Preliminary characterisation of the neuropeptide melanin concentrating hormone in humans.

Jane Naufahu

School of Life Sciences

PRELIMINARY CHARACTERISATION OF THE NEUROPEPTIDE MELANIN CONCENTRATING HORMONE IN HUMANS

Jane Naufahu

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

August 2010

Abstract

Melanin concentrating hormone (MCH) is an orexigenic neuropeptide expressed centrally in the zona incerta and lateral hypothalamus. Evidence from rodent studies implicates hypothalamic MCH in the modulation of varied and diverse physiological functions most notably in energy homeostasis but also emerging as a candidate mediator in reproductive activities. These two aspects of normal physiology are intimately related though the pathways through which they integrate are only just being explored. Currently little is known about the actions, sources or targets of peripheral or circulating MCH. This study is the first to describe normal physiological concentrations of circulating MCH in humans. Particular emphasis has been devoted to aspects which might further understanding of the dual roles of circulating MCH in energy balance and reproductive function. A primary objective was to develop and validate a tool with which to quantifiably measure circulating MCH concentrations since previous work in this area has failed to reconcile this objective. Following the successful validation of a radioimmunoassay (RIA), three studies were undertaken with human subjects drawn from 3 distinct sample populations. The chief objectives were 1) to detect changes in circulating MCH post-prandial in association with other metabolic markers of energy homeostasis; 2) to determine associations between circulating MCH concentrations and resting metabolic rate (RMR); and 3) to detect changes in circulating MCH concentrations during the menstrual cycle. The results indicate that circulating MCH concentrations may be involved in glucose homeostasis since there were associations between the post-prandial MCH and the post-prandial insulin response. Circulating MCH may also convey information regarding the nutritional status of the individual, though this appears to be influenced by a combination of gender and adiposity status. Circulating MCH concentrations remained stable over the course of the menstrual cycle and were not strongly aligned to RMR. Whilst there were emergent patterns and trends there were also inconsistencies between the sample populations. This initial characterisation may be constructive for future exploration of the physiological relevance of circulating MCH in humans.

Acknowledgements

I would like to take this opportunity to thank the following people for their help support, and encouragement throughout my post-graduate studies.

My supervisors Dr Adam Cunliffe, Professor Taj Keshavarz and in particular my Director of Studies, Dr Joanne Murray for all of their invaluable support and guidance in the development of this work.

Dr Tania Murphy for her expertise and assistance with the RP-HPLC work.

To colleagues at the University of Westminster who helped with data collection: Marcela Brito, Barbora Doslikova and especially Fawaz Alzaid for his venepuncture skills and Faridah Shahab for her ongoing help.

To all subjects who gave up their time in order to make this research possible.

Finally to my family and friends for their patience, support and encouragement over the last four years.

Declaration

I declare that the work presented in this thesis is entirely my own unless otherwise stated.

List of Abbreviations

ATP	Adenosine triphosphate
AUC	Area under the curve
B_0	Total binding
BAT	Brown Adipose Tissue
BMI	Body mass index
CNS	Central nervous system
CPM	Counts per minute
CV	Coefficient of variation
EDTA	ethylene diamine tetraacetic acid
GnRH	Gonadotropin-releasing hormone
ICV	Intracerebroventricular
k/o	Knockout
LH	Luteinising hormone
LHA	Lateral hypothalamic area
mAU	milli-absorption units
MCH	Melanin concentrating hormone
MCHR-1	MCH Receptor 1
MCHR-2	MCH Receptor 2
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NPY/AgRP	Neuropeptide Y/Agouti related protein
NEI	Neuropeptide E-I
NGE	Neuropeptide G-E
NRS	Normal rabbit serum
NSB	Non-specific binding
NTS	Nucleus of the solitary tract/Nucleus tractus solitarius
RIA	Radioimmunoassay
RMR	Resting metabolic rate
RP-HPLC	Reverse phase-high performanance liquid chromatography
SD	Standard deviation
SEM	Standard error of the mean
TC	Total count
TSH	Thyroid stimulating hormone
TRH	Thyrotropin releasing hormone
UCP-1	Uncoupling protein-1
v:v	volume:volume
WHR	Waist-hip ratio

Table of Contents

1. Introduction

1.1 Aims of the research

The original research presented in the thesis was designed to develop and validate a novel radioimmunoassay with which to measure and hence describe normal circulating MCH concentrations in discrete human populations under both similar and differing conditions. This preliminary characterisation of the normal patterns of MCH in circulation may serve as a prelude to the further exploration of the physiological relevance of circulating MCH in humans.

These aims were addressed by the following objectives:

1. To confirm that MCH is quantifiably and reliably measurable in human plasma.
2. To establish normal reference ranges for circulating MCH concentrations in humans.
3. To determine whether circulating MCH is a bio-marker of adiposity in humans.
4. To explore associations between circulating MCH concentrations and energy expenditure and energy intake.
5. To determine associations between circulating MCH concentrations and circulating metabolic markers of energy homeostasis and reproductive function.

1.2 Structure of the thesis

The thesis comprises one chapter which describes the development and validation of a radioimmunoassay to measure circulating MCH concentrations in humans (Chapter 4), and three chapters (5, 6, & 7) which describe 3 studies undertaken with 3 different populations of human subjects. The key objectives of the study described in Chapter 5 were to establish a reference

range for circulating MCH concentrations in young healthy adults and to determine associations between circulating MCH concentrations and RMR. The key objective of the study described in Chapter 6 was to investigate the post-prandial MCH response to feeding in association with circulating blood glucose and plasma insulin and leptin concentrations in an older cohort of subjects (40+ yrs). In the study described in Chapter 7 changes in circulating MCH concentrations were investigated in association with circulating progesterone and leptin concentrations over the course of one menstrual cycle in a group of normally cycling women. The findings of all four studies are synthesised in Chapter 8.

