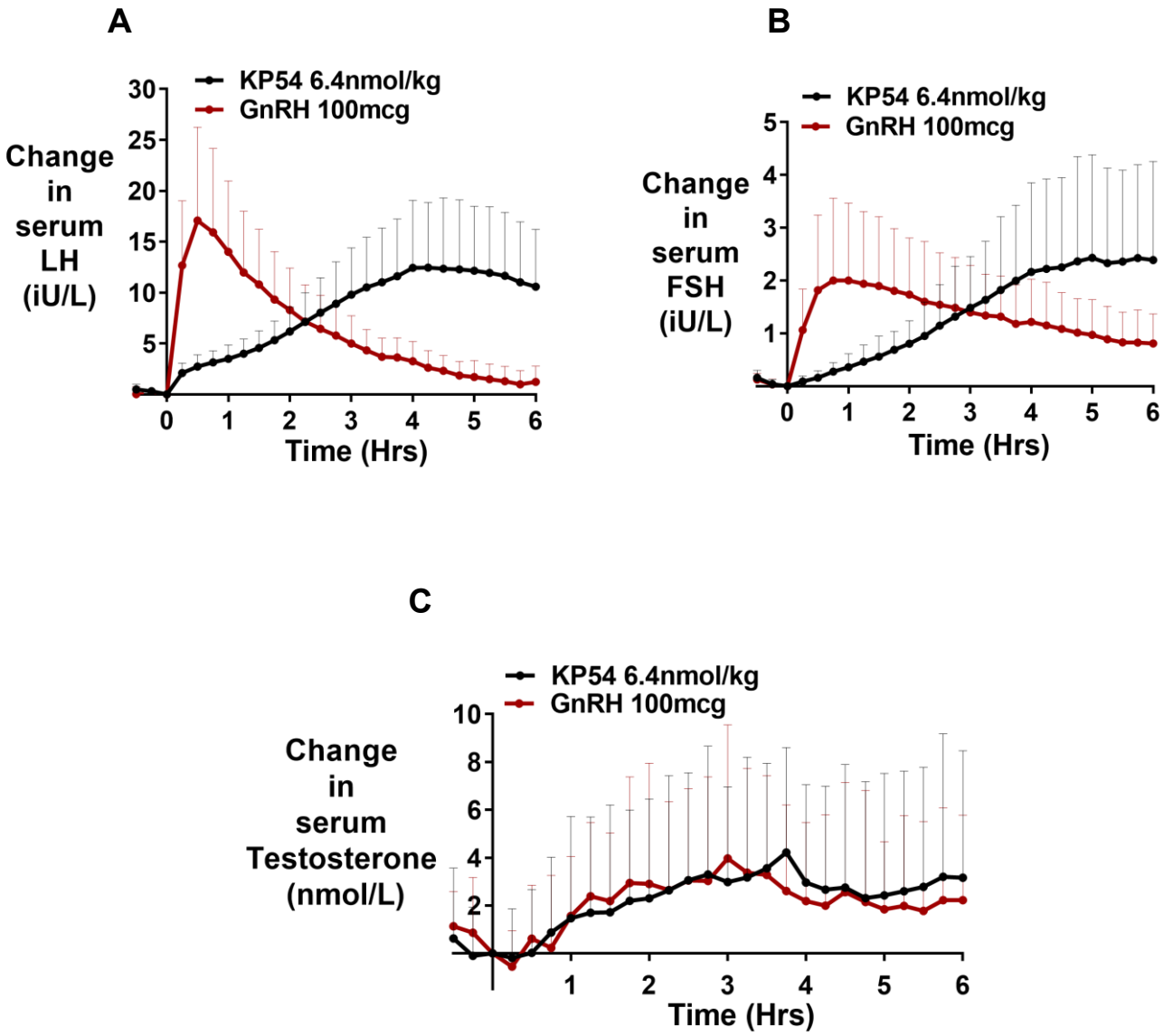


Figure 2.3: Hormone changes after KP54 and GnRH in healthy men

Mean±SD of change of (A) serum LH (iU/L), (B) serum FSH (iU/L), (C) serum Total Testosterone (nmol/L) in healthy men (n=21), over 6 hours following an intravenous bolus of KP54 6.4nmol/kg (in black) and GnRH 100 mcg (in maroon). Groups were compared by two-way ANOVA with repeated measures. Changes in serum LH (p=0.054), FSH (p=0.69) and total testosterone (p=0.92) did not significantly differ between the two intervention arms.

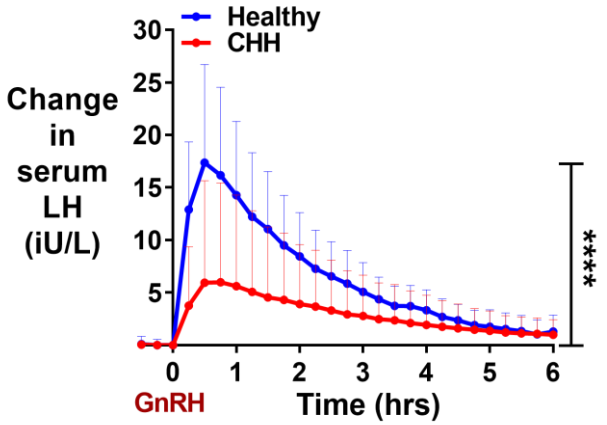
2.4.3 Comparison of gonadotrophin responses between healthy men and men with CHH

2.4.3.1 Serum LH

Following administration of GnRH, the peak LH response in men with CHH was once again observed 30 minutes after administration. However, the maximal mean serum LH reached was 3-fold lower in men with CHH compared to healthy men ($p<0.0001$) **(Figure 2.4A)**. Furthermore, following administration of K54, men with CHH did not exhibit an LH response, such that the LH levels remained undetectable throughout the study duration ($p<0.0001$) **(Figure 2.4B)**.

Healthy men exhibited a median maximal change in serum LH of 12.5 iU/L whereas in men with CHH this was only 0.4 iU/L ($p<0.0001$), after administration of KP54. The smallest rise in serum LH following KP54 in healthy men was 4.1 iU/L, whilst the maximal LH increase in men with CHH was 2.0 iU/L **(Figure 2.5A)**. Therefore, KP54-induced serum LH rise precisely differentiated all men with CHH from the healthy cohort (area under ROC 1.0) (**Figure 2.6A**). With regards to the LH responses following GnRH, the median peak serum LH was 18.2 iU/L in healthy men and 2.0 iU/L for men with CHH ($P<0.0001$) (**Figure 2.5B**). However, there was a great degree of variation in the LH response of healthy men following KP54, with maximal rises in LH as high as 40 iU/L. Therefore, some of the LH responses between the groups overlapped such that the area under ROC was 0.88 (95% CI 0.76 to 0.99) **(Figure 2.6B)**. Thus, the GnRH test differentiated healthy men from those with CHH less accurately compared to KP54.

A



B

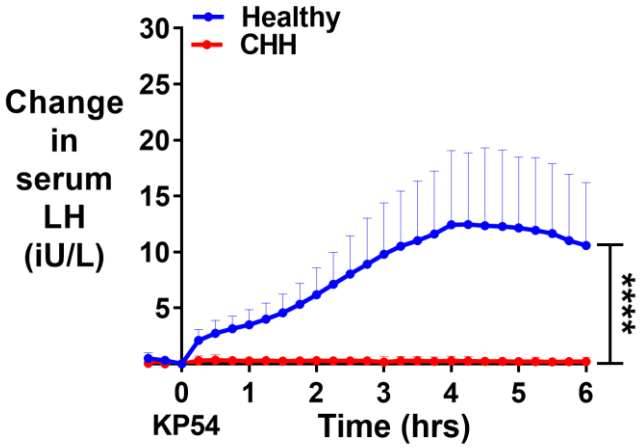
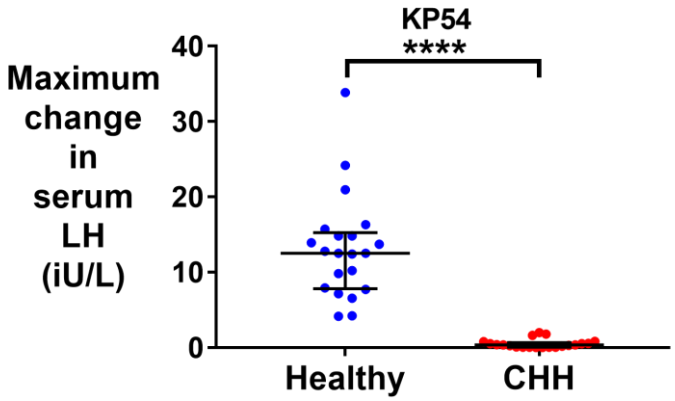


Figure 2.4 LH levels after GnRH and KP54 in healthy men and CHH men

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

A



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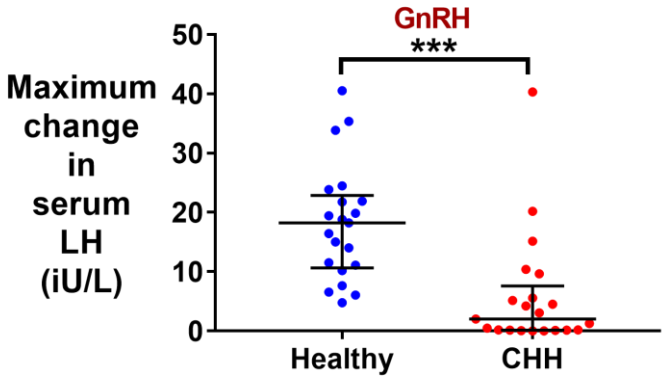
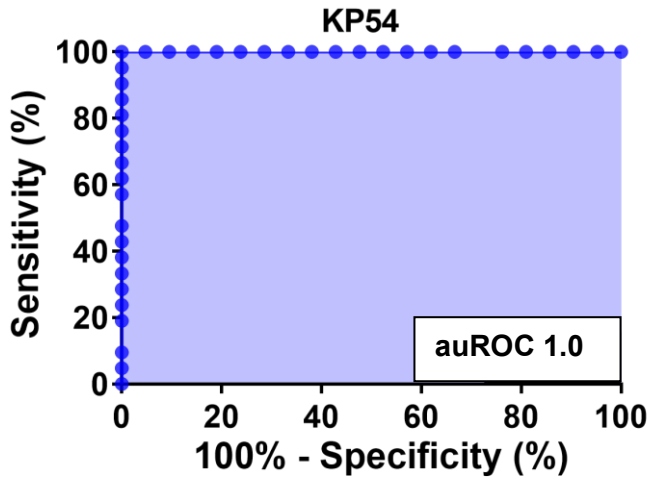


Figure 2.5 Maximal LH levels after GnRH and KP54 in healthy men and CHH men

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

A



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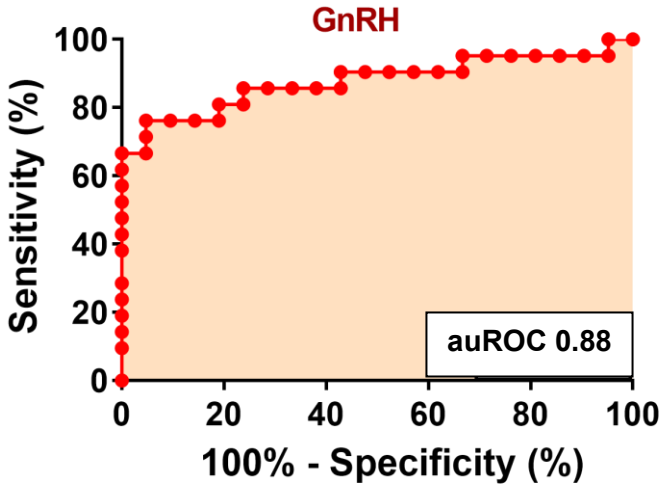


Figure 2.6 Area under ROC (auROC) of LH levels after KP54 and GnRH to differentiate healthy men from those with CHH

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

2.4.3.2 Serum FSH levels

The FSH responses following KP54 showed a similar pattern to the LH levels. The increase in serum FSH levels from baseline was significantly lower in men with CHH compared to healthy men ($p<0.0001$) **(Figure 2.7A)**. The median maximal rise in serum FSH in healthy men was 2.2 iU/L after KP54, which was significantly higher than that observed in men with CHH (0.3 iU/L) ($p<0.0001$) **(Figure 2.8A)**. However, the median maximal change in FSH following administration of GnRH was not significantly different between the two groups (median max FSH-rise in healthy men 1.8 iU/L, median max FSH-rise in men with CHH 1.1 iU/L, $p=0.079$) (**Figure 2.7B and 2.8B)**.

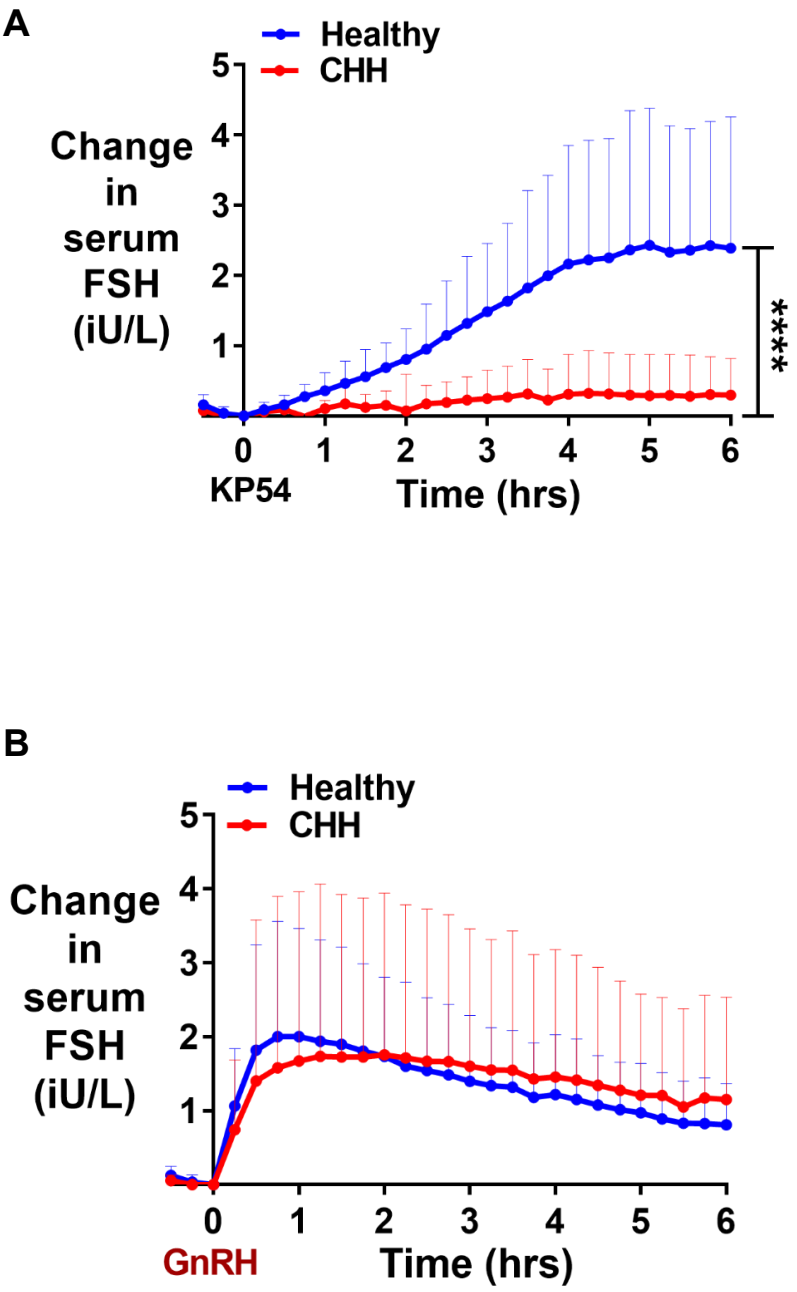


Figure 2.7 Change in serum FSH following KP54 and GnRH in healthy men and men with CHH

Mean±SD of change from baseline of serum FSH in healthy men (n=21) (blue) and men with CHH (n=21) (red) following (A) an intravenous bolus of KP54 6.4nmol/kg (B) an intravenous bolus of GnRH 100 mcg over 6 hours. Groups were compared by two-way ANOVA with repeated measures (**** p <0.0001).

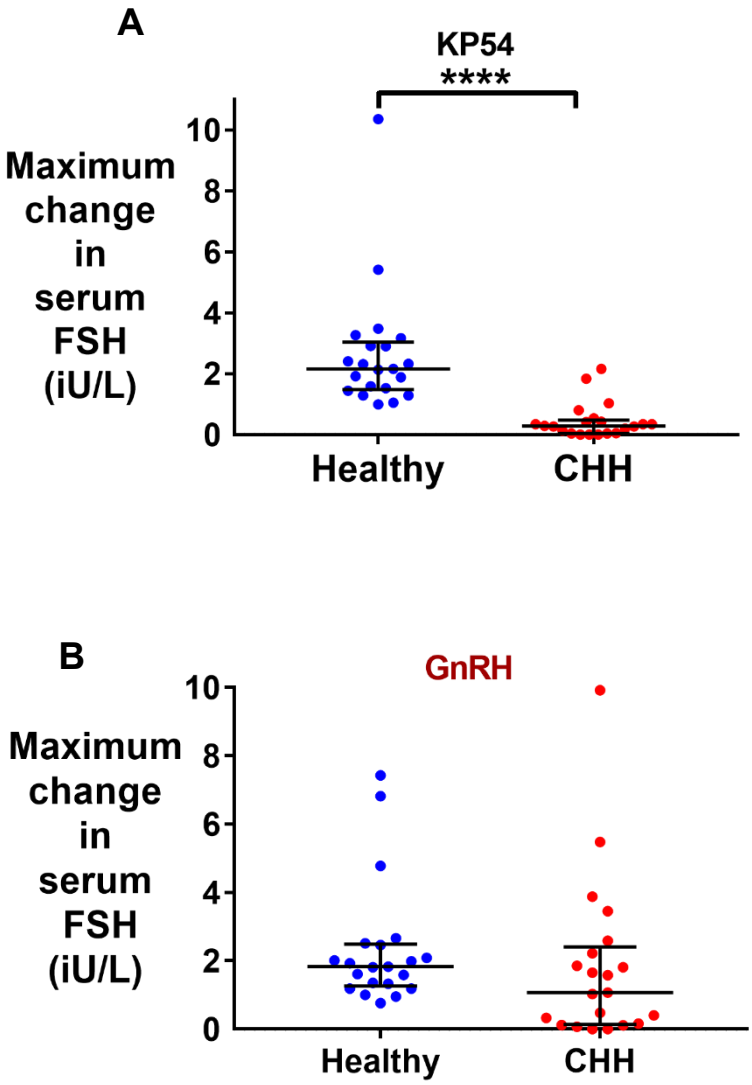


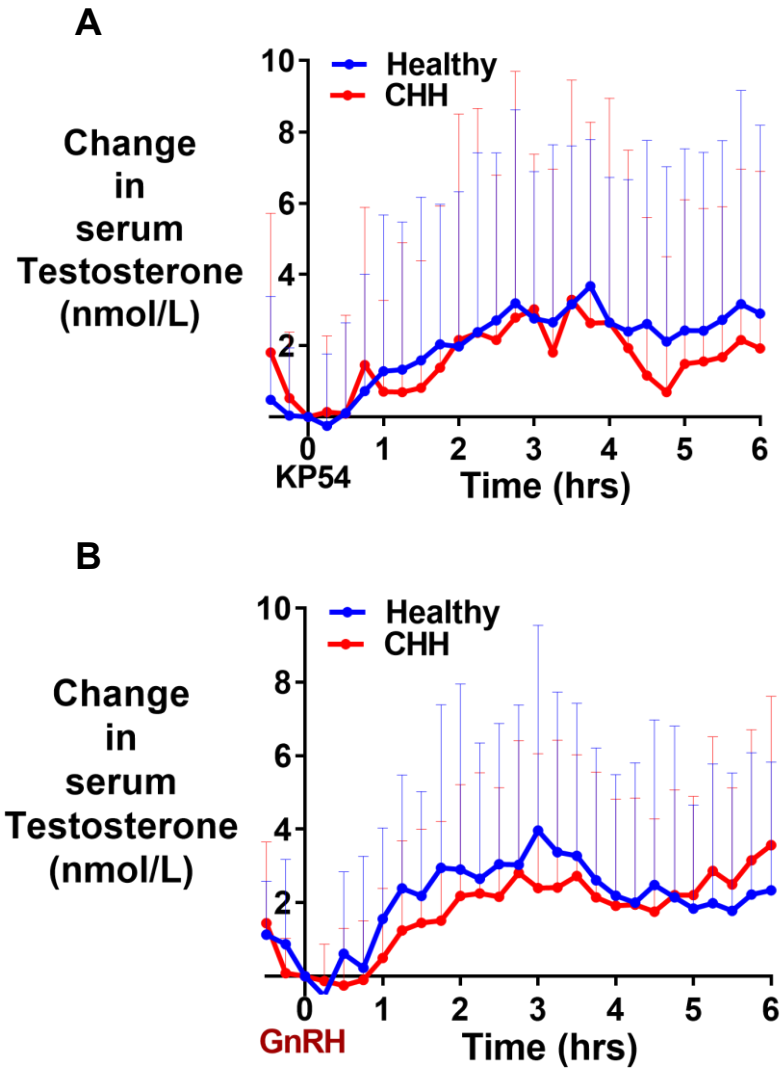
Figure 2.8 Maximum change in serum FSH following administration of KP54 and GnRH in healthy men and men with CHH

Scatter diagram (Median±IQR) of maximum change in serum FSH (iU/L) from baseline in healthy men (n=21) (blue) and men with CHH (n=21) (red) following (A) an intravenous bolus of KP54 6.4nmol/kg (B) an intravenous bolus of GnRH 100 mcg. Groups were compared by the Mann-Whitney U test (**** p <0.0001).

2.4.3.3 Serum Total Testosterone levels

. Despite the distinct gonadotrophin responses between men with CHH and healthy men to administration of KP54, this was not reflected in their testosterone responses, with the change in total serum testosterone between the two groups showing a similar pattern(**Figure2.9A)**. Unsurpringly, there was no difference in the change in total testosterone following administration of GnRH to the two groups (**Figure 2.9B**).

Figure 2.9 Change in serum testosterone following administration of KP54 and GnRH in healthy men and men with CHH



Mean±SD of change from baseline of serum Total Testosterone (nmol/L) in healthy men (n=21) (blue) and men with CHH (n=21) (red) following (A) an intravenous bolus of KP54 6.4nmol/kg

and (B) an intravenous bolus of GnRH 100 mcg over 6 hours. Groups were compared by two-way repeated measures ANOVA (p=0.12).

2.4.4 Other variables affecting gonadotrophin responses

The maximal LH-rise following KP54 correlated with the maximal LH-rise following GnRH in the healthy group (p=0.0005, r^2=0.48) **(Figure 2.10A)**. However, the same was not observed in men with CHH. Men with CHH showed minimal response to KP54, even if they had responded to GnRH. Thus, their LH responses were all distributed to the left of the graph **(Figure 2.10A)**. In men with CHH, the serum LH response was diminished following administration of KP54 even though their LH responses to GnRH were preserved (p=0.007, r^2 =0.33).

Baseline LH levels before KP54 administration predicted the subsequent LH response after KP54 in healthy men **(Figure 2.11A)**. However, whilst this was also true for the LH responses following GnRH in men with CHH **(Figure 2.11B)**, the LH responses following KP54 were attenuated below the projected level due to the lower baseline LH in men with CHH **(Figure 2.11A)**. By comparison, following GnRH, 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<0.001, r^2 =0.71) **(Figure 2.11B).**

The maximum increase following KP54 correlated with the FSH-rise following GnRH in healthy men (p<0.001, r^2 =0.76) **(Figure 2.10B)**. However, this correlation was lost in men with CHH (p=0.70, r^2 =0.008) **(Figure 2.10B)**. In healthy men, baseline FSH levels at the start of the study predicted the subsequent stimulated FSH levels following both KP54 (p<0.0001, r^2 =0.59) **(Figure 2.12A)** and GnRH (p<0.0001, r^2 =0.66) **(Figure 2.12B)**. However, in men with CHH, the FSH response was at least as much after GnRH as in healthy men (p<0.0001, r^2 =0.76) **(Figure 2.12B)**, but not after KP54 (p=0.99, r^2 =0.001) **(Figure 2.12A)**.

Whilst overall the gonadotrophin responses of men with CHH were significantly lower than those observed in healthy men, men with CHH with higher testicular volumes, were found to have higher FSH responses compared to those with lower testicular volumes **(Figure 2.13B**).

Baseline serum AMH levels were consistently lower than 140 pmol/L in all healthy men **(Figure 2.15)**. Five men with CHH on testosterone treatment had high levels of serum AMH (ranging between 145 and 647 pmol/L), despite having physiological replacement testosterone levels with a mean of 14.5±5.6nmol/L. Baseline serum AMH and total testosterone levels did not correlate with the maximal rise in serum LH or FSH levels following either intervention arms in either group **(Figure 2.14, Figure 2.15)**.