2. Review of the literature

2.1 Abstract

Melanin concentrating hormone (MCH) is a comparatively recently discovered anabolic neuropeptide with multiple and diverse physiologic functions including a key role in energy homeostasis. Rodent studies have shown that ablation of functional MCH results in a lean phenotype, increased energy expenditure and resistance to diet-induced obesity. These findings have generated interest amongst pharmaceutical companies vigilant for potential anti-obesity agents. Nutritional status impacts on reproductive physiology and behaviours thereby optimising reproductive success and the ability to meet energetic demands. This complex control system entails integration of direct or indirect peripheral stimuli with central effector systems and involves numerous mediators. Recently a role for MCH in the reproductive axis has emerged giving rise to the premise that MCH may serve as an integratory mediator between those discrete systems which regulate energy balance and reproductive function. Hence this review focuses on published evidence concerning a) the role of MCH in energy homeostasis b) the regulatory role of MCH in the reproductive axis. The question as to whether the MCH system mediates the integration of energy homeostasis with the neuroendocrine reproductive axis and if so by what means has received limited coverage in the literature, evidence to date and current theories are summarised here.

2.2 Introduction

Energy balance is inextricably linked to reproduction: the ability to monitor both internal and external energy availability and consequently to modulate reproductive behaviours confers a species-wide reproductive advantage (Schneider, 2004). In times of energetic scarcity survival of the individual takes precedence over an essentially deferrable and energy costly activity, such as reproduction. Suppressed ovulatory cycles may be evidenced in female mammals and in humans self-imposed severe food restriction may

result in persistent amenorrhea (Wade and Jones, 2004). The deleterious consequences of obesity on reproductive function both in terms of ovulatory dysfunction and prenatal complications have been well documented (Loveland *et al.,* 2001; Pasquali *et al.,* 2003; Lashen *et al.,* 2004). These manifestations of impaired reproductive function both in the obese and in the under-nourished are unsurprising from an evolutionary point of view which propounds survival of the fittest. Hence those mechanisms which control reproductive function and nutritional status must be functionally linked (Schneider, 2004). Nevertheless in mammals these links are complex and incompletely understood (Gosman *et al.,* 2006), though it has been argued that it is energy balance and the availability of oxidisable fuels rather than fat mass *per se* which are the critical determinants of fertility status (Wade and Jones, 2004).

Understanding of the central regulation of energy homeostasis has advanced considerably since Stellar's (1954) identification of hypothalamic satiety and hunger centres more than half a century ago. The discovery of leptin in the early 1990s (Zhang *et al.,* 1994) was hailed as a breakthrough in the regulation of body weight. Originally characterised as an anti-obesity hormone (Hamman and Matthaei, 1996), the precise physiological role of leptin is now known to be more complex and its interactions with numerous metabolic modulators are widespread (Moschos *et al.,* 2002). A consenus viewpoint has arisen in which peripheral signals relating to the nutritional status of the individual are centrally integrated and efferent pathways (such as, the neuropeptide Y and agouti related protein (NPY/AGRP) system) respond to maintain energy homeostasis via modulation of appetite and energy expenditure (for review see Schwartz *et al.,* 2000). Recently several hormones and neuropeptides with the potential to link energy balance to reproductive function have been identified (Schneider, 2004). One such neuropeptide is melanin concentrating hormone .

Originally characterised as a circulating hormone modulating the skin colour of teleost fish (Kawauchi *et al.,* 1983), mammalian MCH was subsequently identified in rat hypothalamic tissue (Vaughan *et al.,* 1989). The amino acid sequence of MCH has been found to be identical in all mammals analysed

thus far, notably humans, rats and mice (Nahon *et al.,* 1989; Thompson and Watson, 1990; Presse *et al.,* 1990; Breton *et al.,* 1993). Centrally the neuropeptide is expressed primarily in the lateral hypothalamic area and rostral zona incerta/incerto-hypothalamic area (Bittencourt *et al.,* 1992; Sita *et al.,* 2007): two areas known to be critically involved in the regulation of feeding behaviours (for review see Schwartz *et al.,* 2000). The orexigenic properties of MCH were first proposed in 1996 (Qu *et al.,* 1996), although the findings of a number of subsequent studies suggest that the anti-obesogenic effects of MCH antagonism are mediated through its impact on appetite regulation secondary to its effects on energy expenditure and altered metabolism (Shimada *et al.,* 1998; Chen *et al.,* 2002; Marsh *et al.,* 2002; Segal-Lieberman *et al.,* 2003).

The physiological functions of MCH are not restricted to energy metabolism. MCH and its receptors are implicated in the modulation of complex behaviours such as those involved in motivation and reward (Saito *et al.,* 1999; Saito *et al.,* 2001; Saper *et al.,* 2002), stress response, affective states (Smith *et al.,* 2006), anxiety and depression (for review see Dyck, 2005). Of particular interest to this review are the effects of MCH on the female reproductive axis. To date the known effects are largely those associated with the central effects of MCH on the regulation of luteinising hormone (LH) release (Gonzalez *et al.,* 1997; Murray *et al.,* 2000a; Chiocchio *et al.,* 2001; Williamson-Hughes *et al.,* 2005; Murray *et al.,* 2006). Little is known about its impact on target reproductive organs though disruption of normal gonadotrophin releasing hormone (GnRH) activity produces a cascade of effects throughout the entire reproductive axis (for review see Schneider, 2004). There are conflicting reports in the literature regarding the reproductive consequences of deletion of either MCH or its receptor (Shimada *et al.,* 1998; Alon and Friedman, 2006). Hence this review will focus on published evidence concerning the dual roles of MCH in those discrete yet overlapping systems which modulate energy balance and reproductive function. The intriguing question as to whether the MCH system plays a role in the integration of energy homeostasis with the neuroendocrine reproductive axis and if so by what means has received limited coverage in

the literature (Smith and Grove, 2002, Williamson-Hughes *et al.,* 2005; Yang *et al.,* 2005) and current theories/evidence to date will be summarised here.

2.3 MCH, appetite regulation and energy expenditure

The pharmaceutical industry is currently very interested in targeting the MCH system as a potential therapeutic in the treatment of obesity because of its regulatory role on eating behaviours and energy expenditure. Of particular relevance are studies which report that deletion of either the MCH gene or the MCH receptor (MCHR-1) results in resistance to diet-induced obesity (Chen et al., 2002; Kokkotou *et al.,* 2005; Mashiko *et al.,* 2005;). Although two MCH receptor sub-types have been identified in humans (designated MCHR-1 and MCHR-2), research to date has focussed on MCHR-1 since MCHR-1 has not been detected in rodents (Tan *et al.,* 2002). Consequently research into the physiological relevance of MCHR-1 has been limited. The majority of preliminary intervention studies have been undertaken using rodent models and their relevance to human populations remains to be seen. For a review of factors pertinent to the interpretation and extrapolation of the results of rodent experiments see Young and Kirkland (2007). A summary of the results of the key studies is presented in Table 1.