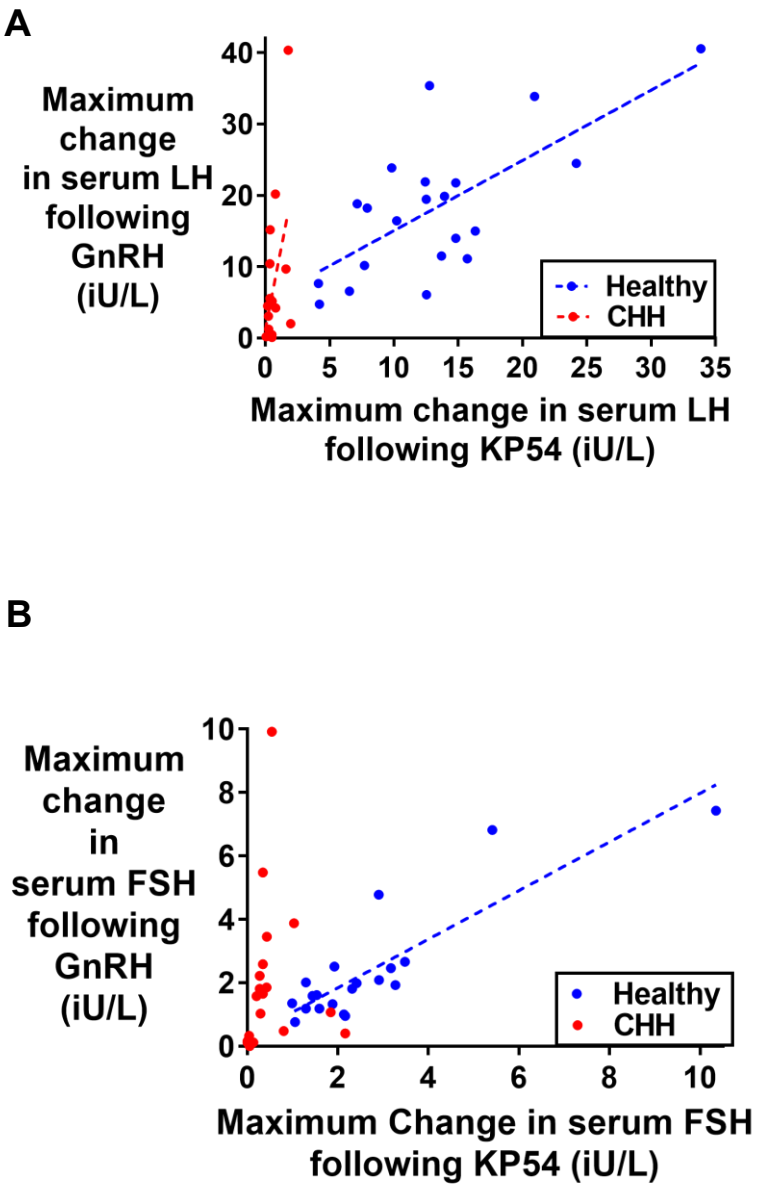


Figure 2.10 Maximum change in serum gonadotrophins following administration of KP54 and GnRH in healthy men and men with CHH

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

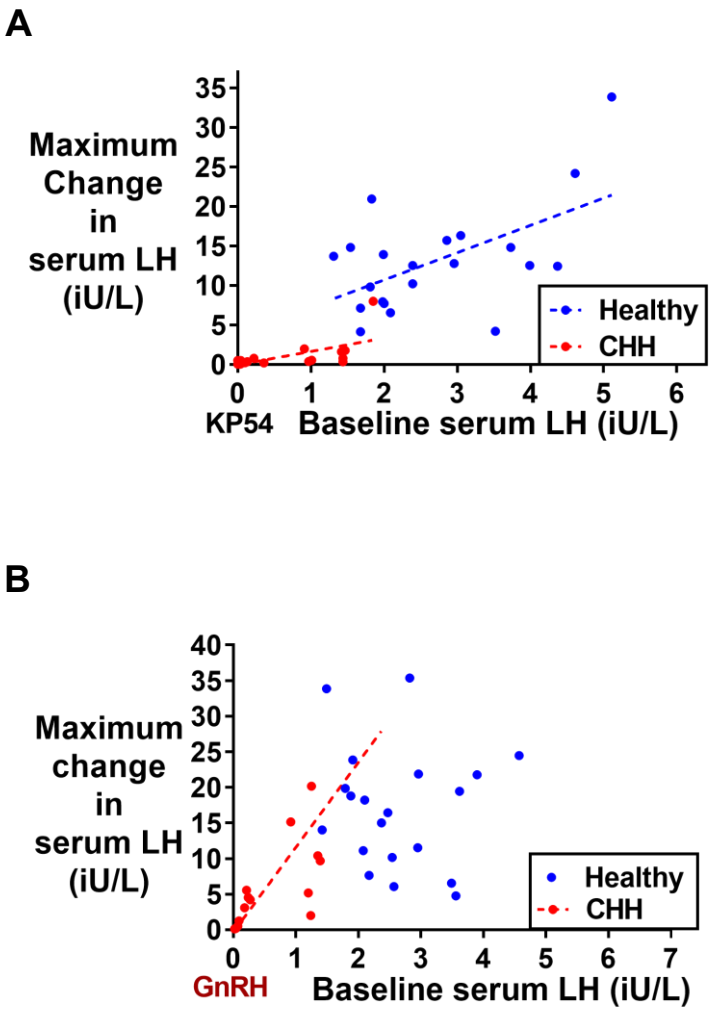


Figure 2.11 Relationship between baseline LH levels and maximum change in serum LH following administration of KP54 and GnRH in healthy men and men with CHH

Simple linear regression of 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=0.009, r^2=0.31) and men with CHH (n=21) in red (p=0.001, r^2=0.41) and (B) following an intravenous bolus of GnRH (100 mcg) in healthy men (n=21) in blue (p=0.17, r^2=0.09) and men with CHH (n=21) in red (p<0.001, r^2=0.71).

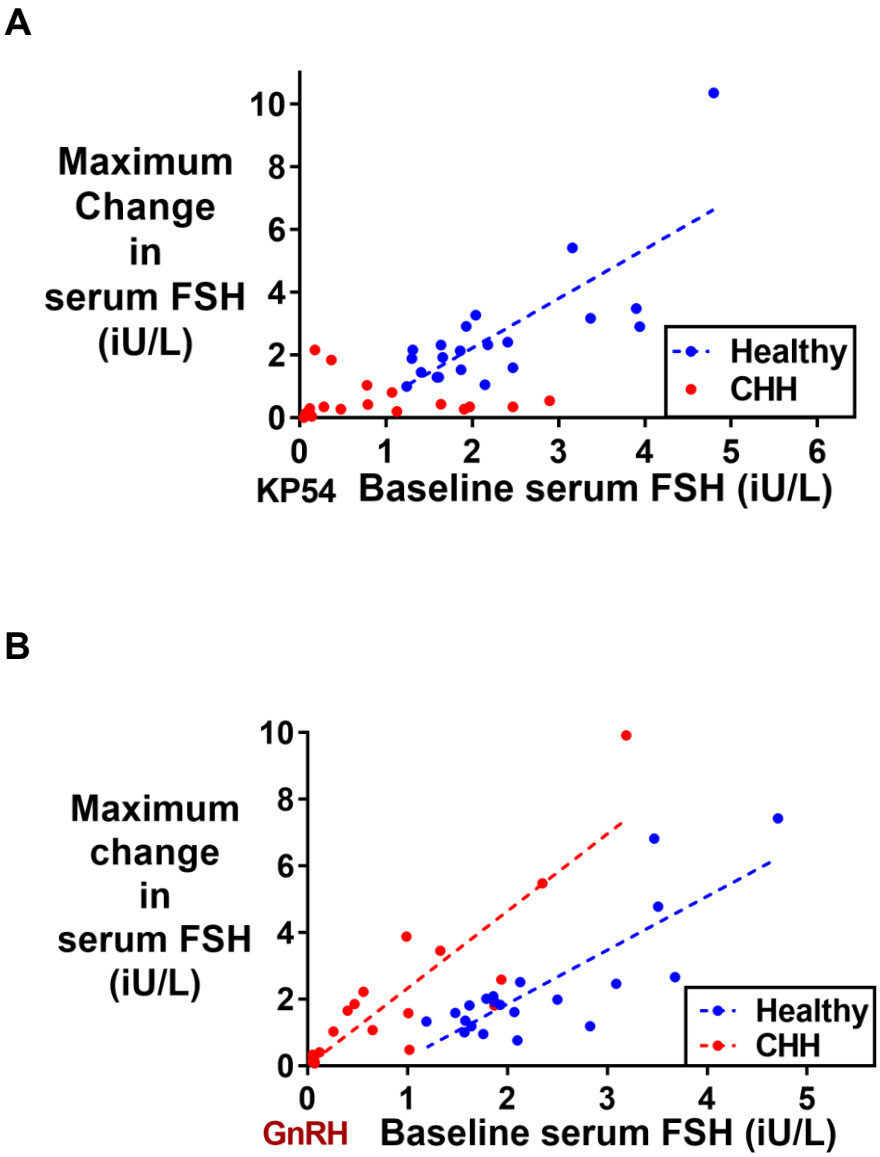


Figure 2.12 Relationship between baseline *FSH* levels and maximum change in serum *FSH* following administration of KP54 and GnRH in healthy men and men with CHH

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

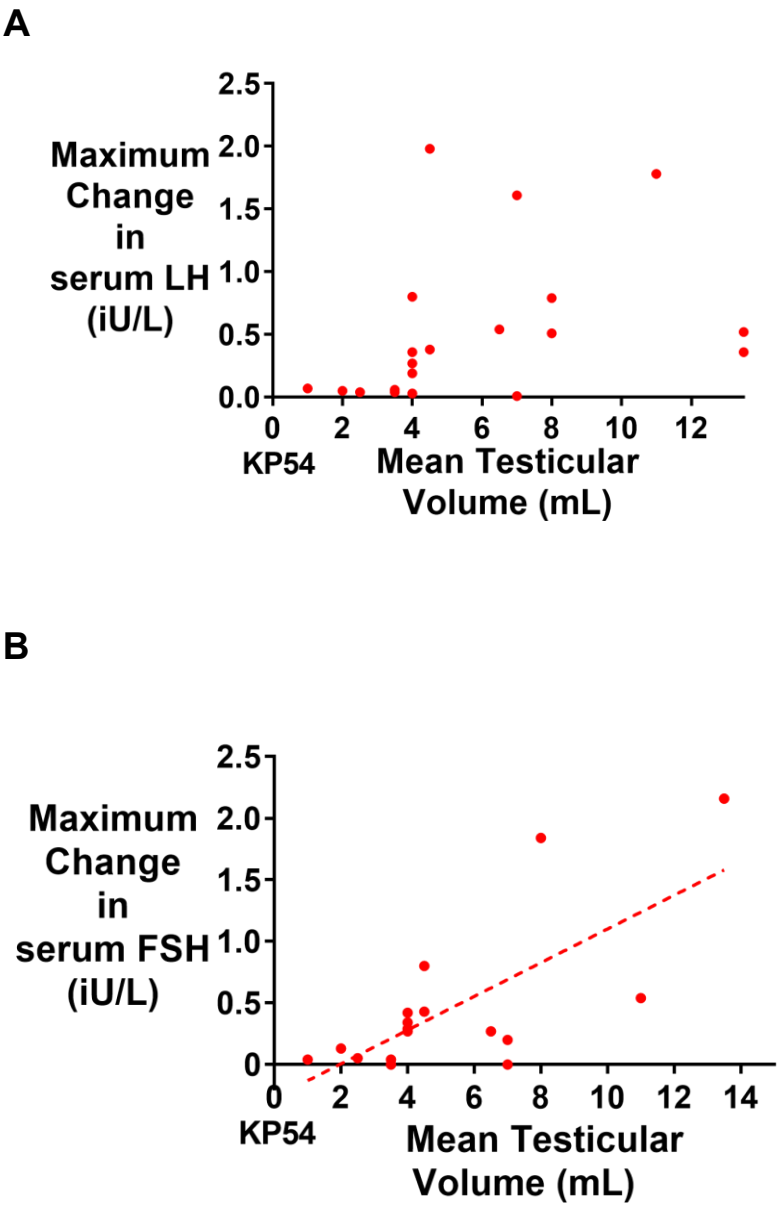


Figure 2.13 Relationship between mean testicular volume and maximum change in serum gonadotrophins following KP54

Simple linear regression of 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 (6.4 nmol/kg) in men with CHH (n=21) (p=0.001, r^2=0.51).

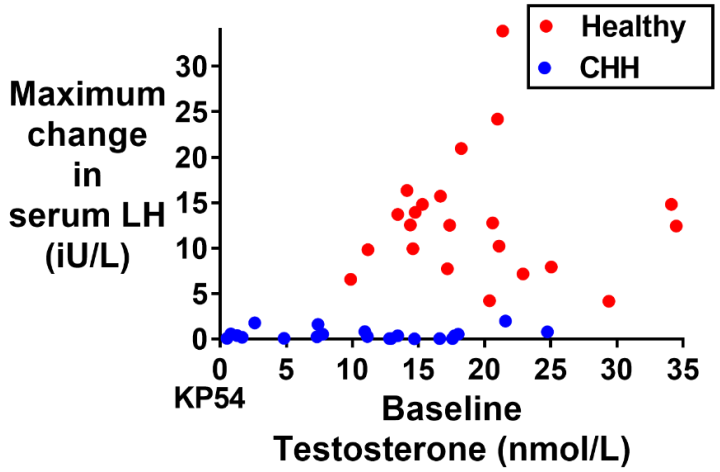


Figure 2.14 Relationship between baseline testosterone levels and maximum change in LH following KP54 in healthy men and men with CHH

Simple linear regression of the maximum change in serum LH (iU/L) from baseline and baseline total testosterone (nmol/L) in healthy men (n=21) in blue and men with CHH (n=21) in red (p>0.86).

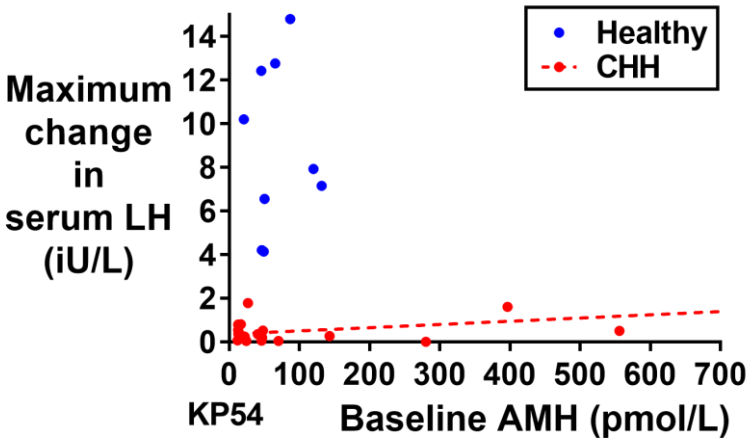


Figure 2.15 Relationship between baseline AMH levels and maximum change in serum LH following KP54 in healthy men and men with CHH

Simple linear regression of maximum change in serum LH (iU/L) from baseline and baseline serum AMH (pmol/L) in healthy men(n=14) in blue and in men with CHH (n=9) in red (p=0.02, r^2=0.02).

2.4.5 Genetic variants in men with CHH

All men with CHH consented to having whole exome sequencing analysis. Four men with CHH had mutations identified as likely pathogenic, five men had variants of uncertain significance (VUS) and ten men had no abnormality identified on genetic testing (**Table 2.2**). Following administration of KP54, the four men with pathogenic mutations were found to have LH rises that were even lower than those observed in CHH men with either no mutations identified or with VUS **(Figure 2.16)** (p=0.04). Furthermore, the maximal LH-rise in the CHH cohort after the KP54 challenge test was also significantly correlated to olfactory status, with the median maximal LH-rise being 0.79 iU/L in normosmic CHH men, 0.22 iU/L in microsmic CHH men and 0.05 iU/L in anosmic CHH men (**Figure 2.17A**). The difference in median maximal LH-rise between the anosmic and normosmic CHH patients was found to be significant (p=0.003) (**Figure 2.17A**). Maximum FSH responses followed a similar pattern, with normosmic CHH men having a median peak FSH-rise of 0.43 iU/L, whilst the equivalent level for microsmic CHH men was 0.38 iU/L and 0.045 iU/L in anosmic CHH men **(Figure 2.17B).**

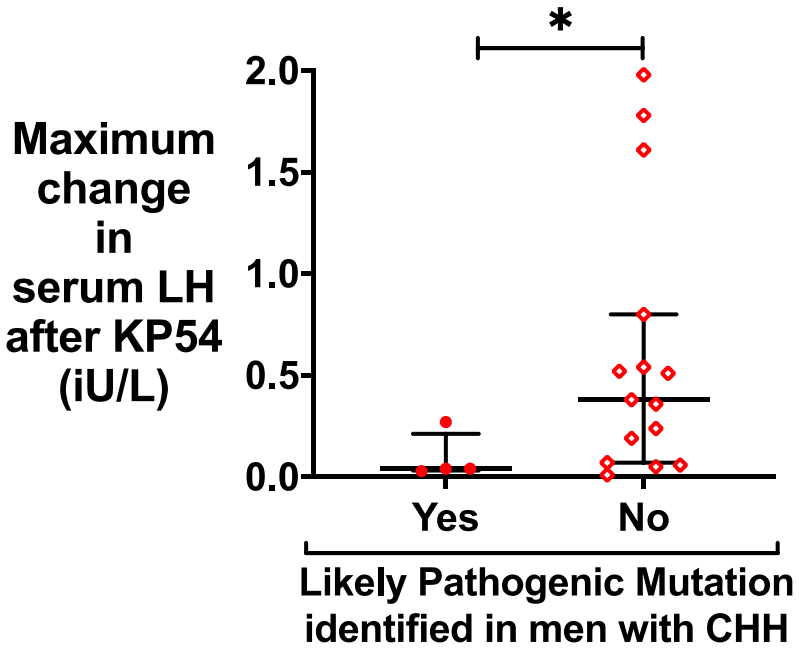


Figure 2.16 Maximum change in serum LH according to results of genetic analysis in men with CHH

Scatter diagram of median (±IQR) change in serum LH following KP54 (6.4 nmol/kg) in men with CHH with likely pathogenic mutations (n=4) (in closed circles), and those with variants of uncertain significance (VUS) or no abnormality detected (n=16) on genetic testing (open circles) (* p=0.04).

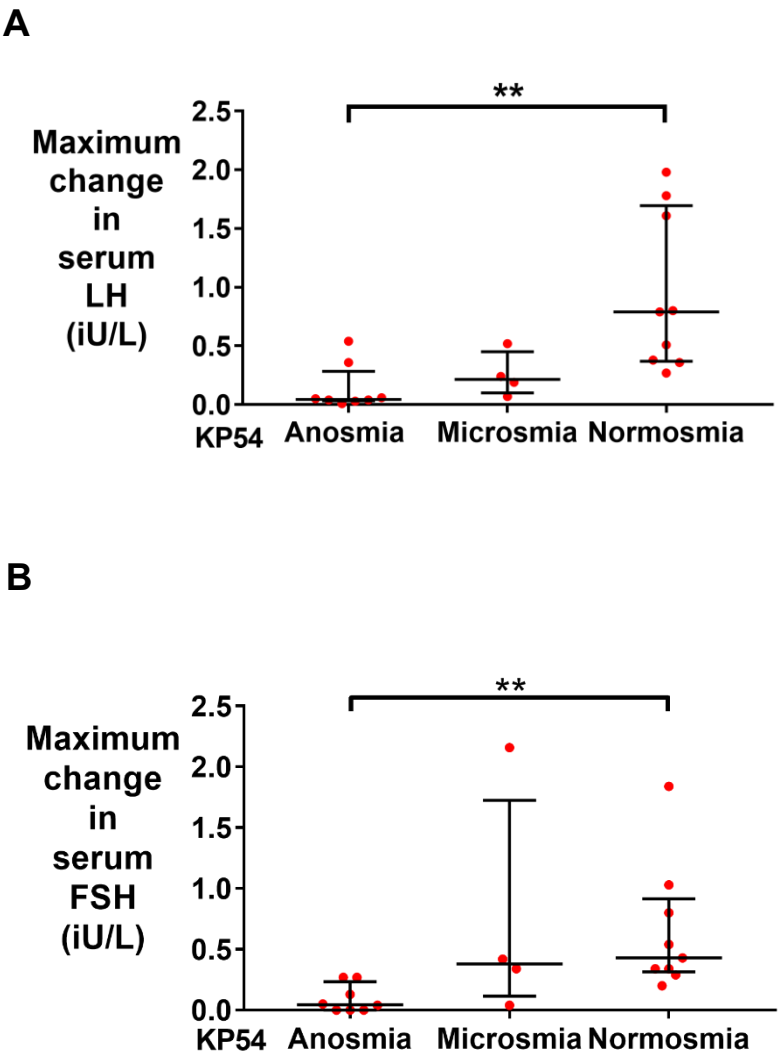


Figure 2.17 Maximum change in serum gonadotrophins following KP54 according to olfactory status in men with CHH

Scatter diagram of median (±IQR) maximal change in (A) serum LH (iU/L), (B) serum FSH (iU/L) in men with CHH with anosmia, microsmia and normosmia determined by the 40-item UPSIT. Groups were compared by Kruskal-Wallis test with post hoc Dunn's multiple comparison test (** $p < 0.01$).

2.5 Discussion

This study investigated the potential of a kisspeptin test to identify and differentiate men with CHH from healthy men, and assessed its performance compared to the currently available GnRH test. All men with CHH exhibited attenuated gonadotrophin responses to KP54, consistent with impaired hypothalamic function, irrespective of their underlying genetic status. This response was universal in all CHH patients, even in those who had evidence of pituitary responsiveness to GnRH. This finding underscores the importance of KP54 as an interrogator of hypothalamic function in men with CHH. The KP54 challenge test accurately distinguished men with CHH from healthy men and exhibited higher diagnostic performance compared to a GnRH test. Following an intravenous bolus of KP54, all men with CHH had a peak LH rise of <2 iU/L, whereas all healthy men had an LH rise >4 iU/L, thus KP54 managed to completely distinguish men with CHH from healthy men. However, the same did not apply to the LH responses following GnRH, where there was considerable overlap between men with CHH and healthy men. Men with CHH had a variable response to GnRH. Thus, the diagnostic performance of the KP54 test with an auROC of 1.0, was superior to that of the GnRH test which had an auROC of 0.88.

In healthy men, the pharmacodynamic effect of GnRH and KP54 on serum testosterone levels was similar, as testosterone secretion reflected changes in serum LH levels. Surprisingly, the peak change in testosterone levels occurred at around the same time (approximately 3-4 hours post peptide administration), irrespective of the timing of the LH peak (30 min following GnRH vs 4 hours following KP54). This observation remains currently unexplained and warrants further investigation, with longer protocols that would explore whether a further peak is seen after administration of KP54 that was not captured within the short duration of the current protocol. Furthermore, given our current knowledge of the presence of testicular *KISS1R* expression, it would be interesting to probe the direct gonadal effects of KP54 in animal models, where changes in testosterone can be monitored following administration of KP54 with and without GnRH antagonism.

Furthermore, the testosterone responses of men with CHH following KP54 were also unexpected, and once again raise the possibility of KP54 having a direct effect at the level of the testis. Moreover, the change in testosterone seen in this patient cohort might also be due to background steroid administration. To investigate this in more detail, future studies should involve a vehicle arm, so that we can compare these patients' ambient testosterone profiles to those elicited following KP54.

The findings of this study are in keeping with previous data from other groups whereby KP10 elicited diminished gonadotrophin responses in men with CHH. KP10 was previously used in eleven patients with CHH at a dose of 0.24nmol/kg. This dose had previously resulted in an LH increase of 5 iU/L at 30minutes in healthy men (Yee Ming Chan et al. 2011), but did not elicit a rise in serum LH in any of the patients with CHH, regardless of their individual genotypic variants (Chan et al. 2020). Repeated KP10 doses, at doses as high as 2.4 nmol/kg, and GnRH priming also failed to elicit an LH response (Chan et al. 2014). Studies have previously suggested that KP54 is able to cross the blood brain barrier, in contrast to KP10 that may not (Comninos et al. 2017; De Tassigny, Jayasena, Murphy, Dhillo, and Colledge 2017), and KP54 could therefore reach GnRH neuronal cell bodies, rather than GnRH neuronal terminals at the median eminence (De Tassigny, Jayasena, Murphy, Dhillo, and Colledge 2017). Moreover, when considering the two isoforms' half-lives ($t_{1/2}$ KP54: 28mins, KP10: 3min) (Waljit S Dhillo et al. 2005), KP54 can be considered the isoform with the most favourable pharmacokinetic profile for intravenous bolus administration during a 'kisspeptin challenge' test (C. N. Jayasena, Abbara, et al. 2015b). KP54 resulted in a median increase in serum LH of 13.2 iU/L in healthy men, whereas KP10 elicited a mean maximal LH rise of 8.3 iU/L when administered to healthy men (Jyothis T. George et al. 2011). KP54's ability to achieve a higher LH rise in healthy participants can be advantageous in difficult cases of partial responders or in patients with milder phenotypes. The ability of KP54 to interrogate hypothalamic function, which is known to be key in the initiation of puberty, could enable it to be used in the investigation of pubertal failure in children presenting with delayed puberty.

Whilst genetic mutations resulting in CHH are quite heterogeneous, they can be broadly categorised into variants causing impaired GnRH migration and those causing impaired GnRH secretion (Boehm et al. 2015). Traditionally mutations resulting in impaired GnRH secretion can produce milder or even partial clinical phenotypes (Boehm et al. 2015). Indeed Young *et al*. investigated the effects of a 12 hour KP10 infusion on the gonadotrophin profiles of four patients with either TAC3 or TAC3R mutations (Young et al. 2013). Kisspeptin resulted in a rise in serum LH (LH following vehicle:0.4 iU/L, LH following KP10: 1.0 iU/L) and serum FSH (FSH following vehicle: 3.1 iU/L, FSH following KP10: 5.0 iU/L) (Young et al. 2013). However, when considering the absolute gonadotrophin levels reached following the KP10 infusion, it is clear that these were markedly reduced compared to the gonadotrophin responses of healthy men treated with the same protocol (healthy men: LH following vehicle 5.2 iU/L, LH following KP10 14.1 iU/L; FSH following vehicle: 2.7 iU/L, FSH following KP10: 5.0 iU/L) (Jyothis T. George et al. 2011). Thus, although patients with mutations causing milder phenotypes, such as those with variants affecting NKB signalling, can respond to KP10, the response is lower than healthy individuals. Indeed, the greater gradation of gonadotrophin responses following KP54 compared to KP10 can be valuable for more accurate differentiation of patients with CHH from healthy individuals.

Following administration of KP54 there was no overlap in LH-responses between men with CHH and healthy men, regardless of the underlying genotypes of men with CHH. However, the four men in our CHH cohort with likely pathogenic mutations exhibited smaller LH-rises following KP54 administration than CHH men who either did not have any abnormality identified on genetic analysis or had variants of unknown significance. Moreover, men with Kallmann syndrome (CHH + anosmia) had significantly lower responses after KP54 compared to normosmic men with CHH. It might be the case that CHH patients with anosmia suffer from disordered GnRH neuronal migration (rather than disordered GnRH secretion) and thus GnRH neurons are not in the required anatomical site to respond to KP54. In congruence with this,

there is evidence that men with Kallmann syndrome exhibit a more severe phenotype of GnRH deficiency than normosmic men with CHH (Bonomi et al. 2018; Quinton et al. 2001).