Whilst the MCH system is unquestionably involved in the control of food intake, the specific effects of MCH administration or ablation are somewhat diverse depending upon empirical methods. Variations in diet, genetic background and environment may all contribute to the miscellaneous effects of MCH on eating behaviours and phenotype which have been reported in the literature. Chronic intracerebroventricular (ICV) infusion of MCH produced no difference in food intake and slightly increased body weight in mice maintained on a regular diet, whilst hyperphagia and substantially increased body weight were observed in those maintained on a high fat diet (Gomori *et al.,* 2002). Chronic ICV infusion of an MCHR-1 antagonist produced differing effects on food intake and phenotype depending on the genetic background of the animal (Mashiko *et al.,* 2005). Kokkotou and colleagues (2005), also reported that the effects of MCH ablation were strain-

specific. $MCH^{-/-}$ mice generated from an obesity-prone strain and from an obesity-resistant strain gained less weight and exhibited reduced fat mass on both high fat diets and regular chow than their wild-type counterparts. These results were amplified in the obesity-resistant strain which gained substantially less weight and exhibited increased energy expenditure when maintained on a high fat diet.

In agreement with the proposed influence of MCH on the reward aspect of feeding, Morens and colleagues (2005) reported that an MCHR-1 antagonist significantly reduced consumption of highly palatable food. This phenomenon may be partly explained by evidence of MCHR-1 expression in the arcuate nucleus, olfactory pathway and nucleus of the solitary tract of the CNS, suggesting a role for MCH in the integration of gustatory and olfactory sensory input (Kokkotou *et al.,* 2001; Saito *et al.,* 2001). Sakamaki *et al.* (2005) reported enhanced sucrose though not saccharin intake in MCH-treated rats (ICV injection). In contrast Baird and colleagues (2008) found that hindbrain administration of MCH produced no increase in licking for water, sucrose or saccharin. Similarly Zheng and colleagues (2005) reported that hindbrain infusions of MCH produced no effect on either food or water intake. Therefore it would appear that the orexigenic properties of MCH require forebrain input and that the MCH response to palatable food is related to the nutritive value of the food as well as to the hedonic aspects of feeding. Taken together these studies serve to illustrate the modal diversity through which MCH may exert its control on eating behaviours. They also exemplify the complexities of the neural control of ingestive behaviours and the essential contribution to energy homeostasis of indirect controls of feeding behaviours as first proposed by Smith (1996).

Results of genetic studies appear to consolidate the putative contribution of the MCH system to energy balance. Mice over-expressing the MCH gene in the lateral hypothalamus displayed a tendency to hyperphagia, obesity and impaired glucose homeostasis (Ludwig *et al.,* 2001). Ablation of the MCH gene led to hypophagia, increased metabolic rate and reduced body mass (Shimada *et al.,* 1998). In contrast disruption of the MCH receptor resulted in hyperphagia with a lean phenotype due to increased energy expenditure

(Chen *et al.,* 2002). It has been suggested that these differences in feeding behaviour may be attributable to ablation of NEI and NGE *(in the MCH knockout (k/o) model),* two distinct peptides which are cleaved from prepro-MCH (Pissios and Flier, 2003; Segal-Lieberman *et al.,* 2003). Whether or not this is the case commonality in these studies is manifest in the reported effects of MCH on energy expenditure. A lack of consistency in terms of the effect of MCH administration/ablation on eating behaviours contrasts with the reported uniformity of its effects on energy expenditure. In those studies reporting on energy expenditure where functional MCH or its receptor were ablated, all report that the animals demonstrated increased energy expenditure, some resulting from increased metabolic rate, others from increased locomotor activity or both. This property of MCH was clearly demonstrated by Segal-Lieberman and colleagues (2003). MCH expression is up-regulated in *ob/ob* mice lacking the leptin gene. Reasoning that MCH ablation in these mice would lead to reduced feeding and attenuate the obese phenotype, double-null animals were bred. Interestingly feeding behaviour was not affected: hyperphagia persisted in these animals even though body weight and fat mass were notably reduced. Increased energy expenditure was evidenced by elevated oxygen consumption and locomotor activity, improved thermogenesis and an increase in the expression of uncoupling protein-1 (UCP-1). The characteristic lean phenotype induced by functional MCH ablation is noteworthy since this property appears to be atypical of other orexigenic mediators such as the orexins (Hara *et al.,* 2005), NPY and AGRP (for reviews see Lin *et al.,* 2004; Flier, 2006) and serves to underline the distinctive role of MCH in energy conservation.

The propensity to conserve energy is more vigorously defended by the human organism than are those responses that resist obesity. In recent years our understanding of the mechanisms which control energy balance has deepened and the role of the hypothalamus as an integrative centre of both centrally and peripherally derived signals reflecting energy intake and stores has become apparent (for review see Schwartz *et al.,* 2000). Whilst initial studies have generated a solid body of evidence implicating a key position for MCH in the systemic regulation of energy homeostasis, specifically as a

promoter of positive energy balance, the specific molecular pathways through which it mediates its effects are yet to be fully elucidated. Evidence that MCH may be a modulator of autonomic nervous system activity has been reported (Astrand *et al.,* 2004; Messina and Overton, 2007). Increased basal heart rate which was independent of increased dark-phase body temperature and locomotor activity were observed in $MCH^{-/-}$ mice. These mice were also leaner and more resistant to diet-induced obesity than their wild-type counterparts. A switch to predominance of sympathetic over parasympathetic activity in MCHR-1 deficient mice lead to speculation that MCH is a significant regulator of autonomic nervous system control (Astrand *et al.,* 2004). In broad concord with these findings two recent studies found that centrally administered MCH induced bradycardia and reduced mean arterial pressure in rodents (Brown *et al.,* 2007; Messina and Overton, 2007). In the former study MCH was injected into the Nucleus Tractus Solitarius (NTS) whilst in the latter chronic ICV infusion not only induced the bradycardic and hypotensive response but the customary tachycardia which occurs in the dark-phase feeding was suppressed by acute infusion of MCH at a dose which would normally promote hyperphagia for 2 – 4 hours. Therefore the tandem consequences of acute MCH administration would appear to be consistent with energy conservation. Taken together these studies suggest that MCH is involved in the tonic suppression of sympathetic nervous system activity, though these results were inconsistent with earlier similar chronic infusion work with sheep (Parkes, 1996).

The production of uncoupling proteins in skeletal muscle and adipose tissue increases metabolic rate by inhibiting adenosine triphosphate (ATP) production thereby enhancing the rate of cellular respiration. There is some evidence that the production of uncoupling proteins in adipose tissue may be associated with MCH activity. Uncoupling protein-1, expressed in brown adipose tissue (BAT) in rodents stimulates thermogenesis by increased cellular respiration and heat production (Nicholls and Locke, 1984). Reports of reduced UCP-1 mRNA activity following chronic MCH ICV infusion and elevated UCP-1 expression in MCH-/- models may provide mechanistic evidence of the metabolic effects of MCH (Ito *et al.,* 2003; Segal-Lieberman

et al., 2003). In cold-exposed rats blockade of MCH lead both to increased BAT mass and UCP-1 expression; the magnitude of those increases were significantly greater than those induced by cold in intact rats. Hence in these conditions loss of functional MCH appears to result in the dysregulation of thermogenic mechanisms involved in energy expenditure (Pereira-da-Silva *et al.,* 2003). Consistent with these observations sympathetic innervation of BAT originates in particular hypothalamic regions including the MCH-rich LHA (Oldfield *et al.,* 2002). Presumed MCH-mediated lipogenesis in liver and white adipose tissue following chronic MCH infusion, in addition to reduced expression of 2 key enzymes in the beta-oxidation pathway (Ito *et al.,* 2003) would appear to augment the obesity-inducing effects of MCH.

Given the reported impact of MCH on both autonomic nervous activity and uncoupling activity in adipose tissue it would seem logical to investigate the presence of any neurochemical link between the MCH system and the adrenergic system, the latter being responsible for lipolysis in adipose tissue. However there is little published work in this area. In rat hypothalamic slices Bayer and colleagues (2005) report a hyperpolarising effect of noradrenaline on MCH neurones. Since the MCH-rich lateral hypothalamus receives inputs from noradrenergic neurones this group speculates that this inhibitory effect may be responsible for decreased energy expenditure due to diminished arousal and activity. More recently Kerman and colleagues (2007) mapped a group of pre-sympathetic premotor neurons which project polysynaptically to both skeletal muscle and adrenal gland and were thus hypothesised to have both autonomic and somatomotor functions. This group identified a distinct population of neurons which express MCH giving rise to the premise that MCH may be active in the co-ordinated response between skeletal muscles and autonomic activity in stress situations. This hypothesis is similar to the one proposed by Brown and colleagues (2007), (study discussed earlier), their finding that microinjections of MCH into the NTS induced bradycardic and depressor responses but that injection of a potent antagonist alone produced no response provoked the suggestion that MCH may only be released into the NTS in particular conditions, such as in stress situations when MCH would increase parasympathetic activity in order to negate the

deleterious effects of stress. Clearly such lines of enquiry require further investigation before more robust conclusions can be drawn but studies like these serve to highlight the extent of understanding yet to be gained regarding the MCH system.

Somewhat surprisingly given the metabolic significance of the MCH system only one study to date has examined the role of this peptide in the thyroid axis. *In vivo* plasma thyroid stimulating hormone (TSH) concentrations were reduced following ICV injection of MCH. *In vitro* both MCH and NEI were reported to inhibit release of thyrotropin releasing hormone (TRH) from hypothalamic explants. MCH did not affect basal TSH release from dispersed pituitary cell cultures though it did significantly inhibit TRH stimulated release of TSH from these cell lines (Kennedy *et al.,* 2001). Without further investigation the significance of these findings is limited though initial conjecture would point to a role for MCH in the maintenance of energy homeostasis via this circuit.

Although MCH is centrally expressed, there is some evidence that it circulates both in rodent (Bradley *et al.,* 2000; Stricker-Krongrad *et al.,* 2001) and in humans (Gavrila *et al.,* 2005). However there has been some published debate concerning the validity of detection methods in the latter (Waters and Krause, 2005; Takahashi, 2005). The physiological role of circulating MCH is unknown at present. Prepro-MCH mRNA and MCH have also been reported in various peripheral tissues; for example, rodent stomach, intestine and testis (Hervieu and Nahon, 1995). The MCH receptor (MCHR-1) has been detected in a limited range of tissues including skeletal muscle, eye, ovary and testis (for review see Schlumberger *et al.,* 2002). In humans, the MCH precursor has been identified in immune cells (Verlaet et al., 2002; Sandig et al., 2007) and MCH and its receptors transcripts have been detected in skin endothelia (Hoogduijn *et al.,* 2002). However the physiological relevance of peripherally derived MCH is largely unknown. Of particular relevance to this review, and in accord with the known effects of MCH on feeding behaviours, a growing body of evidence implicates both central and peripheral MCH in glucose homeostasis (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005; Pissios *et al.,* 2007). Short-term acute ICV

administration of MCH produced a dysregulation of glucose homeostasis; specifically, glucose intolerance, reduced glucose-stimulated insulin secretion and insulin resistance. These phenomena were unrelated to fluctuations in body weight though they were accompanied by modulation of insulin signal transduction (Pereira-da-Silva *et al.,* 2005). Perhaps less compelling, aging MCH $^{-/-}$ mice did not develop insulin resistance and impaired glucose tolerance compared to wild-type controls but this effect may well have been secondary to their sustained lean phenotype, increased locomotor activity and basal metabolic rate (Jeon *et al.,* 2006). Enhanced insulin sensitivity was also observed in double null mice (MCHR-1 knockout *ob/ob*) compared to *ob/ob* controls (Bjursell *et al.,* 2006), although both strains demonstrated comparable body weight gain, food intake, lipoprotein profile and energy expenditure. Both were severely obese. However the double null animals had higher locomotor activity, lower percentage body fat and increased lean body mass which could partly explain the improved insulin and glucose response in the double null animals. Nevertheless the fact that both types were severely overweight and differences in body composition were relatively modest would suggest a role for the MCH receptor in the regulation of glucose homeostasis. This conjecture is supported by a recent study which detected both MCH and its receptor in mouse and human pancreatic islets and in clonal β-cell lines (Pissios *et al.,* 2007). MCHR-1 was detected both in human and mouse islets though at comparatively low concentrations to that found in brain tissue, whilst substantially higher expression levels were detected in the β-cell line. MCH was also detected and localised in both human and mouse islets. Although the authors do not report expression of MCH and MCHR-1 specifically from β-cells the inference is clearly suggestive of the presence of MCH on those cells. Two further findings from this study are that: MCH k/o mice fed a high-fat diet demonstrated reduced β-cell mass compared to controls, this is in contrast to mice over-expressing MCH which demonstrated islet hyperplasia (Ludwig *et al.,* 2001); and exogenous MCH provoked increased insulin secretion both in cultured mouse and human islets and in mouse β-cell lines. Taken together these findings indicate a broader physiological role for MCH in the systemic regulation of energy homeostasis which invites further scrutiny.