Indeed, in keeping with the issue of the spectrum of GnRH deficiency, this study also demonstrated a positive correlation between testicular volume and gonadotrophin responsiveness. Larger testicular volume can be indicative of partial puberty and thus a milder clinical phenotype. In line with this observation, Nabi et al. previously demonstrated that gonadotrophin responses to KP10 increase with increasing Tanner stage in young people (Nabi et al. 2018). Whilst healthy men had LH-predominant responses to both GnRH and KP54, men with CHH exhibited an FSH-predominant response to GnRH but not to KP54. Variations in GnRH pulsatility can explain differential gonadotrophin responses in the context of physiological pulsatile secretion. However, it is unlikely that this can be explained in the context of bolus stimulation. One explanation might be that men with CHH, who have a history of partial or absent puberty, have lower inhibin B levels, and thus reduced inhibin B-associated negative feedback on FSH secretion. Indeed, Pitteloud et al. has previously demonstrated that men with absent puberty exhibit FSH predominant responses, whereas men with partial puberty and higher serum inhibin B have LH-predominant responses following a single intraveous bolus of GnRH (25 ng/kg) (Pitteloud et al. 2009).

The study also demonstrated that whilst healthy men's LH responses to KP54 were correlated with their respective baseline LH levels, this was not the case for men with CHH. The CHH cohort demonstrated attenuated responses to KP54, beyond what would have been expected due to their lower baseline serum LH levels. Thus, KP54 is able to provide additional information regarding the hypothalamic GnRH reserve of an individual, in addition to the knowledge of their baseline LH levels. As previously reported by Bang et al., baseline serum LH levels are closely associated with GnRH stimulated LH responses (Bang et al. 2017), thus GnRH does not provide any additional diagnostic information.

The physiological response to an intravenous bolus of KP54 consists of a short initial LH rise, followed by a plateau between 30 and 75 minutes and a second rise, with a peak occurring

between 4 – 6 hours. When KP54 has been administered in a variety of different participant populations and settings, such as healthy men, healthy women, hypothalamic amenorrhoea and IVF treatment, the gonadotrophin profile is remarkably similar (Jayasena et al. 2010; 2014; 2015b Abbara et al. 2014, 2017;). Serum FSH has a longer half-life (3.9 hours) than LH (20 minutes) and this can explain why serum FSH reaches its peak later than the LH peak. In healthy men, the maximal LH level reached following GnRH was similar to that reached after KP54, despite the difference in the two peptides' half-lives ($t_{1/2}$ GnRH: 3 minutes, $t_{1/2}$ KP54: 28 minutes). Even though it was statistically not significant, the peak FSH response following KP54 seemed to surpass that following GnRH, conceivably due KP54's longer duration of action and also due to the longer time it might take for KP54 to cross the blood brain barrier. This does not apply to GnRH which acts at the level of the pituitary, that resides outside the blood brain barrier.

It is now recognised that approximately 20% of patients with CHH can undergo spontaneous reversal of their hypogonadal state. This may present with reversal of hypogonadal symptoms and normal total testosterone levels after cessation of therapy. Patients experiencing reversal of their CHH also regain the ability to positively respond to a KP10 challenge test (Lippincott et al. 2016). Interestingly, spontaneous reversal was not found to be associated with the severity of GnRH deficiency (usually associated with mutations affecting GnRH neuronal migration) (Lippincott et al. 2016).

Some participants in the CHH group were on long acting testosterone treatment (testosterone undecanoate) at the time of their recruitment. The study protocol permitted this in order to avoid asking patients to discontinue vital treatment that would take months to wash out. This decision was based on previous work by Lippincott et al. demonstrating that sex-steroid milieu does not alter responses to kisspeptin in men with CHH (Lippincott et al. 2018). Indeed, this was also observed in our cohort, where gonadotrophin responses to kisspeptin were not associated with sex-steroid replacement or baseline total testosterone levels. Most importantly, this observation highlights kisspeptin's potential to expedite the assessment of hypothalamic

GnRH function in patients with CHH suspected of having hypogonadism reversal, negating the need to stop their treatment.

In summary, this study demonstrates KP54's potential to specifically interrogate hypothalamic GnRH neuronal function in patients presenting with secondary hypogonadism. With a longer half-life than KP10, it is increasingly recognised as the kisspeptin isoform with the most favourable pharmacokinetic profile, both for practical reasons (bolus administration vs need for infusion) and also for its ability to provide greater granularity in LH responses when assessing borderline or milder clinical phenotypes. The gradation of responses after KP54 is more useful than shorter acting peptides (KP10) in identifying patients with CHH from healthy individuals. Thus, KP54 has the potential to become an important diagnostic tool in the accurate investigation of men with hypogonadotrophic hypogonadism.

2.5.1 Limitations

Kisspeptin stimulates the hypothalamus to secrete endogenous GnRH which in turn stimulates gonadotrophin secretion at the level of the pituitary. Thus, responses to kisspeptin rely on a responsive pituitary gland. The lack of GnRH priming in patients with CHH could theoretically impair their pituitary response to both GnRH and kisspeptin. In this study this issue was negated by comparing the gonadotrophin responses after a GnRH test. Thus, the lack of gonadotrophin responses following KP54 in the CHH cohort is not due to a lack of previous GnRH priming or exposure, but rather due to hypothalamic dysfunction. This is because all of our patients with CHH exhibited gonadotrophin responses following the GnRH test. Furthermore, GnRH priming is not readily available on a national level in the UK. Most importantly, there is evidence that GnRH priming does not affect how patients with CHH are categorized following a KP54 challenge (YM et al. 2018).

In healthy women, kisspeptin responsiveness changes throughout the menstrual cycle with an augmented response observed in the preovulatory phase when oestradiol levels are the

highest (Y. M. Chan et al. 2012; Waljit S. Dhillo et al. 2007). In rhesus monkeys, kisspeptin responsiveness changes as sex steroid milieu change across pubertal development (Seminara et al. 2006). Therefore, it might still be the case that sex steroids may affect gonadotrophin responses to GnRH or KP54. For this reason, the hormonal studies were undertaken at trough levels while patients were on testosterone undecanoate or 1 week without transdermal testosterone gels.

2.5.2 Conclusion and future work

This study has highlighted kisspeptin's huge diagnostic potential; a KP54 challenge test can specifically interrogate hypothalamic GnRH reserve and functionality. One group of patients that could benefit from such as test are men with CHH suspected of having a spontaneous reversal of their hypogonadism. The gradation of their gonadotrophin response may allow more accurate distinction between healthy and abnormal responses which would be particularly valuable in patients with milder or partial phenotypes. Another patient group that stands to benefit from a KP54 challenge test are women presenting with primary amenorrhoea where the distinction between functional idiopathic hypogonadotrophic hypogonadism and CHH can be difficult to make. With the incidence of eating disorders rising in the UK and the mean age of patients affected also reducing, a test that can test the hypothalamic reserve of these patients can revolutionise the way these patients are treated. Following along the same lines, a kisspeptin challenge test can fundamentally change the way young people with delayed puberty are investigated and managed, as it could better differentiate young people with CHH from those with constitutional delay of growth and puberty (CDGP). Furthermore, a larger study recruiting a greater number of healthy participants would also pave the way for the determination of a reference range of serum gonadotrophin responses following a KP54 challenge test in eugonadal men.

Chapter 3

The effect of Kisspeptin-54 and its receptor agonist, MVT602, on gonadotrophin responses in healthy women

CHAPTER 3: THE EFFECT OF KISSPEPTIN-54 AND ITS RECEPTOR AGONIST, MVT602, ON GONADOTROPHIN RESPONSES IN HEALTHY WOMEN

3.1 Introduction

Since the recognition of kisspeptin as a pivotal regulator of reproductive physiology, a wealth of data has emerged exploring the use of kisspeptin-based therapies for the investigation and treatment of reproductive disorders. The vast majority of animal and human studies investigated the effects of either KP10 or KP54 on gonadotrophin hormone secretion. However, the utility of these two isoforms is limited by their rapid enzymatic degradation in the blood circulation (H Matsui and Asami 2014). KP10, being the shortest of the four kisspeptin isoforms, has a half-life of just 3.8 ± 0.3 minutes in healthy men and 4.1 ± 0.4 minutes in healthy women (Jayasena et al. 2011). In contrast, KP54 is a longer peptide with a half-life of 27.6 ±1.1 minutes, but this also increases its manufacturing costs (Dhillo et al. 2005; Jayasena et al. 2015a). Furthermore many studies involving the repeated administration of high doses of kisspeptin resulted in tachyphylaxis, a phenomenon characterised by diminished receptor sensitivity in response to consistent stimulation by a drug agonist (Webb 2011). Tachyphylaxis to kisspeptin is well documented, and the desensitisation is associated with prolonged and non-pulsatile kisspeptin exposure, either via more frequent administration, or higher doses of kisspeptin (Abbara et al. 2013). Therefore, the development of a longer acting kisspeptin receptor analogue offering increased resistance to enzymatic degradation and improved metabolic stability, whilst also retaining agonistic activity at the kisspeptin receptor, could revolutionise translational studies for kisspeptin based treatments.

Since 2007, multiple groups have developed such kisspeptin agonists (Asami et al. 2012, 2013; Oishi et al. 2011; Orsini et al. 2007) by modifying KP10, the shortest amino-acid sequence required to activate KISS1R (H Matsui and Asami 2014; Nishizawa et al. 2016; Orsini et al. 2007). One of these experimental products is a nano-peptide kisspeptin receptor agonist called MVT602 (previously known as TAK448), which resulted from the substitution of

five amino acids and the deletion of one amino acid from the KP10 sequence **(Table 3.1, Figure 3.1).** MVT602's half-life of 1.5-3.5 hours is longer than those of KP10 and KP54 (MacLean et al. 2014). Animal studies of this compound have demonstrated robust gonadotrophin stimulation following subcutaneous administration, whereas continuous infusion over several days resulted in a sustained decline in hormone levels consistent with receptor desensitisation (Matsui et al. 2012). Indeed this property was exploited in human studies, when MVT602 was administered to men with prostate cancer with a view to induce chemical castration (MacLean et al. 2014). MacLean et al. demonstrated that a continuous infusion of MVT602 in this patient cohort resulted in downregulation of the hypothalamic-pituitary-gonadal axis, with total testosterone levels falling in the hypogonadal range by day seven post administration (MacLean et al. 2014). Furthermore, a single intravenous bolus of MVT602 in healthy men resulted in a sustained increase of serum LH levels between 8-12 hours, before returning to baseline levels within 72 hours post administration (MacLean et al. 2014). The LH profile elicited after a single injection in healthy men was more prolonged compared to the LH response to KP54. If this effect is also maintained in healthy women, it could potentially result in a sustained LH profile resembling the physiological LH surge of the pre-ovulatory phase. The physiological effects of MVT602 on the gonadotrophin responses of women have not been studied before. This study explored the endocrine profile of MVT602 in the early follicular phase (day 1 to 4) in healthy women. As the response to kisspeptin can be altered by the ambient sex-steroid milieu, I also investigated the impact of oestrogen supplementation on the endocrine responses to MVT602 in healthy women. Finally, I also studied what effects, if any, MVT602 had on blood pressure and heart rate measurements and how these differ compared to the effects of KP54.

Peptides	Amino acid sequence											
	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-	NH2
MVT602	Ac-	D-Tyr	Hyp-	Asn-	Ser-	Phe-	Aza Gly-	Leu -	Arg (Me)-	Phe-		NH_2

Table 3.1: Comparison between the amino acid sequence of KP10 and MVT602

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

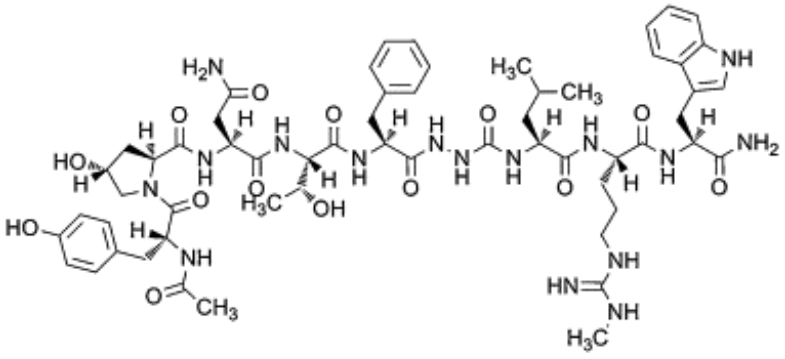


Figure 3.1: The chemical structure of MVT602

(Kuze et al. 2013)

3.2 Aims and Hypotheses

3.2.1 Aims

- To characterise the effects of MVT602 on gonadotrophin secretion during the follicular phase of healthy women
- To compare these effects to those elicited by KP54 (9.6 nmol/kg) and placebo (0.9% NaCl)
- To investigate the effect of oestrogen supplementation on gonadotrophin responses following MVT602 in the early follicular phase of healthy women
- To delineate MVT602's effects on blood pressure and heart rate parameters

3.2.2 Hypotheses

- MVT602 will stimulate gonadotrophin release during the follicular phase of the menstrual cycle.
- MVT602 will achieve a comparable amplitude but will have a more prolonged duration of action compared to KP54.
- Oestrogen supplementation will result in an amplified gonadotrophin response following MVT602.
- MVT602 will not have any effect on blood pressure or heart rate measurements.

3.3 Methodology

3.3.1 Study Overview

I conducted a two-phase prospective, randomised, cross-over physiological study to determine for the first time the effects of MVT602 on the gonadotrophin response of healthy women:

- Phase 1: Randomised, cross-over physiological study comparing MVT602 to KP54 and placebo
- Phase 2: Determination of the effects of oestradiol supplementation on the gonadotrophin response of healthy women following MVT602

3.3.2 Ethical approval

Ethical approval was granted by the West London Research Ethics Committee (Ref:12/LO/0507) and the study was conducted in accordance with the Declaration of Helsinki.

3.3.3 Study Subjects

Study participants were recruited following advertisements in the local press. Interested women were provided with an ethically approved Participant Information Sheet prior to their screening appointment. Eligibility was confirmed following a detailed medical, endocrine and menstrual history, clinical examination, electrocardiography and blood tests including an anterior pituitary panel and serum βhCG level**.** Both study phases had the following inclusion criteria: women aged 18-35 years, regular menstrual cycle history (menstrual cycle length <35 days) and BMI 18-30kg/m^2. Women were excluded if they had any clinically significant past medical history, were on regular or over the counter medication (including hormonal contraception), had a positive pregnancy test, were wishing to seek fertility within two months of completion of the study, had abnormal laboratory test results, or if they had a history of smoking, excessive alcohol use or illicit drug use. All participants provided written informed

consent. Participants were asked to use barrier contraception during the study period and for at least two months after the completion of their final visit.

3.3.4 Study Phase Overview

3.3.4.1 Overview of Phase 1

As discussed earlier, Dhillo et al. previously demonstrated that women are most resistant to the effects of kisspeptin during the follicular phase of the menstrual cycle, coinciding with low levels of circulating sex steroids (Y.-M. Chan et al. 2012; Waljit S Dhillo et al. 2007). Thus, the studies were conducted during the early follicular phase of the menstrual cycle, namely on days 1 to 4, to be able to administer the interventions in a phase of the cycle that is most easily definable.

Participants attended for four study visits, during which they received a subcutaneous injection of:

- MVT602 0.01 nmol/kg
- MVT602: 0.03 nmol/kg
- KP54 9.6 nmol/kg
- NaCl 0.9%

This was a cross-over study therefore all study participants received all four interventions, in random order. The randomisation was performed using an online randomisation tool (random.org).

3.3.4.2 Overview of Phase 2

This phase sought to identify any differences in the gonadotrophin responses of five healthy women to MVT602 when this was given during their follicular phase but with oestradiol pre-treatment to increase ambient oestradiol levels at the time of administration. Given that the

response to kisspeptin is greatest during the pre-ovulatory phase of the menstrual cycle, this study can highlight how the gonadotrophin responses are influenced by a higher sex steroid environment (Dhillo et al. 2007). Five of the nine healthy women already recruited for Phase 1 were randomly selected to take part in phase 2. The participants applied a topical oestradiol patch on Day 1 of their menstrual cycle and returned for a study visit 24 hours later. They all received a subcutaneous bolus of MVT602 at a dose of 0.03 nmol/kg and followed the same study protocol as in Phase 1. The oestradiol patch was removed 72 hours later (Day 4 of their menstrual cycle).

3.3.5 Study Design

All study visits were conducted at the Clinical Research Unit of Charing Cross Hospital (Imperial College Healthcare NHS Trust).

3.3.5.1 Phase 1:

Description**:** Prospective, randomised, cross-over study where each participant attended for one study visit per menstrual cycle. All study participants completed four study visits over four consecutive months. Each study visit involved the administration of one intervention, selected randomly.

Sample size: Nine healthy women were recruited to this phase of the study. The sample size was determined based on data from previous physiological studies on kisspeptin with similar study designs. These studies involved five to six study participants. Due to the intensive nature of the study (described below) we anticipated a drop-out rate of up to 30% and therefore recruited nine volunteers.

The study protocol for kisspeptin-54 and 0.9% Saline study visits is shown in **Figure 3.2A** and for MVT-602 visits in **Figure 3.2B**.

Schedule for KP54 and NaCl 0.9% study visits:

1. Pre-visit: Participant informs research team of menstrual bleeding. Participant asked to refrain from strenuous exercise, caffeine, and alcohol consumption for the 24 hours prior to the start of the study
2. Study Day 1 (SD1):
    a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling
    b. Blood sampling for serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30, 0, 5, 15 minutes post injection and then every 30 minutes for 10 hours. Blood sampling for KP54 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.
    c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for 10 hours
3. SD2: blood sample at 24 hours post drug administration
4. SD8: telephone review for any symptoms or side effects 7 days post drug administration

Schedule for MVT602 study visits:

1. Pre-visit: Participant informs research team of menstrual bleeding. Participant asked to refrain from strenuous exercise, caffeine, and alcohol consumption for the 24 hours prior to the start of the study
2. Study Day 1 (SD1):
    a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling
    b. Blood sampling for serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30, 0, 5, 15 minutes post injection, then every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours. Blood sampling for MVT602 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.
    c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours
3. SD2: blood samples at 28, and 32 hours post drug administration
4. SD 3: blood sample at 48 hours post drug administration
5. SD8: telephone review for any symptoms or side effects 7 days post drug administration

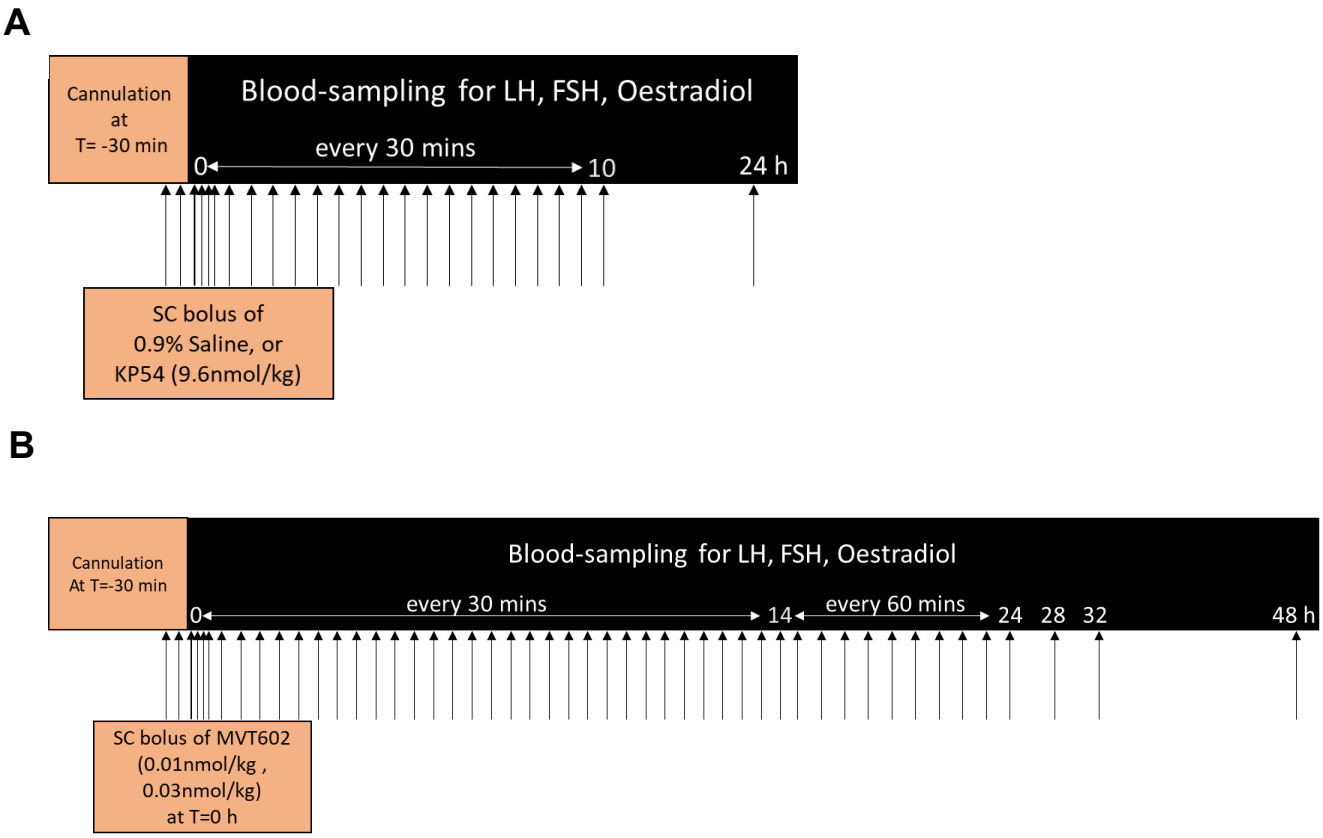


Figure 3.2: Diagram of study protocol for Phase 1

Study participants were admitted to the Clinical Research Facility at 8 am on the morning of each study visit. An intravenous cannula was inserted into one antecubital fossa and blood was sampled at T -30 min, T -15 min and T = 0 h prior to administration of each intervention to determine the basal hormonal values. A subcutaneous (SC) bolus of (A) KP54 or 0.9% saline or (B) of MVT602 was administered at T = 0 h.

Figure 3.2A- Study protocol diagram for the KP54 and 0.9% saline visits. After a SC bolus of KP54 (9.6 nmol/kg) or 0.9% saline at T = 0 h, serum hormone levels (LH, FSH, oestradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 10 h, and additionally at 24 h.

Figure 3.2B- Study protocol diagram for MVT602 visits. After a SC bolus of MVT602 (0.03 nmol/kg) was administered at T = 0 h, serum hormone levels (LH, FSH, oestradiol and

progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 14 h, and then every 60 min until 24 h and additionally at 28, 32 and 48 h.

3.3.5.2 Phase 2:

Description**:** Prospective, open label study where each participant received MVT602 at a dose of 0.03 nmol/kg during the follicular phase of their menstrual cycle, 24 hours after the application of a transdermal oestradiol patch (200μg/day).

Sample size: Five out of the nine healthy volunteers who completed Phase 1 were randomly selected to take part in Phase 2.

Study schedule:

1. Pre-visit: Participant informs research team of menstrual bleeding. Participant asked to refrain from strenuous exercise, caffeine and alcohol consumption for the 24 hours prior to the start of the study. Participant attends research unit for application of oestradiol patch and a baseline blood test.
2. Study Day 1 (SD1):
    a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling.
    b. Blood sampling for serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30, 0, 5, 15 minutes post injection, then every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours. Blood sampling for MVT602 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.

c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours.

3. SD2: blood samples at 28, and 32 hours post drug administration.
4. SD 3: blood sample at 48 hours post drug administration. Oestradiol patch removed at the same time.
5. SD4: blood sample at 72 hours post drug administration
6. SD8: telephone review for any symptoms or side effects 7 days post drug administration.

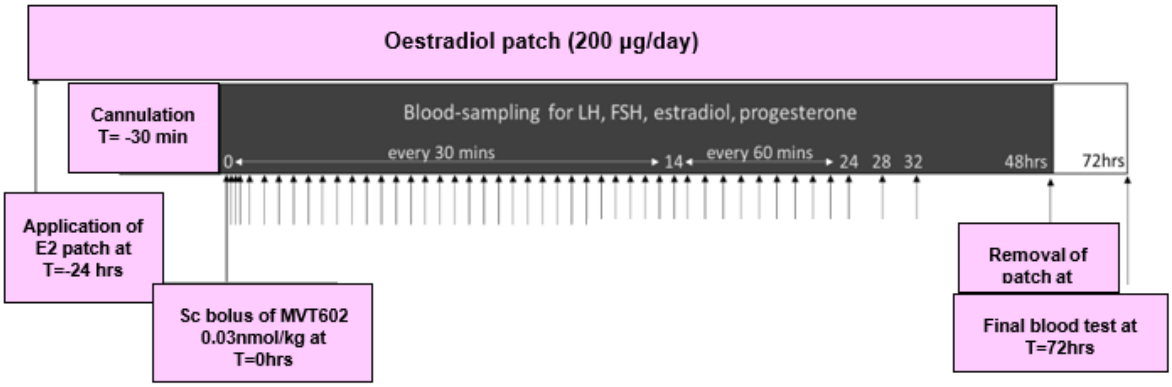


Figure 3.3: Diagram of study protocol for Phase 2

Study participants attended the Clinical Research Facility 24 h prior to the start of the study for application of the oestradiol patch (200mcg/day). They were then admitted to the same unit at 8 am on the morning of the study visit. An intravenous cannula was inserted into one antecubital fossa and blood was sampled at T -30 min, T -15 min and T = 0 h prior to administration of each intervention to determine the basal hormonal values. A subcutaneous (SC) bolus of MVT602 was administered at T = 0 h. Serum hormone levels (LH, FSH, oestradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 14 h, and then every 60 min until 24 h and additionally at 28, 32, 48 and 72 h. The patch was removed at T=48 h.

3.3.6 Peptides

MVT602 was synthesized by Myovant Sciences Ltd. (Virginia, USA) with animal toxicology testing before administration in humans. Human KP54 was synthesised by Bachem (Liverpool, UK). Peptide bioactivity and animal toxicology testing were confirmed following a negative Limulus amebocyte lysate assay test for pyrogen (Associates of Cape Cod, Liverpool) and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital). Both peptides were prepared in accordance with the Good Manufacturing Practice.

3.3.7 Hormone Assays

Blood samples for estimation of serum LH, FSH, oestradiol and progesterone were collected in simple Vacutainer tubes (Becton Dickinson, Franklin Labs, New Jersey). Blood samples were left to clot for at least 1 hour before being centrifuged at 3000 rpm for 10 minutes. Blood samples for plasma kisspeptin measurement 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 centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) at 4000 rpm for 4 minutes. Plasma and serum samples were separated and frozen at -20ºC. Blood samples for estimation of MVT602 levels were collected in chilled K2EDTA tubes (Becton Dickinson, Franklin Labs, NJ) and spun for 10 minutes at 1200 rpm at a temperature of 4ºC, before being stored at -20ºC. Serum LH, FSH, oestradiol and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Inter-assay 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 (iU/L), 2-10 (follicular), 20-60 (mid cycle), 4-14 (luteal); FSH (iU/L), 1.5-8 (follicular and luteal), 10-50 (mid cycle) and oestradiol (pmol/L), <300 (early follicular), 400-1500 (mid cycle), 200-1000 (luteal). Plasma kisspeptin immunoreactivity (IR) was assessed using an established RIA. The antibody cross-reacted 100% with human KP54, kisspeptin-14, and KP10 and <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.

3.3.8 Statistical analysis

Statistical analysis was done using Graphpad Prism version 8.3. Parametrically distributed data were presented as mean ±SD, and non-parametric data were presented as median ±IQR. Statistical comparison across intervention groups was done using one-way ANOVA with post hoc Tukey's multiple comparison test or Kruskal-Wallis test with post hoc Dunn's test, as appropriate. Logistic regression analysis was used for categorical data. A p-value <0.05 was considered to be statistically significant. Area under curve analysis (AUC) was used to assess quantitative size of effect.

3.4 Results

3.4.1 Baseline Characteristics

Following screening nine women were recruited to Phase 1 of the study. Their mean age (+SD) was 28.2 (±5.0) years, BMI was 23.6 (±2.8) kg/m^2 and their median menstrual cycle length prior to the study was 28 (±1.3) days. The baseline demographic characteristics and reproductive hormone profiles are displayed in Table 3.2.

Participant	Age (years)	BMI (kg/m^2)	Cycle length (days)	LH (iU/L)	FSH (iU/L)	Oestradiol (pmol/L)	Progesterone (nmol/L)
1	33	22.2	28	4.8	2.2	100	1
2	24	28.2	28	2.6	4.1	194	1
3	22	25.1	28	3.9	1.8	608	19
4	33	25.5	28	1.8	2.2	251	36
5	25	24.4	30	5.7	5.1	314	8
6	32	21.5	28	1.1	3	760	49
7	35	20.1	28	3.9	5.7	134	1
8	27	25.5	25	4.3	3.52	519	2
9	23	20.1	28	4	2.4	552	36
MEAN (±SD)	**28.2 (±5.0)**	**23.6 (±2.8)**	**27.9 (±1.3)**	**3.57 (±1.47)**	**3.34 (±1.38)**	**381.3 (±234.5)**	**17 (±18.79)**

Table 3.2: Baseline characteristics of participants

Baseline characteristics of participants (n=9). Mean (±SD) is presented for age, body mass index (BMI), menstrual cycle length, serum LH (iU/L), serum FSH (iU/L), serum oestradiol (pmol/L) and serum progesterone (nmol/L) at screening.

3.4.2 Results of Phase 1

3.4.2.1 Gonadotrophin responses following MVT602

Following a single subcutaneous dose of KP54, the median maximal rise in serum LH was 7.8 iU/L, which occurred at 5.5 hours. Both doses of MVT602 potently stimulated serum LH responses in all nine healthy women, with a median maximum change from baseline of 6.2 iU/L (following a dose of 0.01nmol/kg) and 6.6 iU/L (following a dose of 0.03 nmol/kg) at 24 hours **(Figure 3.4A)**. Whilst the amplitude of the LH response following KP54 was comparable to those achieved following MVT602 0.01 nmol/kg and MVT602 0.03 nmol/kg, the time to peak LH response occurred much later with MVT602 at around 22 to 24 hours. Thus, MVT602 greatly extended the area under the curve for LH-rise, by at least three-fold (p=0.029) **(Figure 3.5A)**. The LH profile following both doses of MVT602 showed an initial plateau, prior to a rise in serum LH. Following KP54, there was a small initial LH rise, followed by a plateau between 30 and 75 minutes before a robust LH peak between 4-6 hours.

Serum FSH responses corresponded to LH-rises following administration of both peptides. However, the peak serum FSH reached after MVT602 was smaller to that achieved following KP54 **(Figure 3.4B)**, such that the area under the curve for change in FSH following MVT602 was not different to that seen following KP54 **(Figure 3.5B)**. The oestradiol response, however, was greater following administration of MVT602, which was maintained for at least 48 hours after administration **(Figure 3.4C, Figure 3.5C)**.

In summary, MVT602 stimulated an LH response that was of similar amplitude to that observed following KP54, although the peak LH occurred much later, suggesting a longer duration of stimulation.

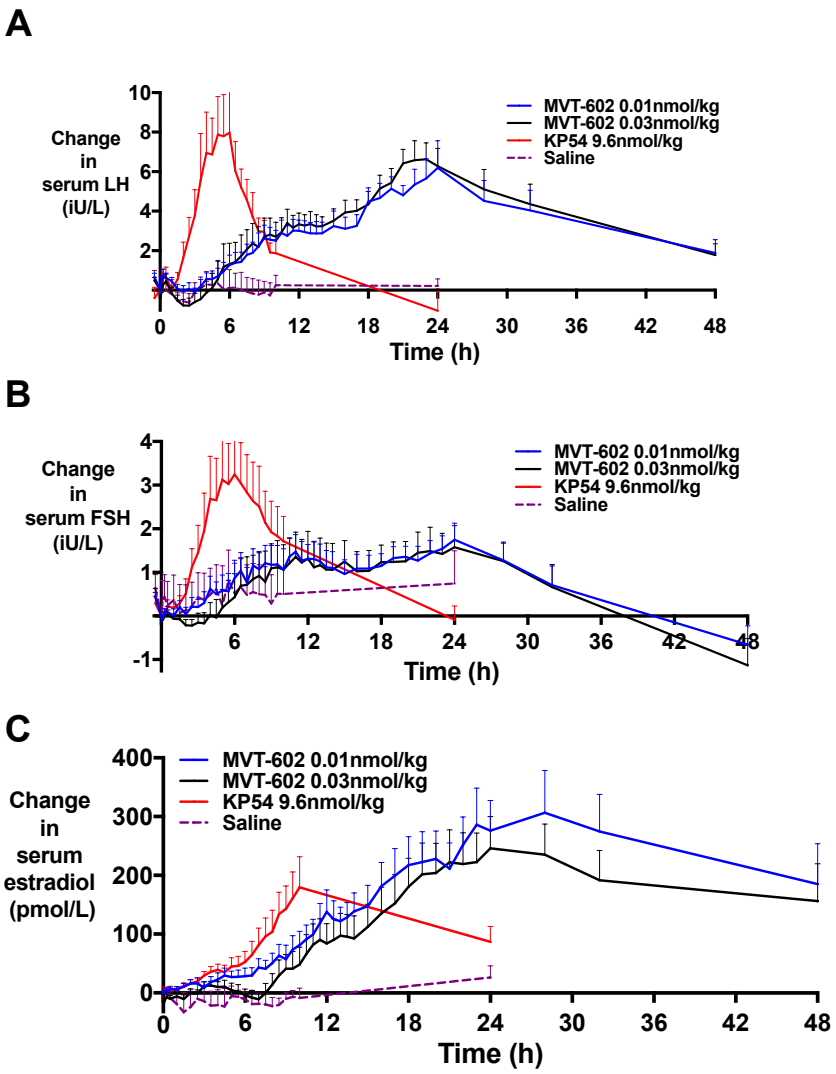


Figure 3.4 Change in serum gonadotrophin and oestradiol levels following administration of MVT602, KP54 and 0.9% Saline

Mean±SEM of change 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 MVT602 at doses of 0.01nmol/kg (in blue), 0.03nmol/kg (in black) over 48 hrs and KP54 9.6nmol/kg (in red) and saline (in purple) over 24 hrs.

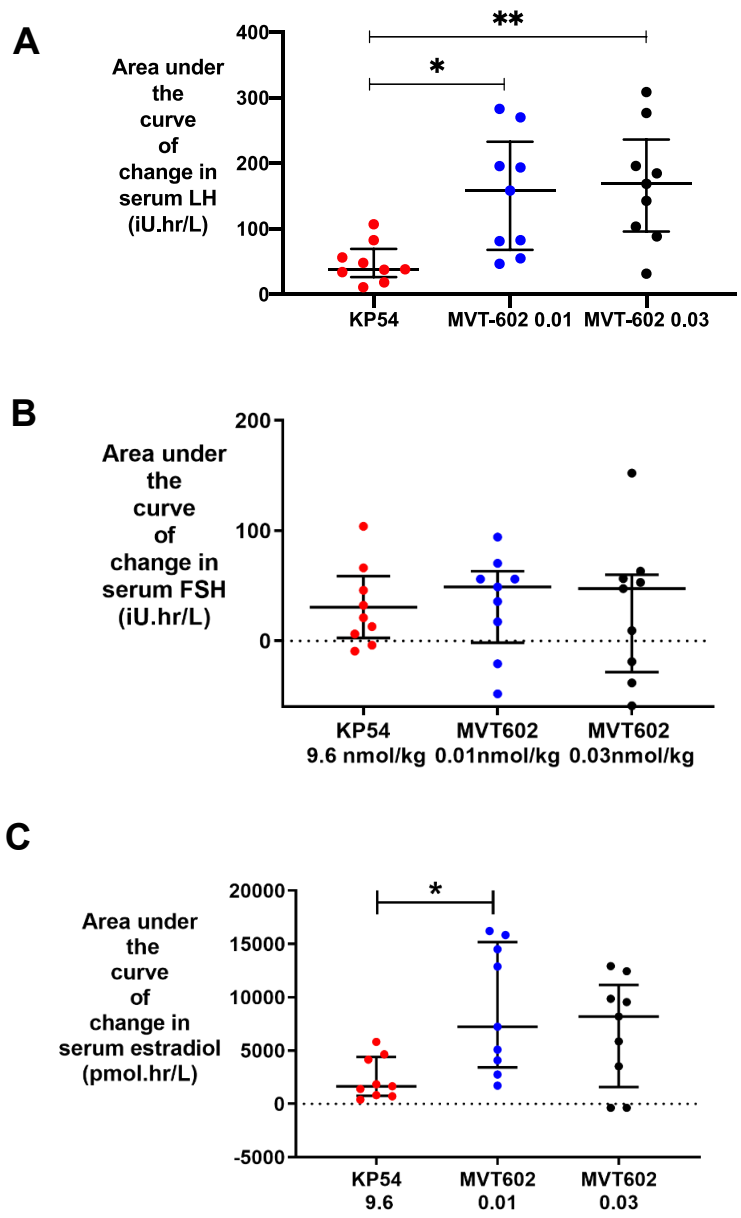


Figure 3.5 Area under the curve of change in gonadotrophins and oestradiol

Scatter diagram of the 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) and (C) serum oestradiol (pmol.hr/L) in healthy women (n=9) receiving KP54 9.6 nmol/kg (in red), MVT602 0.01 nmol/kg (in blue) and MVT602 0.03 nmol/kg (in black). Groups were compared by Kruskal Wallis test with post hoc Dunn's multiple comparison test (* $p<0.05$, ** $p<0.01$).

3.4.2.2 Other determinants of gonadotrophin responses to MVT602

The maximum change in serum FSH was positively associated with the stimulated oestradiol levels following MVT602 administration in healthy women (p=0.04, r^2 =0.45) **(Figure 3.6A)**. The same did not apply between maximal change in LH and maximal change in Oestradiol levels in women receiving MVT602 (p=0.06, r^2=0.41) **(Figure 3.6B)**. Furthermore, there was a significant negative correlation between the participants' baseline Inhibin B levels and their maximal change in serum FSH (p=0.04, r^2 =0.49) **(Figure 3.6C)**. However, their baseline AMH levels did not correlate with the peak stimulated FSH levels reached following MVT602 (p=0.69, r^2 =0.03) **(Figure 3.6D)**.

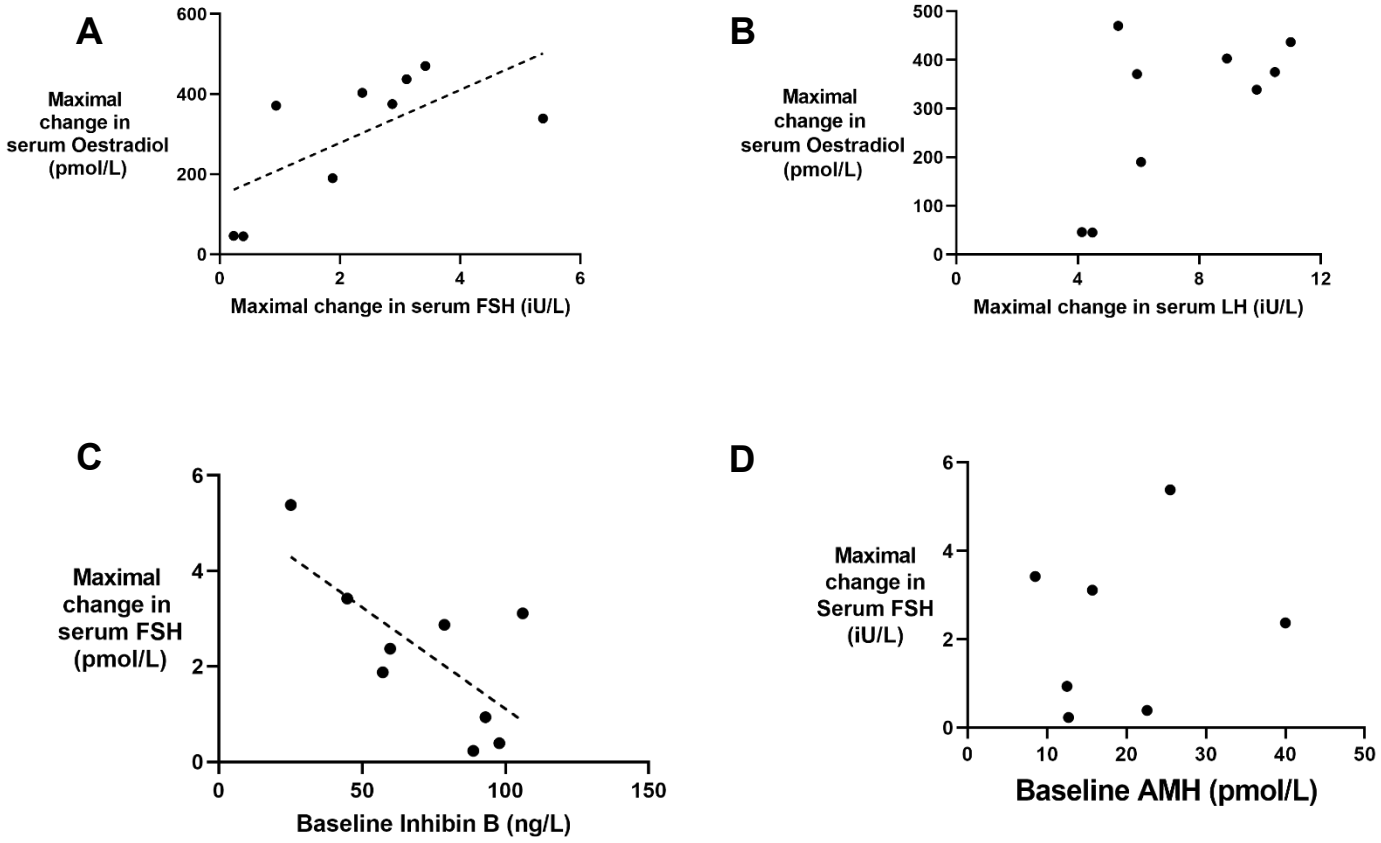


Figure 3.6 Other determinants of gonadotrophin responses to MVT602

Correlation between maximal change in serum oestradiol (nmol/L) and (A) maximal change in serum FSH (iU/L) from baseline levels (p=0.04, r^2 =0.45, equation: y= 66.11*x + 146.1), (B) maximal change in serum LH (iU/L) (p=0.06, r^2=0.41) in healthy women (n=9) following a subcutaneous bolus of MVT602 at a dose of 0.03 nmol/kg. Correlation between maximal change in serum FSH (iU/L) following a single subcutaneous dose of MVT602 (0.03nmol/kg) and (C) baseline inhibin B levels (ng/L) (p=0.04, r^2 =0.49, equation: y=-0.04253*x + 5.365) (n=9), (D) baseline AMH (pmol/L) levels in healthy women (p=0.69, r^2=0.03) (n=7)

3.4.2.3 Effects of MVT602 on Blood Pressure and Heart Rate measurements

The mean systolic blood pressure (SBP) readings of the nine healthy women taking part in the study exhibited similar patterns irrespective of the intervention received (**Figure 3.7A**). The mean SBP remained stable throughout the first 10 hours of the studies. During the two 24-hour studies involving administration of MVT602, there was a fall in BP at ~16 hours after administration of the compound, corresponding to the time the study participants went to sleep. Mean SBP readings then went back to baseline levels at ~24 hours, at the time the study participants woke up. Mixed effects analysis of the data collected during the first ten hours of each study revealed no differences in measurements between the different interventions (P value = 0.86).

The mean diastolic blood pressure (DBP) measurements showed similar trends, with no obvious difference observed following different interventions **(Figure 3.7B)**. Once again, the measurements appeared stable during the first 10 hours of the studies, and decreases were observed approximately 16 hours following administration of MVT602. Diastolic blood pressure readings returned to normal at 24 hours as the participants started waking up. Mixed effects analysis of the data revealed no differences in mean diastolic blood pressure measurements between the different interventions (P value = 0.90).

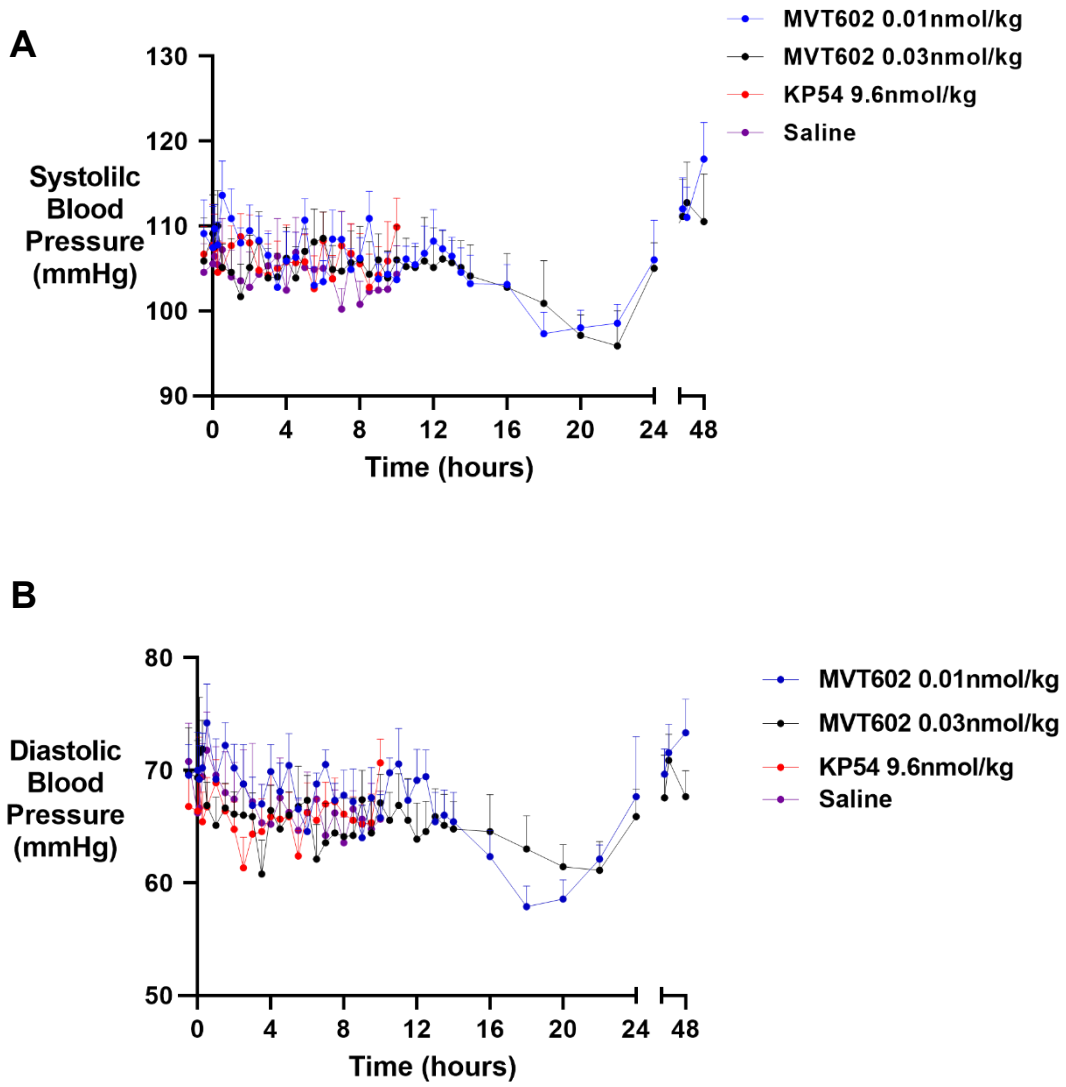


Figure 3.7 Blood pressure measurements during Phase 1

Mean ±SEM of (A) systolic blood pressure and (B) diastolic blood pressure readings following administration of MVT602 0.01 nmol/kg (in blue), MVT602 0.03 nmol/kg (in black), KP54 9.6nmol/kg (in red) and 0.9% saline (in purple) in healthy women (n=9). Groups were compared by mixed effects model (P= 0.86 for systolic BP, and P=0.90 for diastolic BP).

Similar trends were observed during analysis of the heart rate (HR) measurements of the nine participants **(Figure 3.8)**. Mean HR measurements were stable for the first 4 hours of the study before rising, during the participants' lunchtime meal. They returned to baseline levels by 10 hours. During the 24-hour studies that involved administration of MVT602, mean heart rate measurements reduced overnight, indicating that the participants were asleep. There were no significant differences in heart rate levels following analysis with a mixed effects model (P value 0.99).

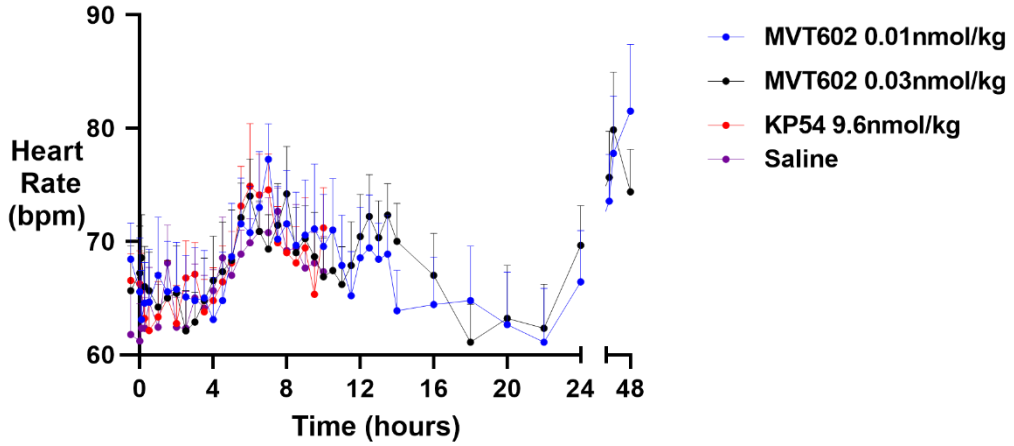


Figure 3.8: Heart rate measurements during Phase 1

Mean ±SEM of heart rate measurements following administration of MVT602 0.01 nmol/kg (in blue), MVT602 0.03 nmol/kg (in black), KP54 9.6nmol/kg (in red) and 0.9% saline (in purple) in healthy women (n=9). Groups were compared by mixed effects model (P=0.99).

3.4.3 Results of Phase 2

Administration of exogenous oestradiol increased baseline serum oestradiol levels, whilst it had the opposite effect on baseline gonadotrophin levels **(Figure 3.9B)**. During oestrogen supplementation, a single subcutaneous bolus of MVT602 0.03 nmol/kg resulted in a mean LH rise of 24.4 iU/L **(Figure 3.9B, Figure 3.10A)**. This was 2.7-fold higher than the amplitude of LH observed when the same dose was given without oestradiol supplementation in the same five women receiving the same dose of MVT602 (mean peak LH 7.5 iU/L) (P<0.0001) **(Figure 3.9B,Figure 3.10A)**. The initial depression in serum LH levels observed following MVT602 in Phase 1 was less pronounced in the oestradiol-treated cohort **(Figure 3.9 A&B, Figure 3.10A).** Maximal FSH-rise was also increased from a mean 1.7 iU/L without oestradiol to a mean 7.6 iU/L following oestradiol supplementation (P<0.0001) **(Figure 3.9A&B, 3.10B)**.