Table 2.1 Key mammalian MCH energy studies

Reference	Species/model	Intervention	Effect on food intake*	Effect on energy expenditure (EE)/metabolic rate*	Effect on phenotype*
Increased availability of physiological MCH concentrations					
Qu *et al.,* 1996	Long Evans Rat	Acute ICV MCH infusion	Hyperphagia	NR	NR
Ludwig *et al.,* 2001	Male MCH-OE mice (MCH overexpression)	Genetic disruption, high fat diet	Hyperphagia	NR	Increased body mass and adiposity
Gomori *et al,* 2002	Male C57BL/6J mice	Chronic ICV infusion of MCH (a) regular diet (b) high fat diet	(a) No effect (b) Hyperphagia	NR	(a) Slightly increased body weight (b) Substantially increased body weight and fat mass
Santollo and Eckel, 2008	Male/female Long Evans rats	Chronic ICV infusion of MCH	Hyperphagia greater in males than females	↓ locomotor activity	NR
Decreased availability of physiological MCH concentrations					
Marsh *et al.,* 2002	Male/female MCHR-1 $^{-/-}$mice	Genetic disruption	Hyperphagia	↑ EE secondary to ↑ motor activity	Reduced fat mass, increased lean mass
Chen *et al.,* 2002	Male/female MCHR-1 $^{-/-}$mice	Genetic disruption	Hyperphagia	↑ EE in males, no difference in females	Reduced fat mass, constant lean mass
Mashiko *et al.,* 2005	Male diet-induced obese mice	Chronic ICV infusion of MCHR-1 antagonist	Hypophagia	NR	Reduced body weight and adiposity
Pereira-da-Silva *et al.,* 2003	Male Wistar rats	MCH protein synthesis blockade, cold exposure	No effect	None	Reduced body weight, increased fat mass, decreased lean mass

Reference	Species/model	Intervention	Effect on food intake*	Effect on energy expenditure (EE)/metabolic rate*	Effect on phenotype*
Bjursell *et al.*, 2006	Male MCHR-1$^{-/-}$ ob/ob mice (double-null)	Genetic disruption	Hyperphagia compared to WT but not to ob/ob	↑locomotor activity but not EE compared to ob/ob	Lower fat mass, increased lean mass compared to ob/ob but not to WT
Kowalaski *et al.,* 2006	Male/female diet-induced obese mice	Short-term oral administration of MCHR-1 antagonist Chronic oral administration of MCHR-1 antagonist	Hypophagia Hypophagia	NR NR	Reduced body weight and fat mass Reduced body weight and fat mass
Endogenous MCH absent					
Shimada *et al.,* 1998	Male/female MCH$^{-/-}$ mice	Genetic disruption	Hypophagia	↑metabolic rate	Reduced body weight and adiposity
Segal-Lieberman *et al.,* 2003	Male MCH$^{-/-}$ ob/ob mice (double-null)	Genetic disruption	Hyperphagia compared to WT no diff to ob/ob	↑ EE compared to ob/ob	Reduced body weight and adiposity
Alon and Friedman, 2006	Male/female MCH/ataxin-3 transgenic mice	Genetic disruption	None	↑ EE in males, not females	Late onset reduced body weight, adiposity length, lean mass
Jeon *et al.,* 2006	Male/female C57BL/6 MCH$^{-/-}$ mice	Genetic disruption	No significant difference	↑ REE + locomotor activity	Attenuation of weight gain Reduced fat mass

* in comparison to controls, NR: Not reported, ICV: Intracerebroventricular, EE: Energy Expenditure, REE: resting energy expenditure WT: wild-type mice, ↑ increased, ↓ decreased

2.4 MCH and reproductive function

Whilst the orexigenic effects of MCH have been well-documented, reports concerning the role of MCH in the reproductive axis have been less prolific. The female reproductive cycle is critically dependent on GnRH which stimulates the pulsatile release of LH. Over the last decade several groups have investigated a potential role for MCH in the regulation of LH release either directly at the level of the pituitary or indirectly via stimulation of hypothalamic GnRH. Early work indicated a stimulatory role for MCH on LH secretion when the peptide was injected into the hypothalamic medial preoptic area or median eminence of ovariectomised rats (Gonzalez *et al.,* 1997; Murray et al., 2000a). However injection into the third ventricle using a different rat model inhibited LH release (Tsukamura *et al.,* 2000). Disparity in empirical methods including diverse oestrogenic milieu and sampling methods were proposed as possible explanations for the conflicting results (Tsukamura *et al.,* 2000). Subsequent studies indicate that this neural control of LH release would appear to be both site-specific (within the hypothalamus) and oestrogen-status dependent. Murray and colleagues (2006) report that MCH administration induced either a stimulatory or inhibitory effect on LH release depending on both the injection site and endocrine milieu of the rat model.

In support of this work a potential neuroanatomical framework through which MCH may act to link energy balance to reproductive behaviours has been proposed and evidenced by the close apposition of MCH fibres to hypothalamic GnRH neurones (Smith and Grove, 2002; Williamson-Hughes *et al.,* 2005), and by the detection of the MCH receptor mRNA in GnRH neurones (Williamson-Hughes *et al.,* 2005). The latter group reported that 85 – 90% of GnRH neurones were in contact with MCH immuno-positive projections. This is in agreement with a recent study, albeit in males, which reported 86% of hypothalamic GnRH neurones were in close apposition with MCH-immunoreactive fibres. Some anatomical overlap was also observed between MCH-immunoreactive projections and GnRH neuroterminals in the median eminence (Ward *et al.,* 2009). *In vitro* Yang and others (2005) reported detection of MCHR-1 mRNA on GT1-7 cell lines (cell model of

hypothalamic GnRH neurones) indicating a functional role for MCH at GnRH nerve terminals though the stability of cultured lines should be considered.

Chiocchio and colleagues (2001) reported a stimulatory effect on both LH and Follicle Stimulating Hormone from pituitary explants but only in proestrous pituitaries. MCH has also been reported to moderate production of other pituitary hormones *in vitro*, namely human and rodent growth hormone (Segal-Lieberman *et al.,* 2006) and rodent TSH (Kennedy *et al.,* 2001). The detection of MCHR-1 in both human and rodent pituitary tissue further supports the significance of the MCH system in the hypothalamic-pituitary-gonadal axis (Saito *et al.,* 1999; Takahashi *et al.,* 2001; Segal-Lieberman *et al.,* 2006).

Speculation concerning a potential feedback loop involving oestrogen and MCH has been strengthened by the results of several recent studies. Conjecture that an oestrogen sensitive mechanism may be involved in the regulation of MCH gene expression has been investigated. Murray and others (2000b) report that oestrogen treatment inhibited the expression of prepro-MCH cell bodies specifically in the medial zona incerta of ovariectomised female rats. Similarly administration of 17-β estradiol depressed hypothalamic MCH gene expression in male rats (Mystkowski *et al.,* 2000). In ovariectomised primates oestrogen exposure provoked fluctuations in the synthesis of hypothalamic MCH and NEI which were coincidental with the LH surge indicating that MCH expression in the hypothalamus may be oestrogen dependent (Viale *et al.,* 1999). This notion of oestrogen/MCH cross-talk is also supported by evidence from in vitro studies which suggest that MCH activity is involved in cyclical GnRH release specifically at proestrous; that is, an oestrogen-rich milieu (Chiocchio *et al.,* 2001, Gallardo *et al.,* 2004). However Murray and colleagues (2000b) report no difference in prepro MCH mRNA expression between ovariectomised and intact rats at either proestrous or dioestrous suggesting that the impact of other ovarian products in this context should also be evaluated. Although MCH neurones do not express the oestrogen receptor there is considerable anatomical overlap in the distribution of oestrogen receptor (ERα) labelled cells and MCH neurones in the paraventricular nucleus, zona incerta and

lateral hypothalamus, which is suggestive of an indirect regulatory mechanism, possibly via an intermediary neurone or ligand which consequently modulates MCH neuronal activity (Muschamp and Hull, 2007).

Speculation that MCH may be involved in the regulation of peripheral reproductive organ function is supported by the detection of the MCH receptor in human ovary, human testis (Mori *et al.,* 2001) and mouse testis (Kokkotou *et al.,* 2001). Additionally retrograde viral transneuronal tracing from the rat ovary resulted in the detection of virus-infected neurones in the MCH-rich lateral hypothalamus and zona incerta though with less intensive labelling than that observed in the paraventricular nucleus (Gerandai *et al.,* 1998). Whilst there appears to be a neuronal connection between MCH-producing areas of the brain and the ovary and MCH mRNA has been detected in human and rat testis (Viale *et al.,* 1999; Hervieu and Nahon, 1995) a potential role for circulating MCH in the reproductive system has not been investigated.

Clearly the neurocircuitry controlling the hypothalamic-pituitary-gonadal axis is complex and other neuromodulators are known to be involved: leptin, serotonin, dopamine and recently kisspeptin and NEI (cleaved from MCH precursor) for example have all been reported to stimulate gonadotropin release (Yu *et al.,* 1997; Vitale *et al.,* 1993; Mackenzie *et al.,* 1984; Messager *et al.,* 2005; Attademo *et al.,* 2006). Precisely where MCH fits into this neurocircuitry is yet to be defined though it has been proposed that leptin may act through MCH, since administration of MCH antisera into the medial preoptic area (mPOA) prevented an expected leptin-induced rise in LH. Additionally melanocortin 5-R antagonists previously shown to prevent the stimulatory effect of MCH on LH release exerted a similar effect on leptin when injected into the zona incerta (Murray *et al.,* 2000c).

To date the role of MCH in male reproductive physiology has been largely neglected. Early work reported the detection of MCH mRNA at the periphery of testicular rat seminiferous tubules and in isolated mice Sertoli cells (Hervieu and Nahon, 1995), whilst a subsequent study localised these products in rodent immature germ cells, spermatagonia and early

spermatocytes (Hervieu et al., 1996). The latter group report similar staining in spermatagonia and early spermatocytes in the testicular tissue of a human adult (Hervieu *et al.,* 1996). This line of enquiry however has not been pursued.

2.5 MCH, energy balance and fertility

The threat to human health imposed by the obesity epidemic continues to grow in England and the UK (NHS Information Centre, 2010) whilst both under and over-nutrition in developing and developed countries impacts negatively on reproductive health (ESHERE Capri Workshop Group, 2006). In the case of over-nutrition this applies to both male and female fertility. Adipose tissue is no longer regarded as merely an energy depot, rather it has emerged as an endocrine organ with an important regulatory function in energy homeostasis (Bradley *et al.,* 2001; Ahima, 2006). Dysregulation of adipose tissue (obesity) is a major risk factor in the development of chronic and potentially fatal health conditions, such as certain cancers, cardiovascular disease, hypertension, stroke and type 2 diabetes (WHO, 2003). On the other hand menstrual dysfunction, poor fecundity and pregnancy complications are consequences of malnutrition at either end of the energy balance spectrum whilst obesity is a risk factor for miscarriage (ESHERE Capri Workshop Group, 2006).