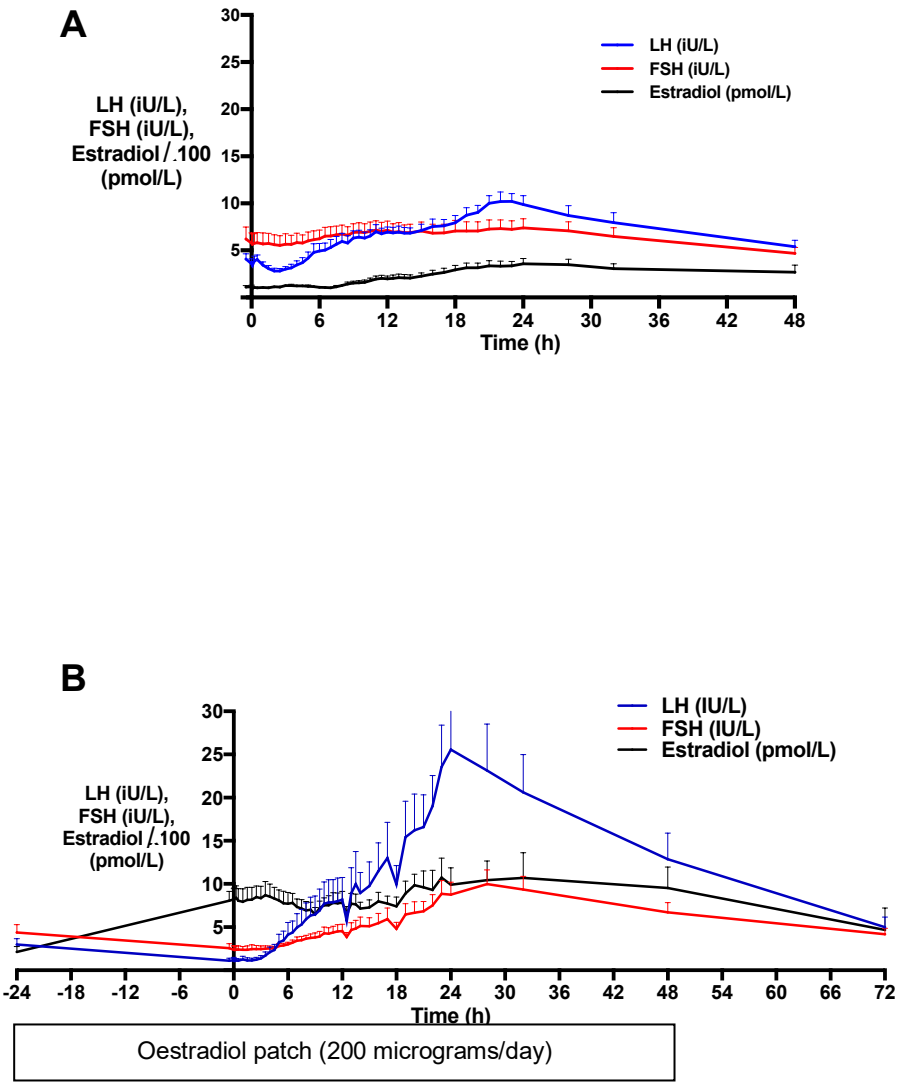


Figure 3.9 Serum gonadotrophin and oestradiol levels following MVT602 with and without Oestradiol supplementation

Mean±SEM of serum LH (iU/L) (in blue), serum FSH (iU/L) (in red), serum oestradiol (pmol/L) (in blue) over time (in hours) in healthy women (n=5) (A) without oestradiol supplementation (B) with oestradiol supplementation.

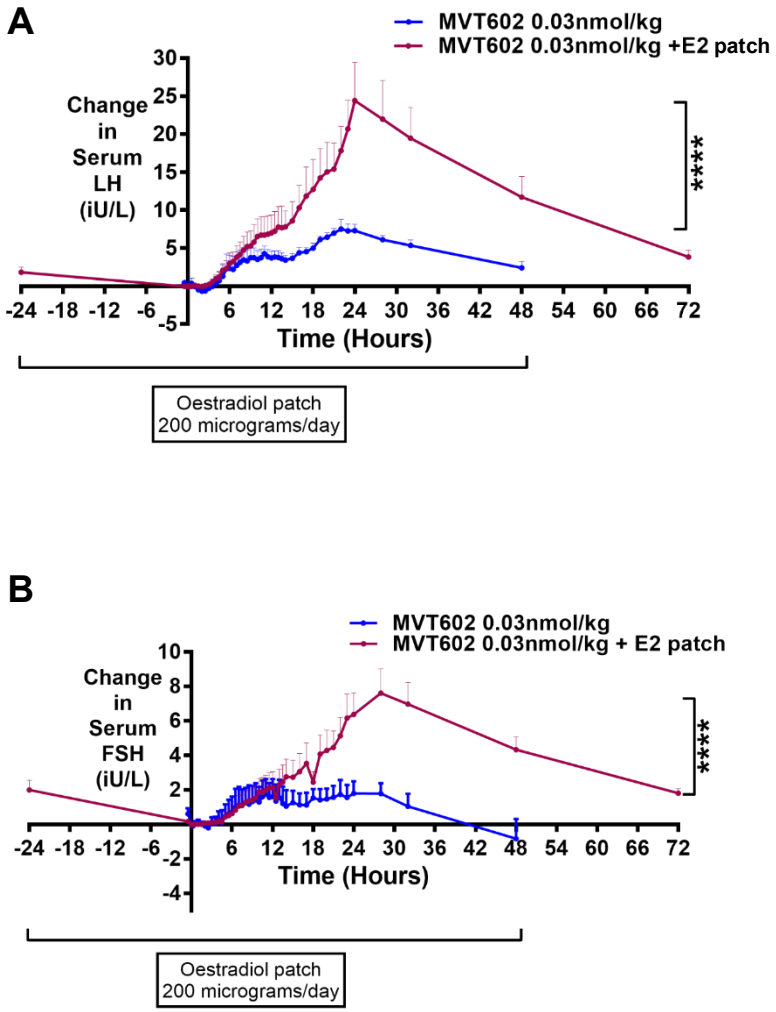


Figure 3.10 Change in serum LH and FSH following MVT602 with and without Oestradiol supplementation

Mean±SEM of change from baseline levels in (A) serum LH (iU/L) in healthy women (n=5) receiving a single subcutaneous bolus of MVT602 (0.03nmol/kg) with oestradiol supplementation (in maroon) and without oestradiol supplementation (in blue) for 72 hours, (B) serum FSH (iU/L) in healthy women (n=5) receiving a single subcutaneous bolus of MVT602 (0.03nmol/kg) with oestradiol supplementation (in maroon) and without oestradiol

supplementation (in blue). Groups were compared by two-way ANOVA with repeated measures. **** denotes P-value <0.0001.

3.4.4 Adverse Events

All participants tolerated KP54 and MVT602 well and no adverse events were reported during phases 1 or 2 of the study.

3.5 Discussion

Kisspeptin has emerged as a critical modulator of reproductive physiology and there is an increasing number of studies confirming that it can be used therapeutically to manipulate the HPG axis. This is the first study eliciting the effects of a novel kisspeptin receptor analogue, MVT602, on the gonadotrophin responses of healthy women. The LH responses elicited by MVT602 were of similar amplitude to those resulting from native KP54 but had an extended duration. The extension of the time to peak LH from 5.5 hours following KP54 to 24 hours following MVT602 greatly extended the area under the curve of LH exposure.

The two doses of MVT602 used in this study were selected following a preliminary dose-finding study in three healthy women conducted by our group in 2017. The study demonstrated that doses lower than 0.01nmol/kg resulted in less robust LH responses and doses higher than 0.03 nmol/kg had no additional effect on the magnitude of gonadotrophin responses. This might explain why the two doses of MVT602 did not result in a significantly different area under the curve of LH response. The dose of KP54 (9.6 nmol/kg) was previously established to be clinically effective to trigger oocyte maturation in the setting of *in Vitro* Fertilisation (Abbara et al. 2015). This dose of KP54 was chosen based on previous work by our lab, whereby a phase 2, multi-dose, adaptive design dose allocation clinical trial of women at high risk of developing OHSS during IVF showed that a dose of 9.6nmol/kg resulted in the highest pregnancy rates (Abbara et al. 2015). KP54 had endocrine profile that suggests that it could be more clinically efficacious compared to other shorter isoforms of kisspeptin (De Tassigny et al. 2017).

The amplitude of LH rise following MVT602 was similar to that observed following KP54, which is consistent with both peptides activating the same kisspeptin receptors expressed on GnRH neurons.

In keeping with previously published data, KP54 elicited a rise in both LH and FSH concentrations, with peak LH being observed at approximately 4-6 hours, whilst peak FSH occurred 1 hour later (Dhillo et al. 2005). Furthermore, our data is in line with previous research demonstrating that exogenous administration of KP54 has a greater stimulatory effect on LH than FSH (Dhillo et al. 2007). Indeed, whilst administration of MVT602 resulted in a higher area under the curve (AUC) of LH secretion (compared to KP54), it did not have a significantly greater AUC of FSH release than KP54. These findings are consistent with previously published data from animal studies (Navarro et al. 2005), healthy women (Dhillo et al. 2007) and healthy men (Dhillo et al. 2005; George et al. 2011). This pattern can be explained by the constitutive mode of FSH secretion, which is primarily basal and less reliant on GnRH pulsatility (Muyan, Ryzmkiewicz and Boime, 1994; Padmanabhan *et al.*, 1997).

However, even though the rise in serum FSH was less robust than the rise in LH, it was sufficient to produce significant increases in the levels of serum oestradiol following both doses of MVT602, that were significantly higher than the rise associated with KP54.

The LH profile elicited following MVT602 in the follicular phase of healthy women differed to that observed in healthy men, in whom the peak LH levels occurred earlier at 6 to 12 hours following administration (MacLean et al. 2014). In contrast to this profile, administration of MVT602 to healthy women resulted in an initial depression/plateau in serum LH during the first four hours, followed by a subsequent rise in LH. This plateau in LH could be due to a variety of reasons; firstly, it is possible that MVT602 may stimulate other G-protein coupled receptors (GPCR) whilst at the same time retaining equipotent activity to KISS1R. Neuropeptides with a common C-terminal RF amide motif (such as kisspeptin) can be divided into five families: Gonadotrophin-inhibitory hormone (GnIH), Neuropeptide FF (NPFF), Pyroglutamylated RF amide peptide (QRFP), Prolactin-releasing peptide (PrRP), and Kisspeptin (Elphick and Mirabeau 2014). These all share an Arg-Phe-NH_2 sequence at the

common carboxyl C-terminal (Lyubimov et al. 2010; Oishi et al. 2011; Quillet et al. 2016). Each family has its own cognate receptor; for example GnIH binds to its cognate receptor GPR147 (Ubuka et al. 2009) to inhibit GnRH stimulated gonadotrophin secretion (Tsutsui et al. 2000). However, cross-signalling amongst the RF amide peptides is well recognised. Indeed, there is evidence that kisspeptin can activate NPFF1R and NPFF2R expressed in cell lines (Elhabazi et al. 2013; Oishi et al. 2011) and cause an inhibitory effect on LH secretion (Murakami et al. 2008; Clarke et al. 2009; Kriegsfeld et al. 2010). NPFF receptors are expressed in the brain, including in areas rich in kisspeptin fibres (Poling et al. 2013). Furthermore, ~40-80% of GnRH neurons possess GnIH receptors in rodents (Kriegsfeld et al. 2005) and primates (Ubuka et al. 2009). *In vitro* work by Ubuka et al. also identified GnIH receptor (GPR147) mRNA expression in the human hypothalamus and pituitary (Ubuka et al. 2009). It is therefore possible that MVT602 can simultaneously activate multiple receptors within the same RF amide lineage. Moreover, it has been established that kisspeptin can activate the NPFFR and trigger neural firing in the absence of KISS1R (Liu and Herbison 2015). Undoubtedly, the interaction between Kisspeptin and RF amide receptors warrants further investigation, for example with experiments on Kiss1r knock out mice or involving the use of NPFFR receptor blockers (Kim et al. 2015).

Secondly, the LH responses observed following MVT602 could be explained by differences in intracellular signaling pathways. Previous work by Min et al on chinese hamster ovaries or GT1–7 cells expressing kiss1r demonstrated that KP10 stimulates a biphasic increase in intracellular calcium, characterised by an acute rapid rise in calcium levels lasting 5 minutes and followed by a more prolonged second phase lasting 30 minutes (Min et al. 2013).

Thirdly, it is possible that the LH profile observed was greatly influenced by circulating sex steroid milieu. It is well established that women are most responsive to the effects of kisspeptin during the pre-ovulatory phase of their menstrual cycle, when circulating oestradiol levels are

high (Chan et al. 2012; Dhillo et al. 2007). Indeed, Narayanaswamy et al. confirmed that gonadotrophin responses to kisspeptin administration in the follicular phase correlated with baseline oestradiol levels in healthy women (Narayanaswamy et al. 2016). Previous *in vitro* work on GnRH neuronal cell lines demonstrated that oestradiol augmented GnRH secretion after kisspeptin exposure (Novaira et al. 2009; Tonsfeldt et al. 2011). This hypothesis is further supported by our data from Phase 2 of the study, whereby oestradiol administration ameliorated the initial LH depression following administration of MVT602. This raises the possibility that a certain oestradiol threshold exists to stimulate the enhanced gonadotrophin responses observed. Nonetheless, we could not identify a clear association between baseline oestradiol levels in our cohort and peak gonadotrophin responses, in contrast to previously published work (Narayanaswamy et al. 2016). This might be due to a difference in the range of baseline oestradiol levels between the two studies: in our study all of our participants had a baseline oestradiol level no higher than 200 pmol/L and had their studies from day 1 to 4 of their menstrual cycle. In contrast, in Narayanaswamy's study the participants exhibited baseline oestradiol levels ranging from 90.3 pmol/L to 580.9 pmol/L (mean oestradiol 249 pmol/L) and their studies took place on days 2 to 6 of their menstrual cycles (Narayanaswamy et al. 2016).

MVT602 resulted in a progressive rise in serum oestradiol, with levels at 24 hours being higher than at baseline. Whilst this could be a result of kisspeptin's effects on gonadotrophin secretion, this might also be related to a direct effect of kisspeptin at the level of the ovary. There is evidence that kisspeptin and KISS1R are expressed in the human ovary (Roman et al. 2012) and that KP54 is able to augment ovarian gonadotrophin receptor gene expression in the setting of IVF treatment (Owens et al. 2018).

MVT602 did not have any effect on blood pressure or heart rate measurements in the nine participants studied. This is consistent with previous data from Nijher and colleagues

demonstrating that exogenous KP54 administration in healthy men and women was not associated with alterations in blood pressure or heart rate (Nijher et al. 2010).

In summary, this study investigated the endocrine effects of MVT602 in healthy women for the first time. Administration of MVT602 results in robust rises in serum gonadotrophins that are of similar amplitude to those elicited by KP54, but that occur much later at around 24 hours after drug administration. This extended duration of LH exposure can have huge therapeutic potential in the treatment of reproductive disorders in women.

3.5.1 Clinical significance of findings

Administration of MVT602 resulted in a serum LH amplitude that was comparable to that induced by KP54. Most importantly, the LH surge following MVT602 was longer than that observed with KP54 leading to a greater duration of gonadotrophin exposure following a single dose of the kisspeptin receptor analogue. A mid-cycle LH surge is prerequisite for oocyte maturation and induction of ovulation (Wallach et al. 1995) and a physiological LH surge lasts approximately 48 hours (Hoff, Quigley, and Yen 1983). Indeed, the LH profile observed in the MVT602 treated groups showed remarkable resemblance to the mid-cycle LH surge observed in physiological cycles.

This feature can prove advantageous when used in an IVF setting, where MVT602 could be used as a trigger of oocyte maturation. IVF is a widely used form of assisted reproduction technology, estimated to account for up to 1.5% of all children born in the United States in 2010 (Sunderam et al. 2013). It is a supraphysiological process that aims to stimulate all the physiological events that lead to successful conception: follicular development, oocyte maturation, ovulation, fertilisation, and implantation (Ali Abbara et al. 2018). However, IVF can be complicated by the dangerous complication of ‘ovarian hyperstimulation syndrome’

(OHSS) (Aboulghar et al. 2010). This is a serious and potentially life-threatening iatrogenic complication, characterised by hyper-enlarged ovaries, release of vascular endothelial growth factor (VEGF) and resultant increased vascular permeability (Whelan and Vlahos 2000). At least 16% of IVF cycles are complicated by moderate to severe OHSS (Toftager et al. 2016), which is predominantly caused by the use of human chorionic gonadotrophin (hCG) causing non-physiological ovarian stimulation. This has triggered a search for more physiologic therapies to induce oocyte maturation during IVF treatment, associated with a safer side-effect profile.

Previous work from our group has demonstrated that KP54 can be a safe and effective trigger of oocyte maturation in IVF cycles, even in women at high risk for OHSS (Jayasena et al. 2014; Abbara et al. 2015). A single subcutaneous dose of KP54 resulted in a mean stimulated LH level of approximately 40 iU/L, lasting for 12 to 14 hrs. The group treated with KP54 had a 33.6-fold reduced odds ratio for OHSS compared to women receiving hCG (Abbara et al. 2015; Abbara et al. 2018). A follow up study investigating the effect of two doses of KP54 administered 10 hours apart in women at high risk of developing OHSS showed an extended LH surge with improved oocyte maturation and no evidence of OHSS (Abbara et al. 2018). In addition, KP54 has been found to decrease oestradiol-driven VEGF production without affecting ovulation (Cerrillo et al. 2009; Zhai et al. 2017)

It has been known for some time that a longer duration of a low-level LH exposure is more efficacious in ovulation induction than achieving higher concentrations of serum LH for a shorter duration of time (Ishikawa et al.1992). This suggests that there might be a certain threshold above which LH initiates ovulation and the duration of exposure above this threshold is more critical for inducing ovulation and supporting luteal (Ishikawa et al. 1992). In a series of experiments involving female macaques receiving either a GnRH agonist or hCG in gonadotrophin stimulated cycles, an LH exposure lasting between 14 and 48 hours was

needed to induce adequate oocyte maturation, ovulation and corpora lutea development (Chandrasekher et al. 1991). Thus, the longer duration of gonadotrophin exposure could improve oocyte maturation, and potentially pregnancy rates, whilst also reducing the risk of developing OHSS. Consequently, it might prove even more efficacious than native KP54 in ovulation induction protocols.

Furthermore, MVT602 is a promising novel agent for the treatment of women with reproductive disorders. Its longer duration of action allows for more infrequent administration and thus reducing the risk of tachyphylaxis, suggesting that it has great translational potential in the treatment of anovulatory disorders. Groups of women who could benefit from this are women with hypothalamic amenorrhoea, anovulatory polycystic ovarian syndrome and hyperprolactinaemia.

3.5.2 Study limitations

MVT602 had not been previously studied in women, thus the observational study design used in this study was appropriate. Consequently, as the effect size of MVT602 in women was not known, a power calculation could not be carried out and thus a small number of participants was recruited. Our recruitment target number was based on previous proof of concept studies involving kisspeptin (Dhillo et al. 2005; Jayasena et al. 2009) and was increased in anticipation of high drop-out relating to the long duration of the study visits. Given the small sample size, there was considerable variation in reproductive hormone responses amongst the participants, raising the question of the generalisability of the results. A more comprehensive study with a power calculation determining the sample size, is required to confirm response reproducibility.

The gonadotrophin responses observed in the current study occurred in the context of minimal circulating sex steroid exposure. Nonetheless, oestrogen supplementation in phase 2 of the study showed promising results and allows speculation that gonadotrophin sensitivity to MVT602 will be higher during the pre-ovulatory phase of the cycle. Indeed, further work including the administration of MVT602 at various phases of the menstrual cycle and the administration of MVT602 in an IVF setting will enable us to make more reliable observations with greater translational value.

3.5.3 Conclusion and future work

This is the first study to investigate the effects of MVT602, a novel kisspeptin receptor analogue, on the gonadotrophin responses during the follicular phase of healthy women. It resulted in LH responses with a similar amplitude to those observed following KP54. However, due to its longer duration of action the resultant LH profile closely resembles the physiological LH surge occurring during the pre-ovulatory phase of the menstrual cycle. These attributes make MVT602 a promising therapeutic agent that could transform the treatment of female reproductive disorders.

The findings of this study are extremely pertinent when considering MVT602's potential as a therapeutic agent for the treatment of anovulatory disorders. The development of chronic protocols of administration of MVT602 would enable the treatment of women with reproductive disorders. The therapeutic potential of KP54 and KP10 have successfully been explored in disorders such as Hypothalamic Amenorrhoea (Jayasena et al. 2014) and hyperprolactinaemia (Millar et al. 2017). Jayasena *et al.* has eloquently demonstrated that kisspeptin results in augmented gonadotrophin responses in women with HA (Jayasena et al. 2014). However, subsequent studies exploring the efficacy of regular kisspeptin administration

for ovulation induction were hindered by the development of tachyphylaxis. Thus, the longer half-life of MVT602 makes it an ideal agent to investigate in women with Hypothalamic Amenorrhoea. Likewise, the prolonged LH profile associated with MVT602 which closely resembles the mid-cycle LH surge, suggests that it has huge translational potential in ovulation induction protocols. Head to head studies comparing the effects of MVT602, KP54 and current gold standard IVF protocols are warranted to confidently evaluate its translational potential in this therapeutic setting.

Chapter 4

The effect of Kisspeptin-54 and its receptor agonist, MVT602, on gonadotrophin responses in women with Polycystic Ovarian Syndrome, and women with Hypothalamic Amenorrhoea

CHAPTER 4: THE EFFECT OF KISSPEPTIN-54 AND ITS RECEPTOR AGONIST, MVT602, ON GONADOTROPHIN RESPONSES IN WOMEN WITH POLYCYSTIC OVARIAN SYNDROME AND WOMEN WITH HYPOTHALAMIC AMENORRHOEA

4.1 Introduction

4.1.1 Subfertility

Subfertility, defined as 'unwanted non-conception' after twelve months' of regular unprotected sexual intercourse, affects between 10 to 15% of couples (Evers et al. 2002). It does not reflect an absolute state of sterility but rather the likelihood of achieving conception over time (Farquhar et al. 2019; Thurston et al. 2019). The World Health Organisation (WHO) recognizes it as a global public health concern (Boivin et al. 2007) and has identified it as the fifth most serious global disability in the world. In the UK, the commonest causes of subfertility after 'male factors' that are responsible for 30% of the cases, are ovulatory dysfunction (25%), tubal disorders (20%) and uterine or peritoneal pathologies (10%) (National Institute for Health and Care Excellence 2013).

After the exclusion of pregnancy, anovulatory disorders such as polycystic ovarian syndrome (PCOS) and hypothalamic amenorrhoea (HA) are the two commonest causes of secondary amenorrhoea (National Institute for Health and Care Excellence, 2013; Thurston et al. 2019). The prevalence of secondary amenorrhea is 3-5% in the general population, but this can be as high as 69% in athletes (Meczekalski et al. 2014). Menstrual disturbance (such as oligomenorrhoea or amenorrhoea) is often indicative of oligo / anovulation (Burgers et al. 2010). Due to variability in menstrual cycle lengths between individuals, oligomenorrhoea is defined as the frequency of 4-9 cycles per year (Soumpasis, Grace, and Johnson 2020). Amenorrhoea is defined as absent menses for at least 3 months (Gordon et al. 2017a), or the

presence of ≤ 3 menstrual cycles per year (Practice and Medicine 2008). In women with oligomenorrhoea, 80-90% had PCOS, whereas in those with amenorrhoea 40% had PCOS (Teede, Deeks, and Moran 2010).

4.1.2 Polycystic Ovarian Syndrome

Polycystic Ovarian Syndrome (PCOS) is a heterogeneous endocrine disorder characterised by hyperandrogenism, menstrual irregularity and polycystic ovarian morphology on ultrasound (Ndefo, Eaton, and Green 2013). It is diagnosed in the presence of at least two of the following three features: oligo/amenorrhoea (cycle length > 35 days), polycystic ovarian morphology on ultrasound (PCOM), and clinical or biochemical hyperandrogenism (Fauser et al. 2004; Teede et al. 2018). The morphological appearance of PCO ovaries comprises of a central stroma surrounded by peripherally located follicles (Zhu et al. 2016). The most recent international guideline on the diagnosis of PCOS revised the 2003 Rotterdam criteria by increasing the number of follicles per ovary required to define PCOM (from twelve to twenty per ovary), reflecting improvements in ultrasonographic resolution technology (Teede et al. 2018). In 184 women with PCOS and hyperandrogenism, 35% were oligomenorrheic and 41% were amenorrhoeic (Cupisti et al. 2007). The diagnosis of PCOS remains a diagnosis of exclusion of other causes of menstrual irregularity (Teede et al. 2018).

PCOS affects 6–15% of reproductive age women (Fauser et al. 2012) and is the commonest cause of WHO Group II ovulation disorder (dysfunction of the hypothalamic-pituitary-ovarian axis) (Centre n.d.). The pathophysiology of PCOS involves dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis (Diamanti-kandarakis et al. 2006). Women with PCOS have increased GnRH pulsatility that results in preferential secretion of LH to FSH and a raised LH to FSH ratio (Azziz et al. 2016). The data regarding circulating kisspeptin levels in women with PCOS is discrepant (Tang, Ding, and Zhu 2019), likely reflecting current limitations in

measuring plasma kisspeptin at the low levels that are associated in non-pregnant women. Some studies have demonstrated increased circulating levels of plasma kisspeptin in women with PCOS (Chen et al. 2010; Gorkem et al. 2018; Jeon et al. 2013; Yilmaz et al. 2014). Others, including the study investigating the largest number of women (n=250), have found no differences in plasma kisspeptin levels compared to women with regular cycles (Albalawi et al. 2018; Daghestani et al. 2018; Emekci Ozay et al. 2016). Furthermore, the first study to investigate this found reduced circulating kisspeptin levels compared to healthy controls (Panidis et al. 2006). It is also important to note that the discrepancy in these data might also stem from the differences in study design, with some studies recruiting lean PCOS women, whilst others recruiting obese PCOS women. It is well established that kisspeptin levels are negatively correlated with increasing BMI in women (Kołodziejski et al. 2018). Animal models of PCOS also provide conflicting evidence on the regulation of the *Kiss1* system: hypothalamic *Kiss1* mRNA and kisspeptin immunoreactivity are reduced in a dihydrotestosterone-induced PCOS model, whereas the *Kiss1* receptor gene is upregulated in the oestradiol-induced model (Brown et al. 2012; Giannocco et al. 2017).

The effect of exogenous kisspeptin administration on the reproductive hormone secretion of women with PCOS has not been studied before. It would therefore be informative to investigate the role of exogenous kisspeptin administration on the HPG axis in this cohort to identify distinct patterns of gonadotrophin secretion that could be used for diagnostic or therapeutic purposes. Women with PCOS have a higher risk of developing Ovarian Hyperstimulation Syndrome during In Vitro Fertilisation (IVF) and Intracytoplasmic Sperm Injection (ICSI) (Macdougall et al. 1993), prompting exploration of different ovulation induction protocols, such as the use of a GnRH antagonist IVF protocol (Lambalk et al. 2017). Thus, an agent such as MVT602, which shows promising potential as an ovulation induction agent, could be particularly advantageous in a cohort at risk of OHSS.

4.1.3 Hypothalamic amenorrhoea

Hypothalamic amenorrhea (HA) is an anovulatory disorder associated with reduced GnRH pulsatility, low circulating sex-steroid levels and low leptin levels (Jayasena et al. 2013). Traditionally HA was thought to be associated with reduced GnRH pulsatility and Loucks and colleagues have demonstrated that acute reduction of energy availability to below 30 kcal/kg can disrupt LH pulsatility (Loucks, Kiens, and Wright 2011). However, it is now recognised that women with HA might have intact GnRH pulsatility (Perkins et al.1999). In a rodent model of HA, hypothalamic *Kiss1* expression was reduced, whilst kisspeptin receptor mRNA expression was increased (Castellano et al. 2005).