To date the dual roles of MCH in energy homeostasis and reproductive function have been explored principally via animal models and *in vitro* work. The focus has been on hypothalamic MCH; the role of circulating MCH remains largely unexplored though as previously noted there is evidence that the peptide circulates both in rodent and in humans. As discussed in Sections 2.3 and 2.4 it would appear that hypothalamic MCH is well placed to act as an interneurone participating in both energy and reproductive pathways since a) it lies downstream of mediobasal hypothalamic neurones responsive to changes in peripheral energy stores and b) may act directly or indirectly on GnRH (Gonzalez *et al.,* 1997, Murray *et al.,* 2000a; Williamson-Hughes *et al.,* 2005; Ward *et al.,* 2009). However little is known about the

role of circulating MCH in mammals or in humans in either context and since evidence suggests that MCH is active in the periphery it is now important to fully understand whether its peripheral actions are reflective of or dissociated from its central actions.

Nutritional status impacts on reproductive physiology and in many mammals on reproductive behaviours thereby optimising both reproductive success and the ability to meet energetic demands. The complex control system entailing integration of direct or indirect peripheral stimuli with central effector systems involves numerous hormonal and chemical mediators, neuropeptides, neural pathways and detection systems. Hormones and neuropeptides, such as MCH, may act in this domain in one of two ways: a) by operating as ***mediators*** between feeding behaviours and reproductive function (that is, when variability in metabolic fuel availability induces altered hormonal response and the consequent modulation of feeding behaviours and reproductive function) and b) by operating as ***modulators*** affecting the availability of oxidisable fuels thereby influencing reproductive behaviours (Schneider, 2004). Though MCH is active both in the ingestive and in the reproductive contexts, investigation into the metabolic pathways through which it may integrate these dual functions has been limited. The tendency to favour male models in energy balance research (Ludwig *et al.,* 2001; Siegal-Lieberman *et al.,* 2003; Mashiko *et al.,* 2005; Bjursell *et al.,* 2006) and female models in reproductive studies (Murray *et al.,* 2000b; Garcia *et al.,* 2003; Williamson-Hughes *et al.,* 2005; Messina *et al.,* 2006) may be problematic for intergender extrapolation of results. In metabolic studies where both genders have been subject to the same protocol, some sexual dimorphism has been reported. For example increased energy expenditure was observed in both male MCHR-1$^{-/-}$ and male transgenic mice with temporal loss of MCH though not in their female counterparts (Chen et al., 2002; Alon and Friedman, 2006). Since in most species the energy cost of procreation for females far exceeds that of males a potential gender difference in MCH action would merit investigation.

The anorectic consequences of oestrogen treatment are well substantiated (Wade, 1986; Geary, 2000; Eckel, 2004). Expanding on earlier work

investigating the modulatory link between oestrogen and hypothalamic MCH expression (Murray *et al.,* 2000b, Mystkowski *et al.,* 2000), a potential interaction between oestrogen and MCH which may modulate ingestive behaviours has recently been investigated with somewhat conflicting results (Tritos *et al.,* 2004; Messina *et al.,* 2006). Whilst Tritos and colleagues (2004) report that oestradiol-induced anorexia is not mediated via leptin or MCH, Messina and colleagues (2006) found that oestradiol treatment decreased MCH-induced food intake. Santollo and Eckel (2008) also report both exogenous and endogenous estradiol exerts a reductive effect on MCH-induced feeding via decreased meal size. They also report a difference in the magnitude of the orexigenic effect of MCH between differing stages of the oestrous cycle, these effects being attenuated in oestrous rats (following high oestradiol secretion) compared to dioestrous rats. Morton and colleagues (2004) reported that pharmacological doses of oestrogen block the increase in hypothalamic MCH expression customarily induced by caloric restriction. The interesting hypothesis raised by the results of these recent studies, is that the characteristic changes in feeding behaviours at different stages of the oestrous cycle in female rats may partly be mediated by altered MCH signalling (Messina *et al.,* 2006). The conclusion by Tritos and others (2004), that MCH is not involved in oestrogen-induced anorexia since MCH k/o mice displayed significant hypophagia and weight loss in response to oestrogen treatment does not take into account the effects of the multiple anorexigenic mediators which also work in concert with oestrogen to induce anorexia (Messina *et al.,* 2006). Additionally it is unclear as to what extent compensatory mechanisms are initiated in transgenic animals.

Pregnancy and lactation present considerable physiological challenges to the organism. Lactation in particular is characterised by substantially increased energy demands and a marked increase in food intake in rats (Oftedal, 2004). The exaggerated feeding response in the rat model (compared to humans) (Dewey, 1998) may provide valuable insights into the framework in which MCH and other metabolic modulators may operate within the context of hyperphagia, increased energy demands and negative energy balance. Investigation into this area has been limited and has produced somewhat

diverse results. Sun and colleagues (2004) report increased hypothalamic MCH expression in lactating rats 11 – 12 days postpartum though no demonstrable increase in circulating MCH. Conversely Garcia and others (2003) report decreased hypothalamic MCH mRNA expression in pregnant and lactating rats. More recently mice in whom a time-dependent ablation of MCH neurones was orchestrated were not capable of rearing their offspring (Alon and Friedman, 2006), indicating a role for MCH in lactation though $MCH^{-/-}$ mice are fertile, at least when crossed with wt/C57BL/6 mice (Shimada *et al.,* 1998). These observations along with the observation by Knollema and others (1992) that MCH expression is transiently induced in the preoptic area (POA) of the hypothalamus 8 – 21 days postpartum and at no other time pose some interesting questions as to the scope of MCH in the regulation of energy balance during lactation.

2.6 Summary

MCH is a comparatively recently discovered anabolic neuropeptide. The portfolio of its multiple and diverse physiological functions; both metabolic and behavioural, continues to expand. Over the last decade or so its role as a crucial mediator of energy balance has become apparent. The property of central MCH to stimulate food intake is shared by a number of other effector molecules, however this property appears to be secondary to its role in energy conservation. Ablation of functional MCH or its receptor leads to a lean phenotype regardless of inconsistency in its reported effects on feeding behaviours though remarkably the lean phenotype is often accompanied by hyperphagia. The lean phenotype is attributed to increased energy expenditure, manifest as increased locomotor activity, metabolic rate or both. Hence the development of specific MCH antagonists is an attractive proposition for pharmaceutical companies. Whilst a solid body of evidence implicates MCH as a key regulator of energy balance the precise mechanisms and molecular pathways through which it operates are yet to be fully defined. Additionally the functionally distinct and overlapping roles of MCH and its two co-precursor molecules, NEI and NGE are not yet fully delineated. Whereas the role of central MCH has received considerable

scrutiny, the source, targets, expression and functional roles of peripheral and circulating MCH are largely unexplored though emerging evidence suggests a role for the peptide in the hypothalamic-pancreatic, hypothalamic-pituitary-thyroid and hypothalamic-pituitary-gonadal axes.