Our group has investigated kisspeptin's role in the treatment of women with HA in a series of studies. Jayasena et al. demonstrated that 8-hourly infusions of KP54 at various doses not only increased basal and pulsatile LH secretion in women with HA, but also induced a 3-fold increase in LH pulse frequency (Jayasena et al. 2013). Furthermore, women with HA exhibited exaggerated gonadotrophin responses to a subcutaneous bolus of KP54 compared to healthy women in the follicular phase (Jayasena et al. 2010).

However, daily subcutaneous administration of KP54 at a dose of 6.4nmol/kg in women with HA resulted in tachyphylaxis (Jayasena et al. 2009). Receptor desensitization has also been observed following repeated kisspeptin administration in animals (Seminara et al. 2006; Thompson et al. 2006; Ramaswamy et al. 2007). In women with HA, when the frequency of administration of KP54 was reduced to twice a week over a period of eight week there was some restoration of gonadotrophin secretion (C N Jayasena et al. 2009). This study better informed our understanding of the potential mechanism of kisspeptin receptor desensitisation in women with HA, and the data support the view that further reductions in the frequency of administration could potentially ameliorate the risk of tachyphylaxis (Jayasena et al. 2009).

In summary, kisspeptin-based therapies have the potential to be useful in the treatment of women with HA, albeit with careful attention to both the dose used and the length of exposure, such that GnRH responsiveness is optimised. Thus, a longer acting kisspeptin analogue such as MVT602, would be an ideal candidate for restoring physiological gonadotrophin secretion whilst also reducing the risk of tachyphylaxis.

Kisspeptin's ability to influence pulsatile GnRH secretion could be pharmacologically advantageous in the treatment of disorders characterised by abnormal GnRH pulsatility, such as HA. Furthermore, MVT602 with its LH-surge like gonadotrophin profile and its more infrequent administration afforded by its longer duration of action appears to be an ideal candidate to investigate in women with anovulatory disorders. Whilst these two conditions have very distinct pathophysiological features, it is now increasingly recognisable that these they can coexist, thus making the diagnosis and management of these patients challenging. In this final study of my research project I investigated the patterns of gonadotrophin secretion to KP54 between women with HA, women with PCOS and healthy women. I also studied the gonadotrophin and sex hormone profiles elicited after a subcutaneous bolus of MVT602 in women with these two anovulatory disorders. Finally, I compared their responses to those elicited in healthy women in the follicular phase (presented in Chapter 3), in an attempt to delineate differences that could aid in the diagnostic or therapeutic management of these patients.

4.2 Aims and Hypotheses

4.2.1 Aims

(1) To characterise the effects of MVT602 on gonadotrophin secretion during the follicular phase of women with PCOS and in women with HA.

(2) To compare these effects to those elicited following MVT602 administration during the follicular phase of healthy women.

(3) To compare the endocrine profiles following MVT602 to those elicited following KP54 (9.6 nmol/kg), and 0.9% saline.

(4) To investigate what effect oestrogen supplementation has on the gonadotrophin responses following MVT602 in women with PCOS and women with HA.

(5) To delineate MVT602's effects on the blood pressure (BP) and heart rate (HR) of women with PCOS and women with HA.

4.2.2 Hypothesis

- MVT602 will result in an exaggerated gonadotrophin response in women with HA.
- MVT602 administration to women with PCOS will result in gonadotrophin responses similar to those observed in healthy women.
- Women with HA and women with PCOS will exhibit a comparable LH amplitude but will have a more prolonged duration of action following administration of MVT602 compared to KP54.
- MVT602 will not influence the BP or HR parameters of women with HA and PCOS.

4.3 Methodology

4.3.1 Study Overview

I conducted a two-phase, prospective, randomised, cross-over study to determine the effects of MVT602 on the gonadotrophin responses of women with HA and PCOS:

- Phase 1: Randomised, cross-over physiological study comparing MVT602 to KP54, and placebo.
- Phase 2: Determination of the effects of oestradiol supplementation on the gonadotrophin response of women with HA and PCOS to MVT602.

4.3.2 Ethical approval

The study was conducted in accordance with the Declaration of Helsinki, after ethical approval was granted by the West London Research Ethics committee (Ref: 12/LO/0507). All participants provided full written consent.

4.3.3 Subjects

Six women with HA and six women with PCOS were recruited via newspaper advertisements in the local press or Imperial College Healthcare NHS Trust outpatient departments. Interested women were provided with an ethically approved Participant Information Sheet prior to their screening appointment. Eligibility was confirmed following a detailed medical, endocrine and menstrual history, clinical examination, electrocardiography and blood tests including an anterior pituitary panel and a serum βhCG. The inclusion criteria for women with HA were based on American Endocrine Society guidelines (Gordon et al. 2017a) and included: women aged 18-35 years, with a history of secondary amenorrhoea of at least 6 months' of duration, in the presence of either significant weight loss, or vigorous exercise or emotional stress, BMI

of 18-30kg/m^2,with low serum LH and normal or low serum FSH levels and normal MRI of the pituitary gland. Diagnostic criteria for the PCOS group were in accordance to the 2018 International PCOS guideline (Teede et al. 2018), namely: women aged 18-35 years with a history of secondary amenorrhoea or oligomenorrhoea (defined as 3-8 menstrual cycles per year), polycystic ovarian morphology on US scan, with the presence of >20 antral follicles per ovary, and the presence of hyperandrogenism. Women were considered to have clinical hyperandrogenism if they scored >5 on the Ferriman-Gallwey hirsutism scale, whereas biochemical hyperandrogenism was defined as a Total Testosterone level of >0.7 nmol/L or Androstenedione of >2.2 ng/m. Women were excluded if they had any other clinically significant past medical history, were on regular or over the counter medication (including hormonal contraception), had a positive pregnancy test, were wishing to seek fertility within two months of completion of the study, had abnormal laboratory test results, or if they had a history of smoking, excessive alcohol use or illicit drug use. Written informed consent was obtained from all study participants. Participants were advised against conception during the study period and for at least two months after the completion of their final visit. Study participants also agreed to use barrier contraception during the study period.

4.3.4 Study Phase Overview

4.3.4.1 Overview of Phase 1

As previously discussed, women are most resistant to the effects of KP54 during the follicular phase of their menstrual cycle. Thus, the studies for women with PCOS were conducted during the follicular phase of their menstrual cycle, when more reliable observations of the effects of kisspeptin on gonadotrophins can be made. All bar one of the PCOS participants had evidence of amenorrhoea and were given a progesterone challenge to induce a withdrawal bleed

(Medroxyprogesterone 10mg twice daily for seven days). In these five women studies were performed during days 1-4 of the withdrawal bleed. One of the participants with PCOS had evidence of oligomenorrhoea (menstrual cycle length of 42 days) and her studies were thus conducted during days 1-4 of the follicular phase of the menstrual cycle. All of the participants in the HA group had secondary amenorrhoea and their studies were conducted once every month, without the induction of a withdrawal bleed. All 12 study participants attended for four study visits, during which they received a subcutaneous injection of:

- MVT602: 0.03 nmol/kg
- KP54 9.6 nmol/kg
- NaCl 0.9%

All study interventions were administered to all participants, in random order. The randomisation was performed with the use of an online randomisation platform (random.org).

4.3.4.2 Overview of Phase 2

This study investigated the effects of circulating sex steroids on the gonadotrophin responses to MVT602, given our prior knowledge of maximal responsiveness to kisspeptin during the pre-ovulatory phase of the menstrual cycle (Dhillo et al. 2007). During this phase of the study, two women with PCOS and two women with HA were given MVT602 following oestradiol supplementation. An oestradiol patch was applied on Day 1 of the menstrual cycle/withdrawal bleed in the PCOS group and at least 1 month following the previous studies in the HA group. Participants then returned for a study visit 24 hours after application of the patch. They all received MVT602 at a dose of 0.03 nmol/kg and followed the same study protocol as in Phase 1. The oestradiol patch was removed 72 hours later.

4.3.5 Study Design

All study visits took place at the Clinical Research Unit of Charing Cross Hospital (Imperial College Healthcare NHS Trust). For women with PCOS, the studies were conducted during the early follicular phase of their menstrual cycle/withdrawal bleed. All participants with HA received saline (NaCl 0.9%) as their first intervention to ensure absent LH pulsatility, before returning for subsequent study visits.

4.3.5.1 Phase 1:

Description**:** Prospective, randomised, cross-over study where each participant attended for one study visit per menstrual cycle/month. All study participants completed three study visits over consecutive months/cycles, where one intervention was administered randomly.

Sample size: six women with PCOS and six women with HA were recruited to this phase of the study. The sample size was selected based on data from previous kisspeptin studies with similar designs (Jayasena et al. 2009).

Study schedule: Similar to the studies in healthy women, the study schedule differed according to the intervention being administered.

Schedule for KP54 and NaCl study visits:

Pre-visit: Participants with PCOS inform the research team of menstrual/withdrawal bleeding. Participants with HA schedule study visits at least 4 weeks after the completion of the previous

study visits. Participant asked to refrain from strenuous exercise, caffeine, and alcohol consumption for the 24 hours prior to the start of the study.

Study Day 1 (SD1):

a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling
b. Blood sampling for serum reproductive hormone level (LH, FSH, oestradiol and progesterone) measurement done at timepoints -30, 0, 5, 15 minutes post injection and then every 30 minutes for 10 hours. Blood sampling for KP54 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.
c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for 10 hours.

5. SD 2: Blood sample at 24 hours post drug administration
6. SD 8: Telephone review for any symptoms occurred 7 days post drug administration.

Schedule for MVT602 studies:

Pre-visit: Participants with PCOS inform the research team of menstrual/withdrawal bleeding. Participants with HA schedule study visits at least 4 weeks after the completion of the previous study visits. Participants are advised to refrain from strenuous exercise and against consuming caffeine or alcohol for the 24 hours prior to the study visit.

Study Day 1 (SD1):

a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling.
b. Blood sampling for serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) takes place at timepoints -30, 0, 5, 15 minutes post injection, then every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours. Blood sampling for MVT602 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.
c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours

6. SD 2: blood samples at 28, and 32 hours post drug administration
7. SD 3: blood sample at 48 hours post drug administration
8. SD 8: telephone review of study participant for any new symptoms, conducted 7 days after drug administration.

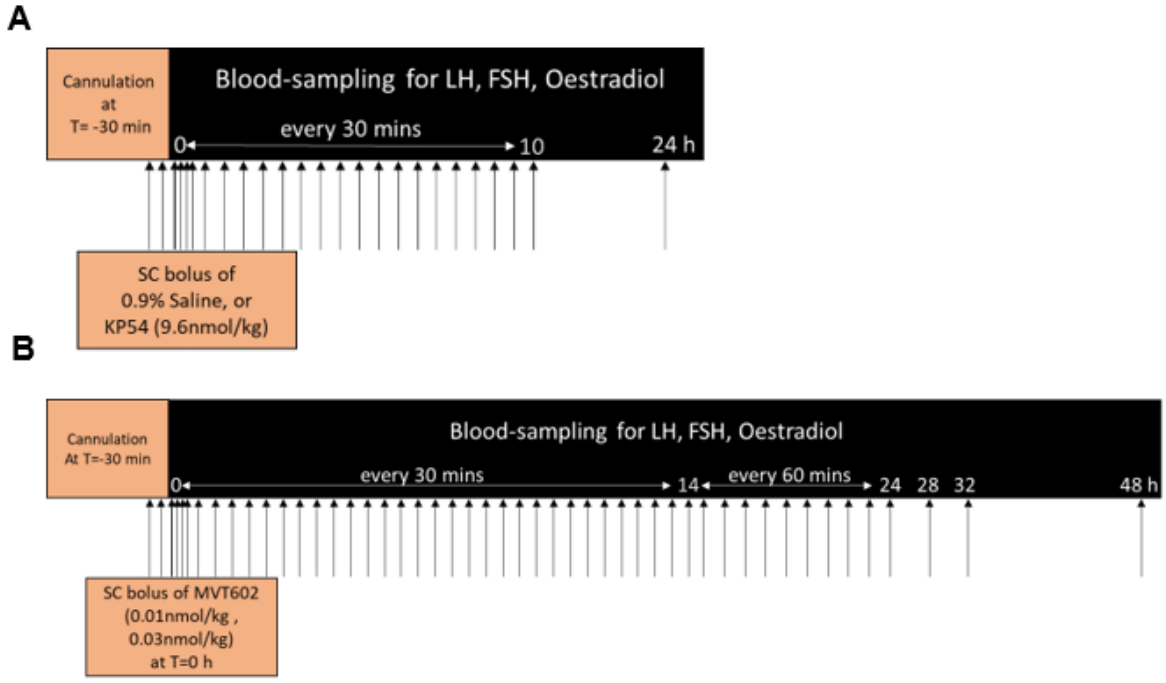


Figure 4.1: Diagram of the protocol for Phase 1

Women with PCOS (n=6) and women with HA (n=6) were admitted to the Clinical Research Facility at 8 am on the morning of each study visit. An intravenous cannula was inserted into one antecubital fossa and blood was sampled at T -30 min, T -15 min and T = 0 h prior to administration of each intervention to determine the basal hormonal values. A subcutaneous (SC) bolus of (A) KP54 or 0.9% saline or (B) of MVT602 was administered at T = 0 h.

Figure 4.1A- Study protocol diagram for the KP54 and 0.9% saline visits. After a SC bolus of KP54 (9.6 nmol/kg) or 0.9% saline at T = 0 h, serum hormone levels (LH, FSH, oestradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 10 h, and additionally at 24 h.

Figure 4.1B- Study protocol diagram for MVT602 visits. After a SC bolus of MVT602 (0.03 nmol/kg) was administered at T = 0 h, serum hormone levels (LH, FSH, oestradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 14 h, and then every 60 min until 24 h and additionally at 28, 32 and 48 h.

4.3.5.2 Phase 2:

Description**:** Prospective, open label study of MVT602 administration (0.03 nmol/kg) with concurrent oestradiol supplementation (topical oestradiol patch, 200μg/day).

Sample size: two women with PCOS and two women with HA who completed Phase 1 were invited to take part in Phase 2.

Study schedule:

Pre-visit: Participants with PCOS inform research team of menstrual/withdrawal bleeding. Participants with HA scheduled study visit to occur at least 4 weeks after a previous study visit. Participants asked to refrain from strenuous exercise, caffeine and alcohol consumption for the 24 hours prior to the start of the study. Participant attended the research unit for application of oestradiol patch and a baseline blood test.

Study Day 1 (SD1):

a. Urinary pregnancy test and weight documented on arrival. Insertion of cannula for blood sampling
b. Serum reproductive hormone levels (LH, FSH, oestradiol and progesterone) were measured at -30, 0, 5, 15 minutes post injection, then every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours. Blood sampling for MVT602 levels was also done at 0, 5, 15, 30, 60, 120, 150, 240, and 360 minutes post injection.
c. Physiological parameters (blood pressure and pulse rate) monitored every 30 minutes for the first 14 hours and then hourly from 14 to 24 hours.

7. SD 2: blood samples at 28, and 32 hours post drug administration

8. SD 3: blood sample at 48 hours post drug administration. Oestradiol patch removed.
9. SD 4: blood sample at 72 hours post drug administration
10. SD 8: telephone review for any new symptoms taking place 7 days post drug administration.

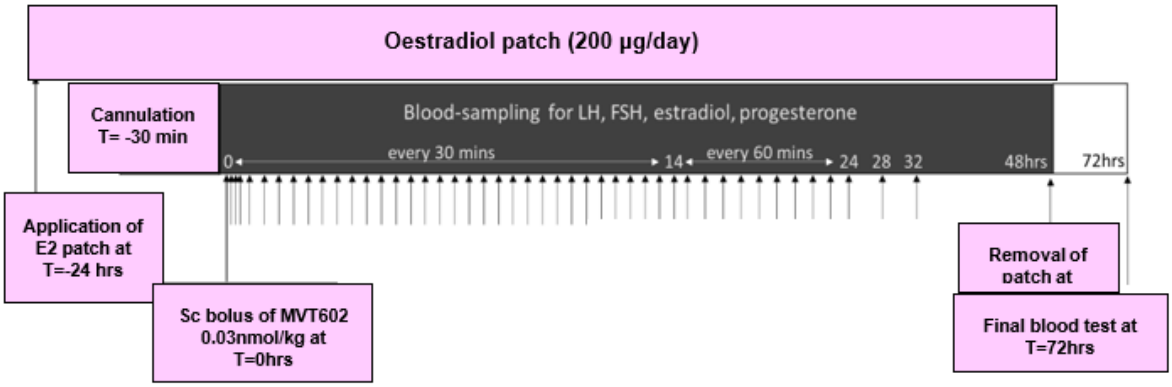


Figure 4.2: Diagram of protocol used for Phase 2

Women with PCOS (n=2) and women with HA (n=2) attended the Clinical Research Facility 24 h prior to the start of the study for application of the oestradiol patch (200mcg/day). They were then admitted to the same unit at 8 am on the morning of the study visit. An intravenous cannula was inserted into one antecubital fossa and blood was sampled at T -30 min, T -15 min and T = 0 h prior to administration of each intervention to determine the basal hormonal values. A subcutaneous (SC) bolus of MVT602 was administered at T = 0 h. Serum hormone levels (LH, FSH, oestradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 14 h, and then every 60 min until 24 h and additionally at 28, 32, 48 and 72 h. The patch was removed at T=48 h.

4.3.6 Peptides

MVT602 was synthesized by Myovant Sciences Ltd. (Virginia, USA), whilst human KP54 was synthesised by Bachem (Liverpool, UK). Peptide bioactivity and animal toxicology testing were confirmed following a negative Limulus amebocyte lysate assay test for pyrogen (Associates of Cape Cod, Liverpool) and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital). Both peptides were prepared in accordance with the Good Manufacturing Practice.

4.3.7 Hormone Assays

Blood samples were analysed as previously described in Section 3.3.7.

4.3.8 Statistical analysis

Analyses were conducted using Graphpad Prism version 8.3. Parametrically distributed data are presented as mean±SD, whereas non-parametric data are presented as median (interquartile range). Statistical comparison across intervention groups was 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. Binary data were compared using 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. The correlations between variables were assessed using Pearman correlation analysis for parametric distribution and Spearman rank correlations for non-parametric distribution.

4.4 Results

4.4.1 Baseline Characteristics

Following screening twelve women were recruited to the study, six with a diagnosis of PCOS and six with a diagnosis of HA. The HA group had a median age of 25 (23, 30.8) years and a median BMI of 20.7(19.4, 23.1) kg/m^2. The PCOS group had a median age of 24.5 (21, 26.3) years and a median BMI of 23.2(18.4,25.1) kg/m^2. The baseline demographic characteristics and reproductive hormone profiles of the study participants are displayed in **Table 4.1**. When the two groups were considered with the nine healthy eumenorrheic women (in the follicular phase), they did not have any significant differences in age, weight, body mass index, baseline gonadotrophin or oestradiol levels **(Table 4.1)**. Women with HA and with PCOS had longer menstrual cycle lengths than healthy women. Serum sex hormone binding globulin (SHBG) was lower in women with PCOS, whilst serum anti-Mullërian hormone (AMH) levels were higher in this group.

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 1.1 Baseline characteristics of the three groups

Mean±SD is used for normally distributed values and median (interquartile centile) is presented for non-parametric values. Kruskal-Wallis Dunn's multiple comparison test was used for comparisons between the different groups.

4.4.2 Results of Phase 1

4.4.2.1 Gonadotrophin responses following KP54

Following a single dose of KP54, the mean maximal rise in serum LH in healthy women was around 8 iU/L, whereas the peak change in serum LH in women with HA and with PCOS was significantly greater, at around 15 iU/L. Furthermore, the time to peak serum LH levels following administration of KP54 was similar in the three groups (around 5.5 hours) **(Figure 4.3A).** However, despite the increased gonadotrophin responses seen in the HA and PCOS groups, the area under the curve for change in serum LH was not different between the groups **(Figure 4.4A)**.

The FSH response in women with PCOS was not different to that observed in healthy women during the follicular phase. However, the rise in serum FSH was more exaggerated in women with HA (P-value=0.04) **(Figure 4.3B)**, but once again this did not reach significance **(Figure 4.4B)**. Whilst KP54 resulted in a higher mean serum oestradiol level in women with HA **(Figure 4.3 C)**, there was no significance between the groups on two-way repeated measures ANOVA analysis **(Figure 4.4C)**.

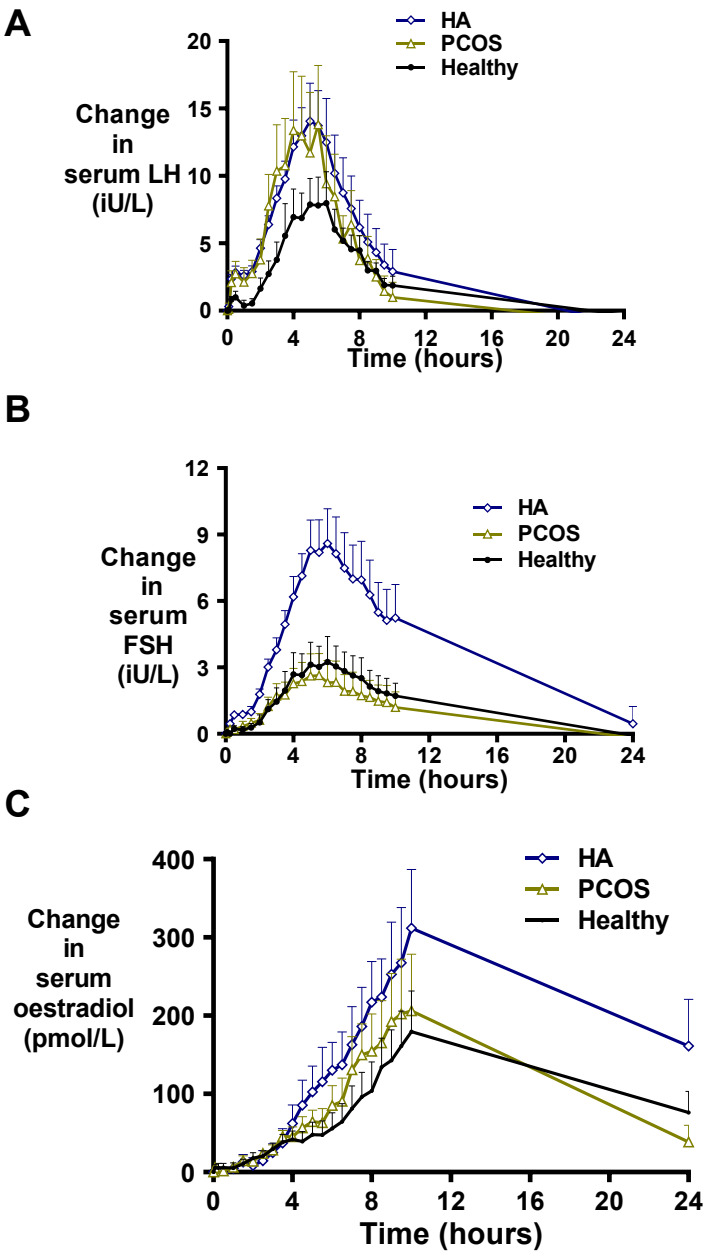


Figure 4.3 Change in gonadotrophins and oestradiol following administration of KP54 in the three groups

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 (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue, following a subcutaneous bolus of KP54 (9.6nmol/kg). Comparison between the groups were made by two-way ANOVA with repeated measures.

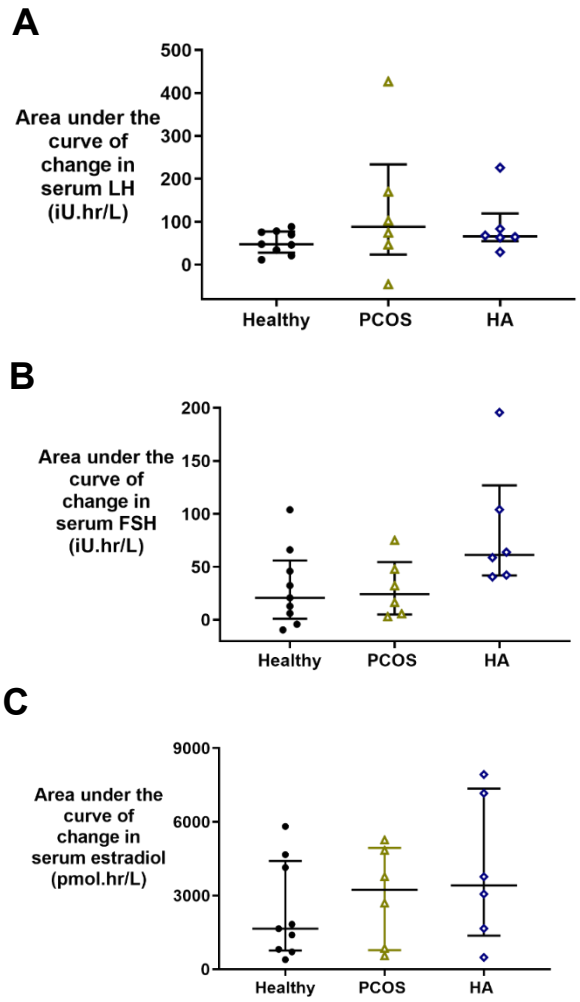


Figure 4.4: Maximal change in serum gonadotrophins and oestradiol following administration of KP54 in the three groups

Scatter diagram (Median±IQR) of maximum change in serum (A) LH (iU/L), (B) FSH (iU/L), (C) oestradiol (pmol/L) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue, following a subcutaneous bolus of KP54 (9.6nmol/kg). Comparison between the groups was made by Kruskal-Wallis Dunn's multiple comparison test.

4.4.2.2 Gonadotrophin responses following MVT602

Following administration of MVT602, the change in serum LH was similar in healthy women and women with PCOS. However, administration of MVT602 in women with HA resulted in a higher LH rise. This also occurred sooner, such that the change in serum LH in women with HA was more than 2-fold higher at 6 hours, when compared to healthy women and women with PCOS **(Figure 4.5A)**. Women with HA also exhibited a second, smaller peak in serum LH levels at around 24 hours, which coincided with the first LH peak of the other two groups **(Figure 4.5A)**. Women with HA reached the LH peak significantly sooner compared to healthy women (**Figure 4.7**).

Women with HA exhibited a dramatic rise in serum FSH levels following MVT602, which was 4-fold higher than the maximal FSH rise observed in the other two groups. This resulted in a significantly greater area under the curve for FSH release **(Figure 4.6B)** and subsequently led to increased serum oestradiol levels **(Figure 4.5C)**.

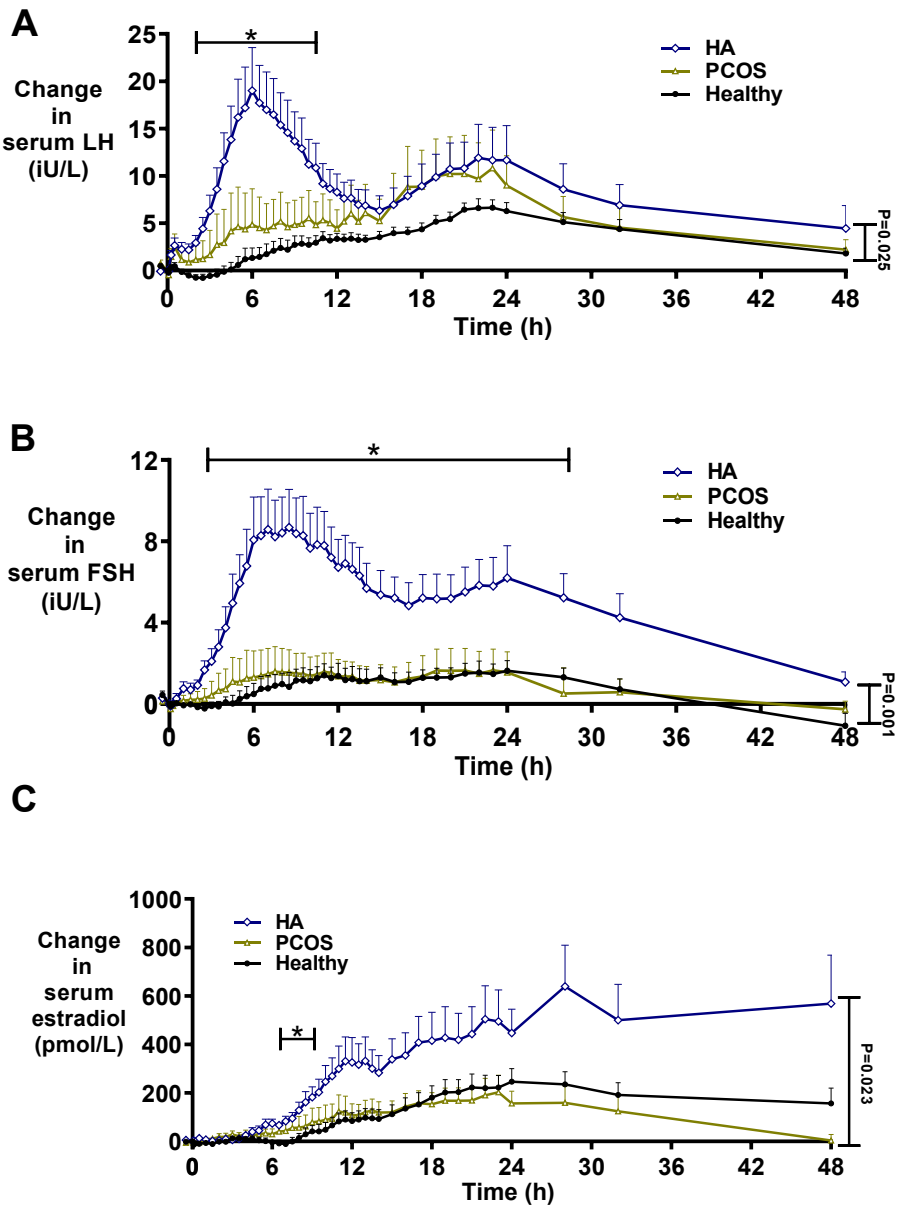


Figure 4.5: Change in gonadotrophins and oestradiol levels in the three groups following administration of MVT602

Mean±SEM 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 MVT602

(0.03nmol/kg). Comparison between groups were made by two-way ANOVA with repeated measures. * P-value <0.05.

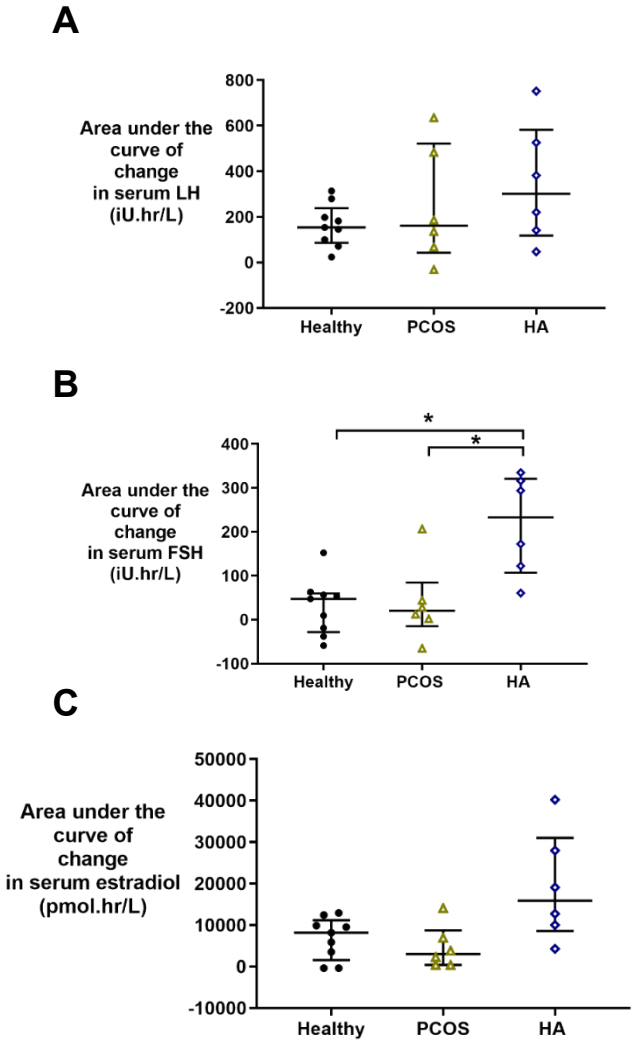


Figure 4.6: Area under the curve of change in gonadotrophins and oestradiol in the three groups following MVT602

Scattergram (Median±IQR) of Area Under the Curve of change in (A) Serum LH (iU.hr/L) (B) Serum FSH (iU.hr/L) (C) Serum oestradiol (pmol.hr/L) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue, following a subcutaneous bolus of MVT602 (0.03nmol/kg). Comparison between the groups were made by using Kruskal-Wallis Dunn's multiple comparison test. * P-value <0.05.

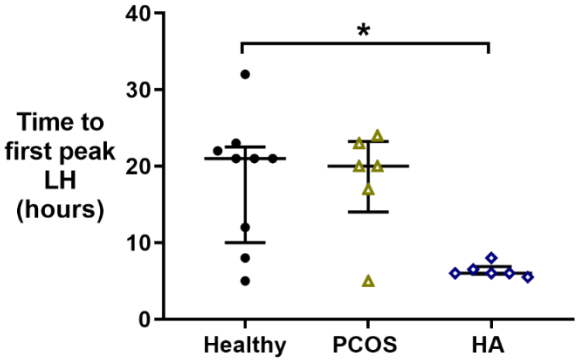


Figure 4.7: Time to first peak LH rise in the three groups following MVT602

Scattergram (Median±IQR) of time to first peak of serum LH rise (in hours) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue, following a subcutaneous bolus of MVT602 (0.03nmol/kg). Comparison between the groups was made by using Kruskal-Wallis Dunn's multiple comparison test. * P-value <0.05.

4.4.2.3 Comparison between gonadotrophin responses to KP54 and MVT602

Within each group, MVT602 (0.03nmol/kg) resulted in a serum gonadotrophin change that was of similar amplitude to that observed following KP54 (9.6nmol/kg). Likewise, the peak gonadotrophin levels following these interventions were of similar amplitude in all three groups of women **(Figure 4.8)**.

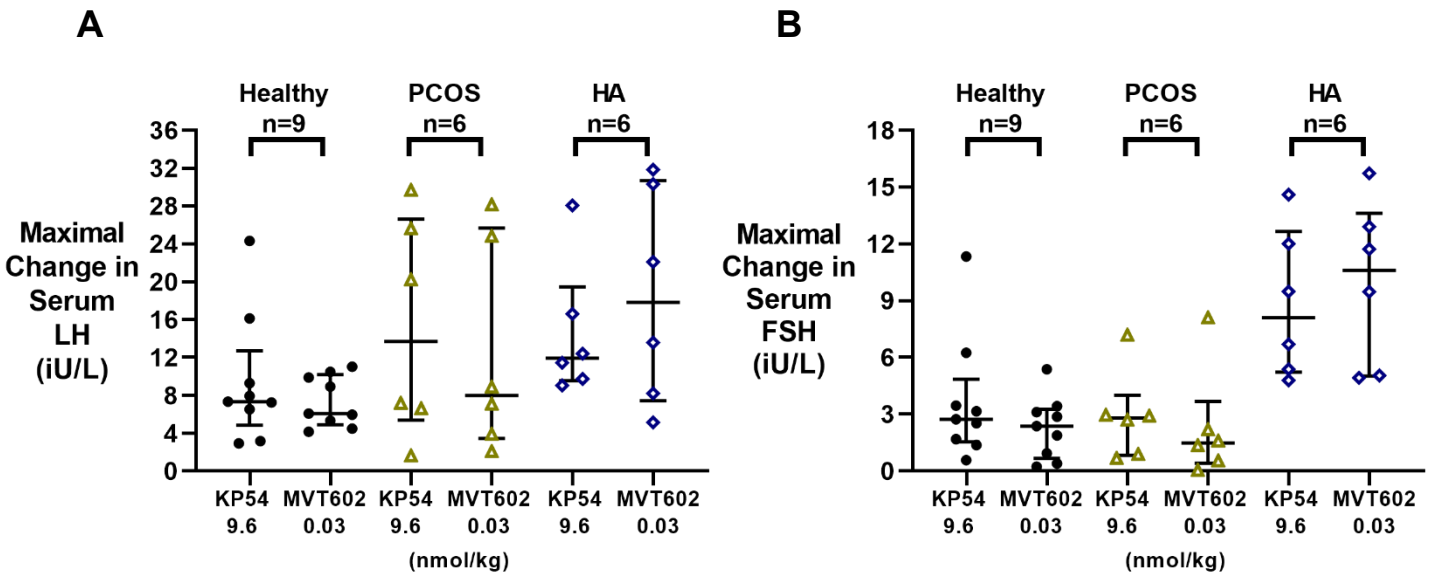


Figure 4.8: Maximal change in serum gonadotrophins following KP54 and MVT602 in the three groups

Scatter diagram (Median±IQR) of maximum change in serum (A) LH (iU/L) and (B) FSH (iU/L) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue, following a subcutaneous bolus of KP54 (9.6nmol/kg) and MVT602 (0.03nmol/kg). Outcomes following the two interventions (MVT602 and KP54) within the same diagnosis group were analysed using the Mann-Whitney test.

4.4.2.4 Determinants of serum gonadotrophin responses following MVT602

Baseline oestradiol levels in women with HA were positively correlated with change in serum LH (P=0.0002, r^2=0.98) and serum FSH levels (P=0.01, r^2=0.82) following MVT602. However, this was not the case in women with PCOS (for LH: P=0.68, r^2=0.05, for FSH: P=0.36, r^2=0.21), or healthy women (LH: P=0.80, r^2=0.009, FSH: P=0.485, r^2=0.05) **(Figure 4.9A, Figure 4.10A)**. Similarly, baseline oestradiol did not seem to influence maximal gonadotrophin levels following administration of KP54 in any of the three groups **(Figure 4.9B, Figure 4.10B)**.

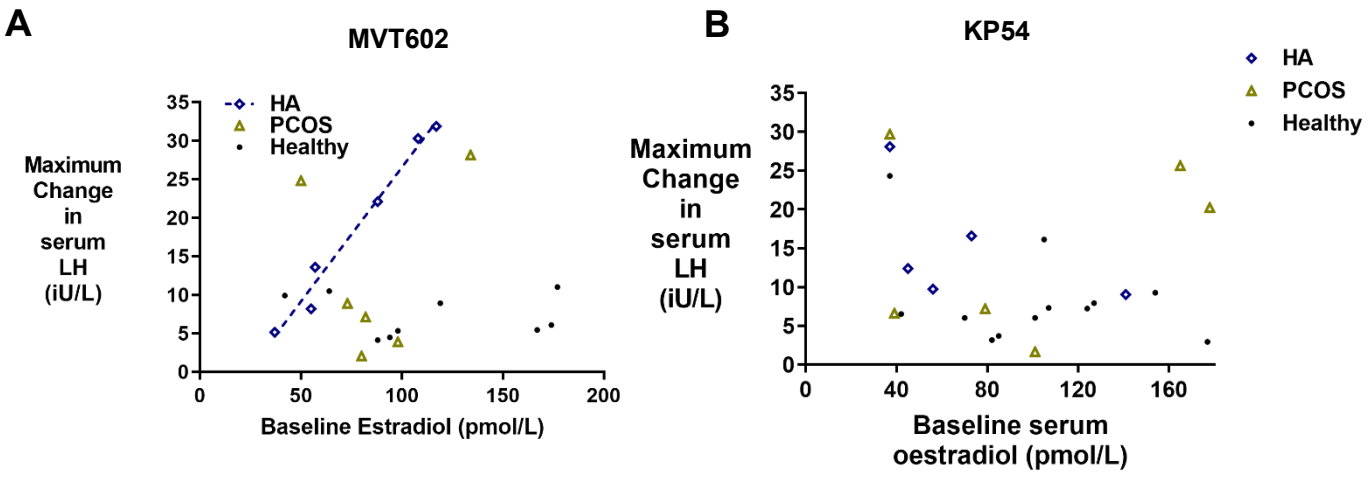


Figure 4.9: Change in LH compared to baseline oestradiol following KP54 and MVT602

Scatter diagram of maximum change in serum LH (iU/L) compared to baseline serum oestradiol levels (pmol/L) following administration of (A) MVT602 (0.03nmol/kg) and (B) KP54 (9.6nmol/kg) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue. Blue dotted line denotes linear regression of the relationship in HA (P=0.0002, r^2=0.98).

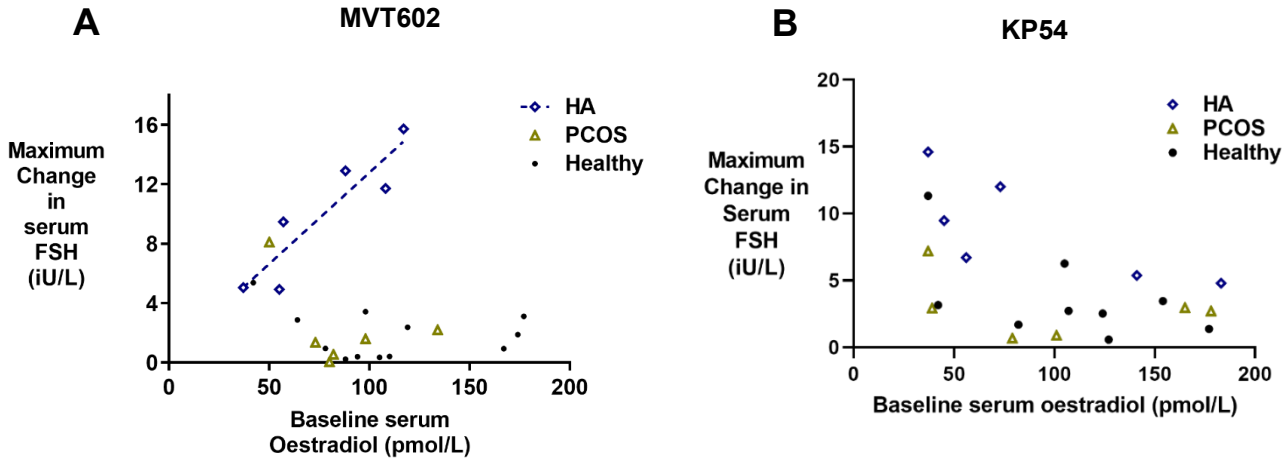


Figure 4.10: Change in FSH compared to baseline oestradiol in the three groups following administration of MVT602 and KP54

Scatter diagram of maximum change in serum FSH (iU/L) compared to baseline serum oestradiol levels (pmol/L) following administration of (A) MVT602 (0.03 nmol/kg) and (B) KP54 (9.6 nmol/kg) in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue. Blue dotted line denotes linear regression of the relationship in women with HA (P=0.01, r^2=0.82).

4.4.2.5 Effects of MVT on Blood Pressure and Heart Rate measurements

Blood pressure and Heart rate measurements in women with HA

The six women with HA had similar mean systolic blood pressure readings between the different arms **(Figure 4.11A)**, with these remaining stable during the first 10 hours of the studies. During the overnight study involving the administration of MVT602, the mean systolic readings of all participants fell, reflecting the period they were asleep. By 24 hours the measurements returned to baseline when the participants woke up. Analysis of the results of the first 10 hours of the studies using a mixed effects model revealed no differences in mean systolic blood pressure measurements between the different interventions (P value = 0.86).

Similar trends were observed when noting the mean diastolic blood pressure measurements, with no obvious difference observed between the various interventions **(Figure 4.11B)**. Once again, the measurements appeared stable during the first 10 hours of the studies, whilst a reduction in diastolic blood pressure was noted 16 hours after the administration of MVT602. Diastolic blood pressure readings returned to normal at 24 hours as the participants started waking up. Mixed effects analysis of the data up to and including the first 10 hours of the studies showed no differences in mean diastolic blood pressure measurements between the different interventions (P value = 0.91).

Heart rate readings showed similar patterns to blood pressure recordings. As expected, the mean heart rate of the participants fell during the overnight study **(Figure 4.11C)**. There were no significant differences in mean heart rate readings between the three interventions (P value = 0.45).

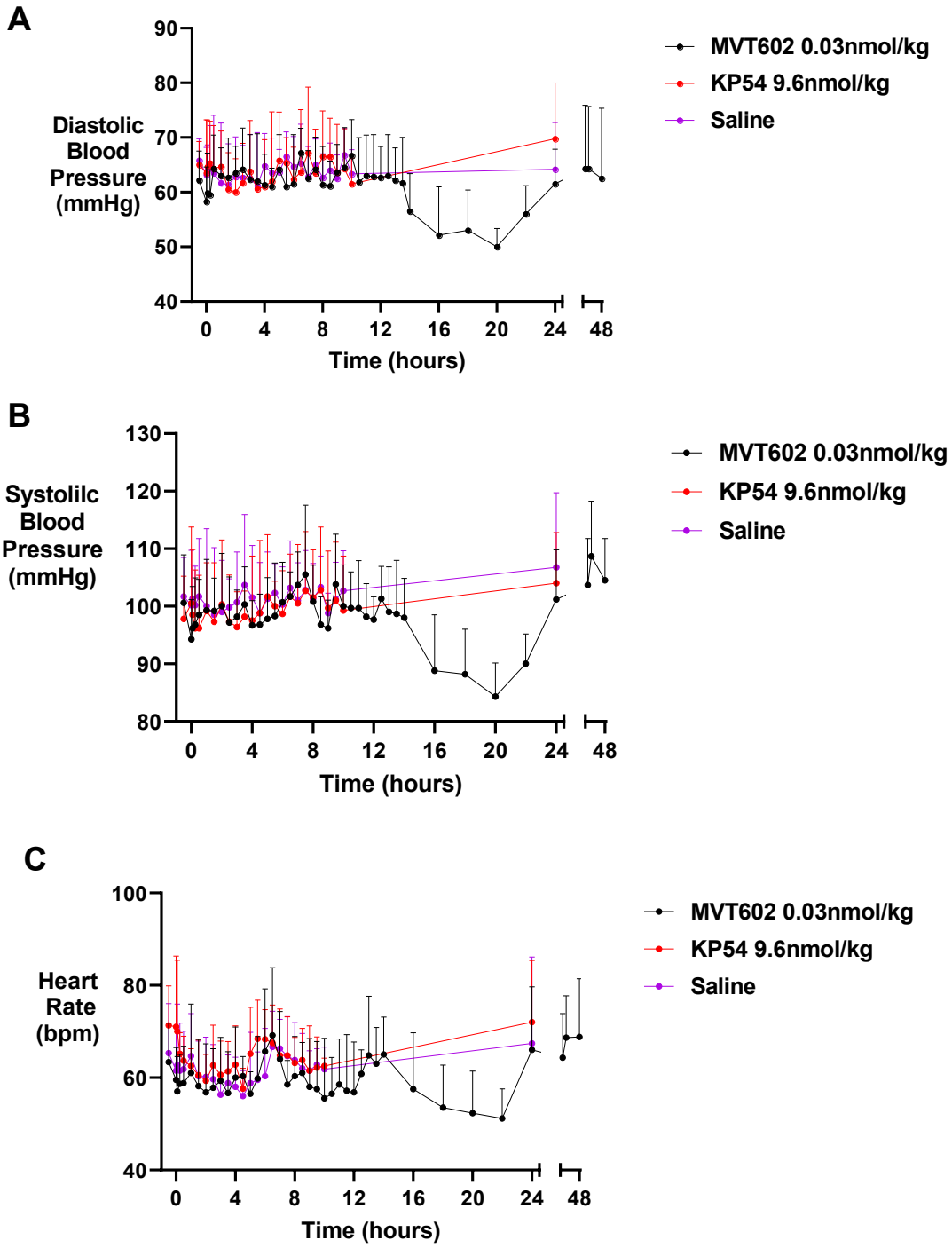

A
Diastolic Blood Pressure (mmHg)
90
80
70
60
50
40
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline
B
Systolilc Blood Pressure (mmHg)
130
120
110
100
90
80
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline
C
Heart Rate (bpm)
100
80
60
40
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline

Figure 4.11 Blood pressure and heart rate measurements in women with HA

Mean ±SEM of (A) systolic blood pressure (mmHg), (B) diastolic blood pressure (mmHg) and (C) Heart rate (bpm) over time (hours) following administration of MVT602 (0.03 nmol/kg) in black, KP54 (9.6 nmol/kg) in red, and 0.9% Saline in purple in women with HA (n=6).

Blood pressure and Heart rate measurements in women with PCOS

Mean systolic and diastolic blood pressure measurements in women with PCOS exhibited similar trends to those seen in women with HA **(Figure 4.12A&B)**. There were no differences in the blood pressure responses between the different interventions following mixed effects model analysis (P value = 0.23 for systolic blood pressure and P value = 0.29 for diastolic blood pressure). Similarly, the mean heart rate readings in women with PCOS were not different between the various interventions (P value = 0.65) **(Figure 4.12C)**.

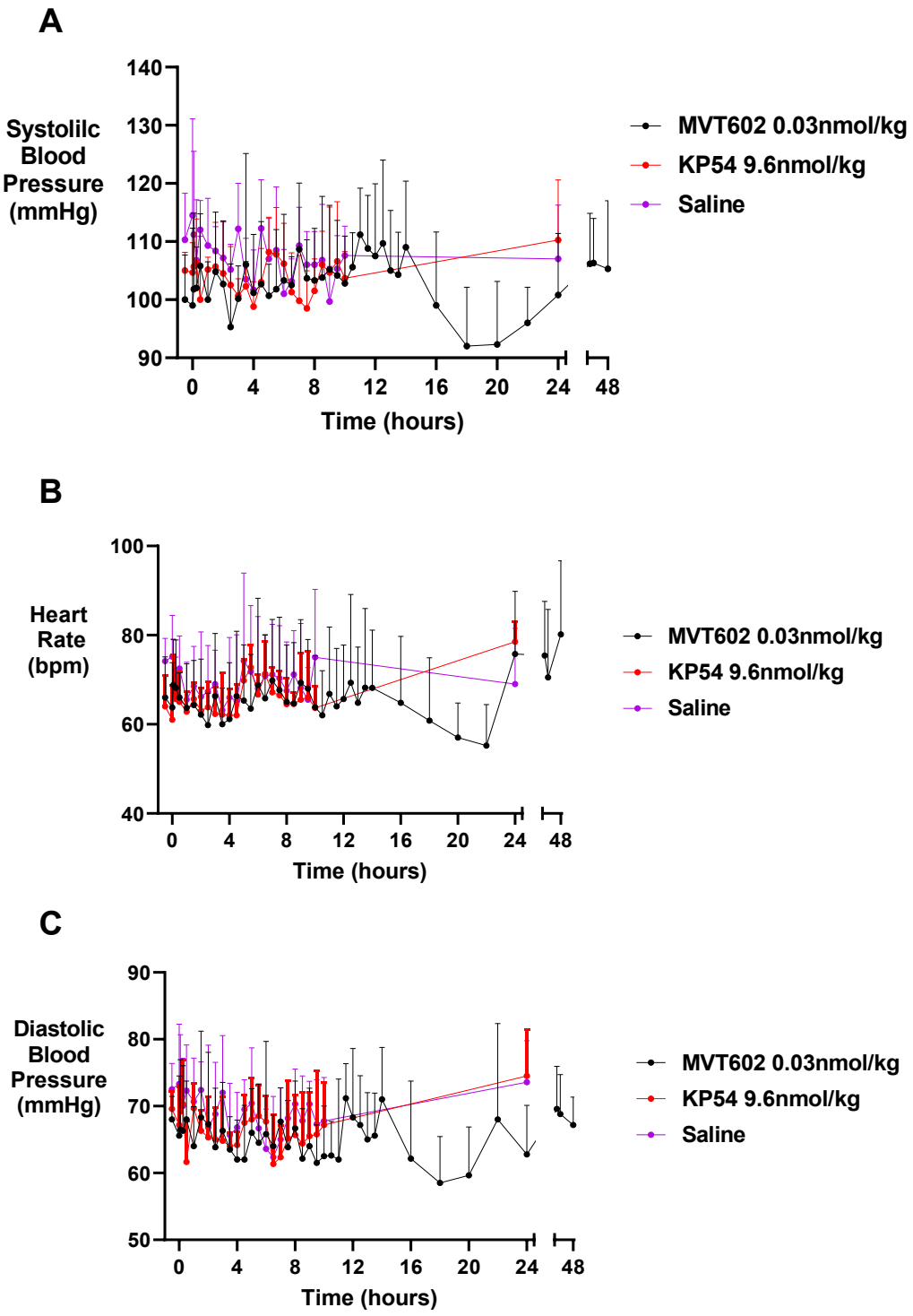

A
Systolilc Blood Pressure (mmHg)
140
130
120
110
100
90
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline
B
Heart Rate (bpm)
100
80
60
40
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline
C
Diastolic Blood Pressure (mmHg)
90
80
70
60
50
0
4
8
12
16
20
24
48
Time (hours)
MVT602 0.03nmol/kg
KP54 9.6nmol/kg
Saline

Figure 4.12 Blood pressure and heart rate measurements in women with PCOS

Mean ±SEM of (A) systolic blood pressure (mmHg), (B) diastolic blood pressure (mmHg) and (C) Heart rate (bpm) over time (hours) following administration of MVT602 (0.03 nmol/kg) in black, KP54 (9.6 nmol/kg) in red, and 0.9% Saline in purple in women with PCOS (n=6).

Comparison of effects of MVT602 on Blood pressure and heart rate measurements in women with normal fertility, PCOS and HA

Following administration of MVT602 0.03nmol/kg, the trends in mean systolic blood pressure were similar among the three groups of women studied. Unsurprisingly, women with HA had lower systolic blood pressure readings at baseline and throughout the studies, however there was no statistically significant difference in the measurements following mixed effects analysis (P value = 0.21) **(Figure 4.13A)**. Similar patterns were observed on analysis of the mean diastolic blood pressure values among the three groups, with women with HA starting from a lower baseline, but overall not having significantly different results compared to the other two groups (P value = 0.28) **(Figure 4.13B)**. Women with HA were also found to have a lower heart rate during the entirety of their study, however on mixed effect analysis of the data there was no difference in the mean heart rate recordings of the three groups (P value = 0.22) **(Figure 4.13C)**.

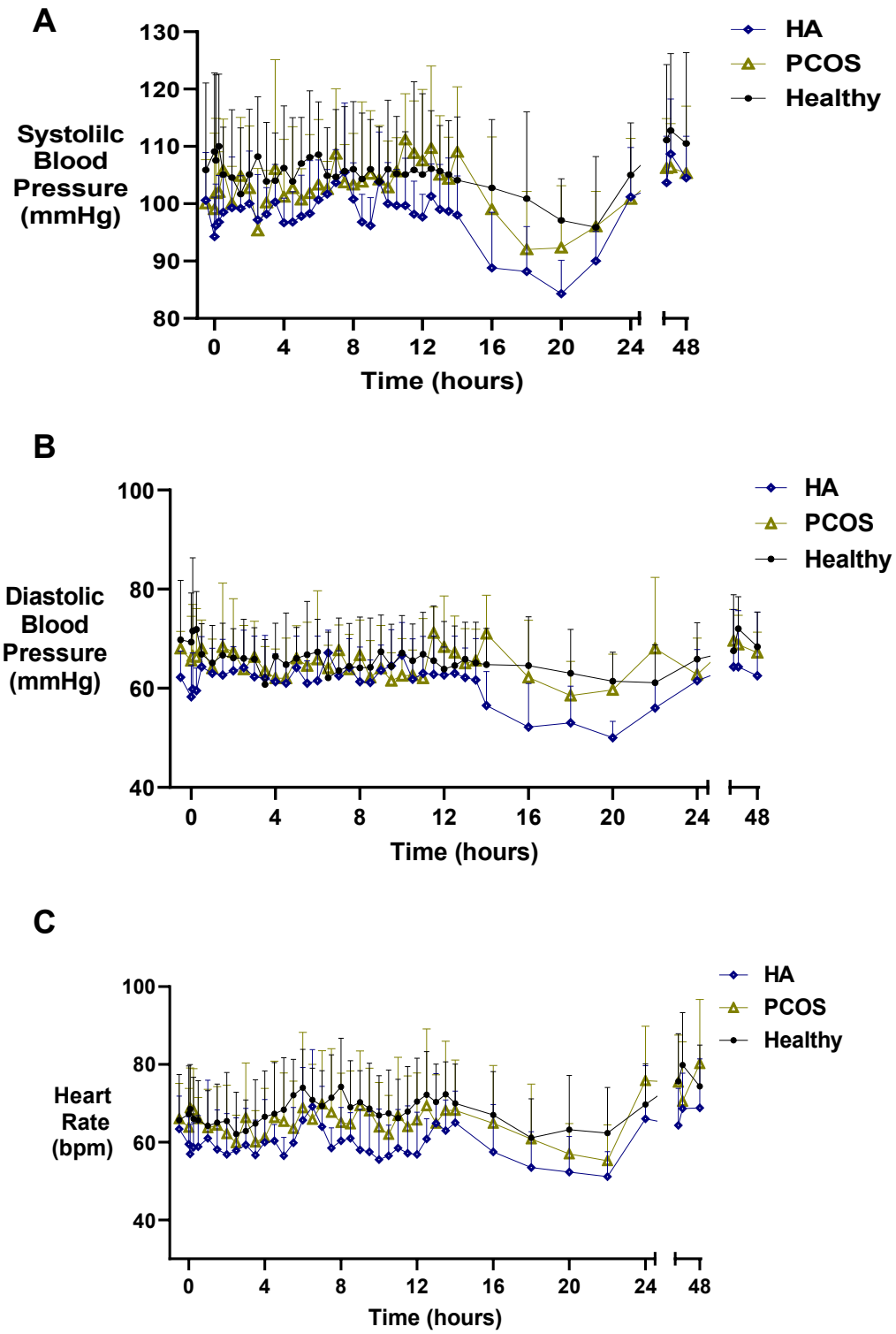

A
Systolilc Blood Pressure (mmHg)
130
120
110
100
90
80
0
4
8
12
16
20
24
48
Time (hours)
HA
PCOS
Healthy
B
Diastolic Blood Pressure (mmHg)
100
80
60
40
0
4
8
12
16
20
24
48
Time (hours)
HA
PCOS
Healthy
C
Heart Rate (bpm)
100
80
60
40
0
4
8
12
16
20
24
48
Time (hours)
HA
PCOS
Healthy

Figure 4.13 Effects of MVT602 on blood pressure and heart rate readings in the three groups

Mean ±SEM of (A) systolic blood pressure (mmHg), (B) diastolic blood pressure (mmHg) and (C) Heart rate (bpm) over time (hours) following administration of MVT602 (0.03 nmol/kg) in healthy women (n=9) in black, women with PCOS (n=6) in olive, and women with HA (n=6) in blue.

4.4.3 Results of Phase 2

As expected, oestradiol supplementation greatly increased the gonadotrophin responses of all the study participants. With oestrogen supplementation, the maximal rise in LH following MVT602 was increased from 7.5 iU/L to 24.4 iU/L in healthy women, from 10 iU/L to 22.3 iU/L in women with PCOS and from 18.1 iU/L to 25.8 iU/L in women with HA, thus extending the duration of the LH secretion by approximately 24 hours (**Figure 4.14A**). The peak FSH rise was also augmented in healthy women (from 1.7 iU/L to 7.6 iU/L) and women with HA (from 2.9 iU to 12.1 iU/L) after oestrogen treatment. However, application of oestrogen had no apparent effect on the peak FSH levels of women with PCOS (**Figure 4.14B**). Furthermore, the timing of maximal gonadotrophin rise was not affected by oestrogen pre-treatment. Considering once more how baseline oestradiol levels correlate with the maximal LH rise achieved in each group, it seems that the positive relationship previously detected in women with HA receiving MVT602 might now be reversed, however our sample size is too small for statistical significance **(Figure 4.15)**.

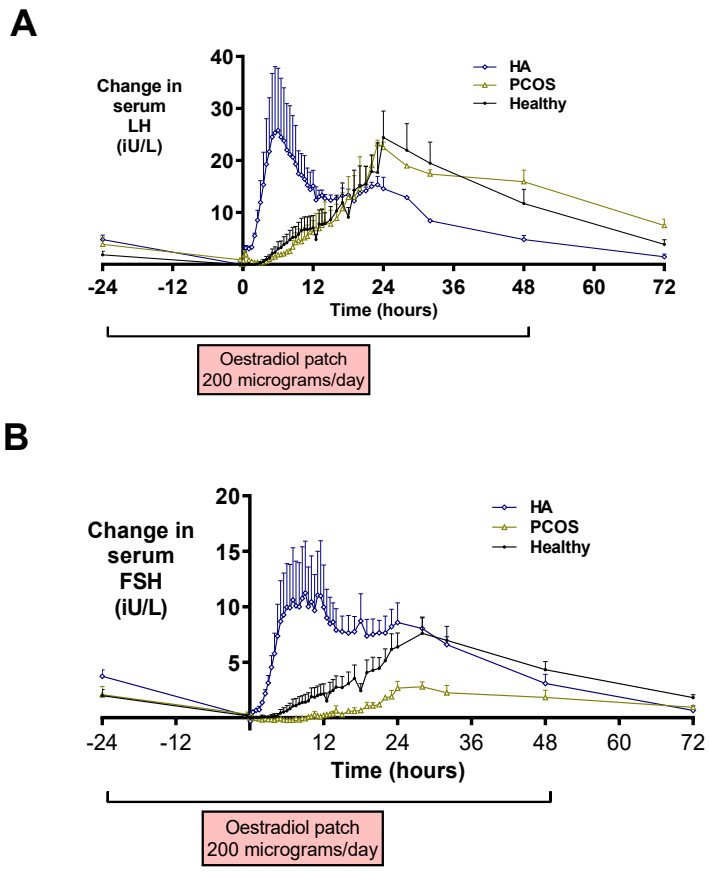


Figure 4.14 Change in serum gonadotrophins following administration of MVT602 in the three groups pre-treated with oestrogen

Mean ±SEM of (A) serum LH (iU/L) and (B) serum FSH (iU/L) over time (hours) following administration of MVT602 (0.03 nmol/kg) in healthy women (n=9) in black, women with PCOS (n=6) in olive, and women with HA (n=6) in blue pre-treated with oestrogen (transdermal oestradiol patch 200µg/day)

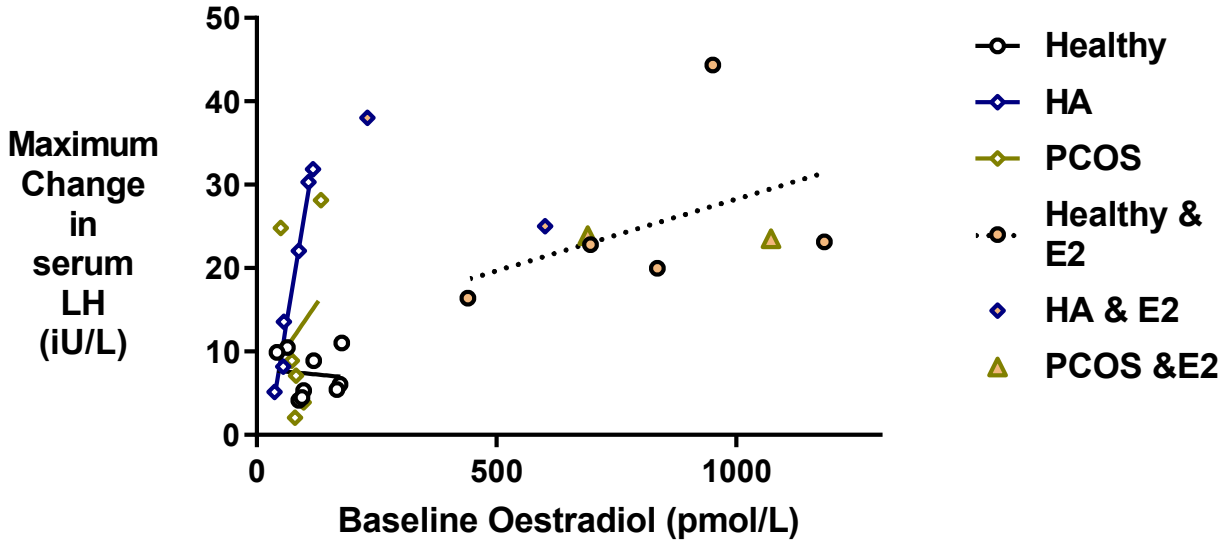


Figure 4.15 Maximum change in serum LH compared to baseline oestradiol following administration of MVT602 in the three groups with and without oestrogen pre-treatment

Scatter diagram of maximum change in serum LH (iU/L) compared to baseline serum oestradiol levels (pmol/L) following administration of MVT602 (0.03 nmol/kg) without oestrogen pre-treatment in healthy women (n=9) in black, women with PCOS (n=6) in olive and women with HA (n=6) in blue and following administration of MVT602 following oestrogen pre-treatment in healthy women (n=5) in black and pink, women with PCOS (n=2) in olive and pink and women with HA (n=2) in blue and pink .

4.5 Discussion

This is the first study investigating the effects of the novel kisspeptin analogue, MVT602, on the gonadotrophin responses of women with the two commonest anovulatory disorders, namely HA and PCOS. Furthermore, the study also compares for the first time the effects of this analogue with KP54. Administration of MVT602 to women with HA resulted in an exaggerated gonadotrophin response and sustained oestradiol rise. In contrast, administration of MVT602 in women with PCOS resulted in gonadotrophin responses similar to those elicited in healthy women. These findings highlight the analogue's potential to differentiate women with HA from women with PCOS, but also provide valuable insights into the mechanistic differences that might explain the disparity in responses. Moreover, the study highlights MVT602's potential for restoring ovulation in women with HA.

Administration of 0.03 nmol/kg of MVT602 resulted in a robust LH response in the early follicular phase of healthy women and was thus chosen as the dose most suitable for administration in anovulatory women. The choice of dose of KP54 was based on previous work from our group, whereby a dose of 9.6 nmol/kg not only resulted in a robust gonadotrophin response, but also led to safe induction of oocyte maturation in an IVF protocol (Abbara et al. 2018).

Women with HA exhibited exaggerated responses to MVT602, whereby a single dose of the analogue resulted in a 2-fold increase in serum LH levels compared to the healthy group. It is interesting to consider why the gonadotrophin responses in women with HA were higher. One possibility is that women with HA have increased hypothalamic sensitivity to the effects of kisspeptin. Previous work by Jayasena et al. has demonstrated that women with HA are more sensitive to KP54 than healthy women receiving the same dose (Jayasena et al. 2009). An animal model seeking to replicate the endocrine and metabolic conditions of HA by imposing

a negative energy balance for seventy-two hours, resulted in significant suppression of hypothalamic *Kiss1* expression and increased *Kiss1r* expression. Furthermore the animals in the same study exhibited enhanced gonadotrophin release to exogenous kisspeptin (Castellano et al. 2010; J. M. Castellano, Navarro, Fernandez-Fernandez, et al. 2005). Female rats undergoing prolonged undernutrition exhibited an augmented and sustained LH release following a KP10 infusion (Roa et al. 2008). Therefore, the gonadotrophin responses of our HA cohort to MVT602 are in line with the available animal data on kisspeptin in this condition. These findings indicate that the amplified LH response might result from a compensatory rise in KISS1 receptors in response to the reduction in circulating kisspeptin levels (Castellano, Navarro, Fernandez-Fernandez, et al. 2005; Iwasa et al. 2010; Matsuzaki et al. 2011).

In keeping with the enhanced LH response, FSH responses to a subcutaneous bolus of MVT602 in women with HA were also exaggerated, such that the peak FSH in this patient cohort was four-fold higher than in healthy women and women with PCOS. This was responsible for the subsequent greater rise in serum oestradiol levels in this group. We have previously shown that twice weekly subcutaneous injections of KP54 in women with HA over 8 consecutive weeks resulted in sustained FSH responses, however these did not translate into ovarian follicular growth (Jayasena et al. 2009). This novel FSH response induced by MVT602 warrants further investigation, as it might be able to restore ovulation in women with HA.

In contrast to this, the serum FSH profiles in women with PCOS revealed an attenuated response, not only to MVT602 but also to KP54. This observation might reveal dissociated gonadotrophin responses in a condition characterised by increased GnRH pulsatility. One explanation for this might be the higher inhibin B levels in women with PCOS that might prevent FSH rises despite the administration of exogenous kisspeptin. It was interesting,

however, to note that baseline inhibin B and serum AMH levels did not correlate with the changes in serum FSH in this study.

The relationship between baseline oestradiol levels and the magnitude of LH responses to MVT602 is indeed intriguing. On the one hand there was a positive correlation between basal oestradiol levels and subsequent LH responses following administration of MVT602 in women with HA, but not in healthy women nor in women with PCOS. It is well known that circulating sex steroids are crucial in determining LH responses to exogenous kisspeptin. Healthy women are more sensitive to the effects of kisspeptin when this is administered during the pre-ovulatory phase, a high oestradiol state (Dhillo et al. 2007). Indeed, this is something that we have also observed in this study, where the mean LH responses of women from all three groups were higher when MVT602 was administered after oestradiol pre-treatment. The exact mechanism behind the disparity in baseline oestradiol levels and subsequent gonadotrophin responses is uncertain, but it might involve discrepant sex steroid feedback pathways in women with HA. Further work specifically probing the sex steroid feedback pathways in Hypothalamic Amenorrhea is warranted.

Regardless of the participants' menstrual cycle length, a single bolus of MVT602 induced a rise in serum LH of similar amplitude as after native KP54 9.6 nmol/kg. This indicates the consistency of kisspeptin secretion following activation of the kisspeptin receptor, irrespective of the ligand.

MVT602 did not affect blood pressure or heart rate recordings in women with HA and women with PCOS. Previous work by our lab has demonstrated that exogenous administration of KP54 in the form of infusions or subcutaneous boluses does not affect blood pressure or heart rate measurements. This is the first study reporting the effects of MVT602 on these

physiological parameters in women and suggests that this analogue would be safe for administration for ovulation induction protocols.

4.5.1 Limitations

The small sample size might affect the statistical significance of some of the observations but also resulted in considerable variation in responses within the different treatment groups. It is important that a larger study with a greater number of recruits is done to confirm reproducibility of the data.

Whilst we speculate that the robust LH profile elicited with MVT602 in women with HA could restore ovulation, the applicability of MVT602 to restore ovulation in HA requires confirmation during chronic protocols of MVT602 administration. Nonetheless, it would be interesting to investigate the effects of this compound on the gonadotrophin axis of women with HA in various frequency protocols.

4.5.2 Conclusion and future work

In summary, this is the first study to investigate the effects of MVT602 on the gonadotrophin responses of women with anovulatory disorders. MVT602 resulted in amplified gonadotrophin response in women with HA, especially FSH responses high enough to induce a rise in serum oestradiol. This signifies the compound's potential as an ovulation induction agent in women suffering from HA. The lack of an effect on blood pressure and heart rate measurements was also reassuring, when planning long-term studies with more regular administration of the compound.

MVT602 has shown great potential as a novel analogue in the field of kisspeptin based therapeutics. It resulted in amplified gonadotrophin and oestradiol responses following administration in women with HA. To begin with, a confirmatory study verifying reproducibility of the results in women with hypothalamic amenorrhoea can help validate our current findings and explore potential variations of gonadotrophin responses in more detail. Moreover, animal models of HA exploring how varying levels of exogenous oestradiol pre-treatment influence MVT602's effects on the gonadotrophin axis can shed some light on the complex mechanisms surrounding sex steroid feedback control of the reproductive axis. Furthermore, it would be appropriate to also explore the gonadotrophin effects of the analogue in chronic protocols involving women with HA. Finally, the discrepant FSH responses to MVT602 between women with HA and women with PCOS warrant further research which might improve our understanding of the gonadal feedback pathways.

Chapter 5

Discussion

CHAPTER 5: DISCUSSION

Kisspeptin is recognised as a crucial regulator of the reproductive axis. Its effects on gonadotrophin secretion are potent and have been the subject of great interest, as scientists and clinicians have recognised its potential as a novel diagnostic and therapeutic agent in the field of reproductive endocrinology.

As kisspeptin stimulates GnRH secretion, it can be used as a specific test of hypothalamic GnRH neuronal function. Indeed, another group has verified the utility of KP10 to discriminate men with CHH and healthy men (Yee Ming Chan et al. 2011). More recently, they have also demonstrated that KP10 could also have a role in the diagnostic work up of young people with delayed puberty, as their discrepant responses to kisspeptin enabled distintion bwteen a diagnosis of Constitutional Delay of Growth and Puberty from CHH (Chan et al. 2020). In our studies we used KP54, that is not only longer acting but also results in more robust gonadotrophin responses. Furthermore, there is evidence that KP54, but not KP10, crosses the blood brain barrier to directly act on GnRH neuronal bodies (Jayasena, Abbara, et al. 2015b). Moreover, the higher magnitude of LH rise induced by KP54 (median LH-rise of 13.2 iU/L) compared to KP10 (mean LH-rise 8.3 iU/L) (George et al. 2011) could facilitate differentiation of hypothalamic function in borderline cases, such as people with milder phenotypes or young people presenting with delayed puberty.

Thus, KP54 is the isoform with the most favourable profile for translation into a diagnostic challenge test, that can be used in clinical settings. Our study on the use of KP54 for interrogation of hypothalamic GnRH function showed that it can accurately distinguish men with CHH from healthy men. Furthermore, its diagnostic performance was superior to that of GnRH, which is the only clinically available test of pituitary function at present.

The gradation in LH responses in men with CHH elicited by KP54 in our study was fascinating, as it correlated with the severity of their genotypic variance. Men with recognised pathogenic variants had lower peak LH responses compared to men with CHH with variants of unknown significance or no abnormalities identified after genetic testing. Indeed, this was also the case when comparing the gradation of LH response to the olfactory status of these patients. Men with Kallmann syndrome (CHH + Anosmia) had lower LH responses compared to men with CHH, suggesting that genetic variants affecting GnRH neuronal migration are more severe and thus less likely to respond to KP54 due to the abnormal anatomical location of the GnRH neurons. The assertion that Kallmann syndrome is more severe than CHH is also supported by the literature (Bonomi et al. 2018; Quinton et al. 2001), albeit the severity of GnRH dysfunction does not affect a patient's likelihood of reversal (Boehm et al. 2015).

A KP54 challenge test would revolutionise the way that children with delayed puberty are investigated and eventually diagnosed and managed. Furthermore, a KP54 test will be invaluable in patients with CHH where reversal of the gonadal dysfunction is suspected, negating the need to stop their hormone replacement therapy to assess the integrity of the axis. Indeed, work from Lippincott et al. has demonstrated that men with reversal of their CHH had higher gonadotrophin responses to KP54 than men with persistent CHH (Lippincott et al. 2016).

The two most studied isoforms of kisspeptin (KP54 and KP10) have been found to stimulate LH and FSH release when given either subcutaneously or intravenously. KP54, being the peptide with the longest amino-acid sequence, has a longer half-life, which makes it a more favourable agent to use for bolus administration than KP10. Kisspeptin receptor tachyphylaxis was recognised almost a decade ago which led to a search for more stable kisspeptin receptor ligands. These might prove instrumental in preserving kisspeptin's potent agonistic characteristics whilst also sparing receptor downregulation.

MVT602 is one such compound, previously studied in men both in healthy and disease states and found to be safe and efficacious (MacLean et al. 2014). Its effects on the gonadotrophin responses in women had not been previously studied and this became the focus of my research. My studies have demonstrated that a single subcutaneous dose of MVT602 elicits robust gonadotrophin responses in the follicular phase of the menstrual cycle in healthy women. The LH profile observed closely resembles the pattern of LH secretion seen in the pre-ovulatory phase during the LH surge. This observation suggests that MVT602 might be a suitable agent for use in ovulation induction protocols and it would be useful to test this in an IVF setting. The gonadotrophin responses to MVT602 in women were distinctly different to those observed in healthy men. In healthy women, there was an initial plateau in LH levels for the first 4-6 hours, followed by a gradual rise that peaked at around 24 hours. It is vital to also examine the effects of MVT602 when administered at different times in the menstrual cycle in order to determine the effect of steroid feedback on gonadotrophin responses. It is possible that the low circulating oestradiol levels might not be the sole driver behind this pattern, however, as women with HA exhibited more robust responses to MVT602, despite having similar baseline oestradiol levels with the control group. This has provided new insights into the pathophysiology of hypothalamic amenorrhea, as it suggests differences in the regulation of the *kiss1* system in women with this disorder, but also alludes to changes in their sex-steroid feedback control. The gonadotrophin responses of women with PCOS were similar to those seen in healthy women. Of particular interest were the FSH responses of women with PCOS to MVT602, which might be explained by gonadal hormones exerting negative feedback control on the pituitary. These findings warrant further investigation and might provide further insights into how ovarian-derived steroids and other hormones adapt in the presence of anovulatory disorders.

MVT602 might also be acting on other, off-target receptors, such as GnIH, that antagonise its effects on the kisspeptin receptor. It is known that kisspeptin can activate other receptors within the RF amide family (Oishi et al. 2011). Furthermore, it is possible that the off-target effects of MVT602 are only evident in females, with George and colleagues reporting discrepant effects of GnIH administration on kisspeptin-stimulated LH release in men and in women (George et al. 2017). Thus, the mechanism of kisspeptin and MVT602 receptor binding should be further investigated.

Overall, MVT602 resulted in FSH responses that were less robust that LH responses. This was not surprising as the studies involving the administration of KP54 and KP10 demonstrated the same preferential rise of LH over FSH (Dhillo et al. 2007; George et al. 2011; Jayasena, Abbara, et al. 2015; Narayanaswamy et al. 2016; Navarro, Castellano, et al. 2004) and provide further confirmation of the constitutive action of GnRH on gonadotrophin secretion.

The findings of these studies are extremely pertinent when considering MVT602's potential in the treatment of anovulatory disorders. We have previously shown that kisspeptin is safe and effective in inducing oocyte maturation in women undergoing IVF treatment, without increasing the risk of OHSS. MVT602 can build on this work, by providing a more sustained duration of LH exposure to more closely resemble the LH surge. In this study we have demonstrated that the area under the curve of LH release was prolonged after administration of MVT602, replicating the conditions required for oocyte maturation. It would be useful to examine MVT602 as a trigger agent in ovulation induction protocols and compare its performance to existing pharmacological agents.

In women with HA, MVT602 resulted in augmented gonadotrophin responses that in turn stimulated oestradiol secretion. This suggests that MVT602 is a promising agent for use for ovulation induction in these patients.

In summary, KP54 and MVT602 have huge potential to improve the diagnosis and treatment of patients with reproductive disorders. Future work will look to realise this potential and bring these agents to the bedside for patient benefit by demonstrating that chronic protocols can cause stimulation without tachyphylaxis and demonstrating a benefit on clinical outcomes.

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