The known activities of MCH in the reproductive axis are chiefly those associated with the regulation of LH release and the impact of oestrogen on MCH expression. MCH may stimulate or inhibit LH release depending on oestrogenic milieu and MCH expression in the hypothalamus may be oestrogen dependent. Furthermore in female rats cyclic modulation of feeding behaviours may be partially mediated by altered MCH signalling. The complexities of the inter-relationship between ingestive and reproductive behaviours are slowly being unravelled and MCH is undoubtedly a key component of this neurocircuitry, though the extent and scope of its molecular interactions are still being documented. As the MCH story unfolds questions are both answered and posed, manipulation of the availability of MCH and its receptor in different animal models has provided some important insights into its physiological relevance. Based on evidence to date it is feasible that the MCH system may provide a link between behavioural aspects of ergotropic vigilance, trophotropic impulse and pleasure drive. The behavioural aspects of energy balance/reproduction in free-living situations may be importantly impacted upon by MCH. Clearly in order to understand these complex phenomena, it is now essential to explore the precise and comprehensive biological role of the MCH system in humans, with particular reference to its largely uncharacterised peripheral and circulatory roles.

3. Materials, Subjects and Methods

3.1 Radioimmunoassay (RIA) to measure MCH in plasma

3.1.1 Reagents for MCH RIA

The following reagents were used in the MCH RIA:

- Buffer 1: see formula in Section 3.1.1.1
- Buffer 2: see formula in Section 3.1.1.2
- Purified MCH, # H1482, Lot 1006273, Bachem, Switzerland
- ([I^{125}]-MCH), # NEX373010UC, PerkinElmer Inc., USA
- Primary antibody: Anti-MCH Antibody, # M8440, Sigma-Aldrich, UK
- Normal Rabbit Serum (NRS), #S-5000, Vector Laboratories Ltd, UK
- Secondary antibody: Anti-Rabbit Sac-Cel, #AA-SAC1, IDS Ltd, UK
- Bovine Serum Albumin: # A3294, Lot 045K0662 Sigma-Aldrich, UK

3.1.1.1 Buffer 1

Add the following reagents to 1L distilled water:

- 0.114 g NaH_2PO_4 H_20
- 1.30 g Na_2HPO_4
- 9.00 g NaCl
- 1.00 g Na azide

Stir until all reagents are fully dissolved then add 1.00 g Bovine Serum Albumin, stir until fully dissolved, adjust pH to 7.4

3.1.1.2 Buffer 2

Add 9.306 g ethylene diamine tetraacetic acid (EDTA) to 1L Buffer 1 ,

stir until fully dissolved, adjust pH to 7.4

3.1.2 RIA Protocol

The development and validation of a RIA to measure MCH in human plasma is described in Chapter 4. Following establishment/optimisation standard curves were derived from a 3 day protocol as follows:

Day 1: A stock solution of MCH was serially diluted with Buffer 1 to give a range of concentrations (12.5 pg/ml – 10 ng/ml). The standard curve was prepared in triplicates and included total count (TC), non-specific binding (NSB) and B_o tubes. As outlined in Table 3.1, Buffer 1 was added to all tubes except the TCs. A 1:300 Buffer 1:Normal Rabbit Serum (NRS; v:v) solution was prepared. This solution was added to each of the NSB tubes (100 μl/tube) and then used to prepare the primary antibody solution. An initial dilution of 1:30,000 Buffer 1 with NRS:MCH antibody solution was prepared and 100 μl added to each tube except TC and NSB. After vortexing all tubes were incubated overnight at 4°C.

Day 2: Radio-labelled MCH (I^{125}MCH) was diluted with assay Buffer 2 to produce 10,000 counts per minute in 100 μl (cpm). The prepared radiolabelled MCH was added to all tubes which were then vortexed and incubated overnight at 4°C.

Day 3: One hundred microlitres of a cellulose suspension coated with anti-rabbit IgG (secondary antibody) was added to all tubes, except the TCs, and incubated at room temperature for 30 mins. Deionised water (1000 μl) was added to all tubes, except the TCs, and then all the tubes were immediately centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was aspirated and the radioactivity of the remaining pellet measured by gamma counter (Wallac Wizard 1470, PerkinElmer Inc, USA).

Table-3.1 RIA Protocol: Summary of volume of reagents and/or unknown added to each tube per day

	Day 1					Day 2	Day 3
Tubes	**MCH standard**	**Unknown**	**Buffer 1**	**NRS**	**AB 1 in NRS**	**I^{125} MCH**	**AB 2**
TC	-	-	-	-	-	100 µl	-
NSB	-	-	200 µl	100 µl	-	100 µl	100 µl
B_0	-	-	200 µl	-	100 µl	100 µl	100 µl
Standards	100 µl	-	100 µl	-	100 µl	100 µl	100 µl
Unknowns	-	100 µl	100 µl	-	100 µl	100 µl	100 µl

AB: antibody, TC: total count, NSB: non-specific binding, B_0: total binding, NRS: normal rabbit serum

Standard Curves were plotted using Assayzap software (Biosoft, Cambridge). Assayzap is a flexible programme which selects the most appropriate fitting method for the shape of a specific standard curve.

3.2 Subjects and experimental protocol

Conditions for experimental procedures were standardised for all studies as follows:

3.2.1 Ethical Approval

All experimental protocols described in Chapters 4, 5, 6 and 7 were approved by the University of Westminster Ethics Committee, (Ethics Application Number 06/07/29) in January 2007 (Appendix 1). All participants were volunteers and received no financial incentive to participate. All volunteers received a copy of an Informed Consent Form and a Subject Information Sheet (examples Appendices 2 & 3) prior to attendance in the laboratory to ensure full participant awareness of all experimental procedures. All volunteers signed a copy of the Informed Consent Form prior to participation which was counter-signed by a witness. Data collected by other researchers

at the University of Westminster has been included in the present analyses (Chapters 4 and 7), for which separate ethical approval was obtained.

3.2.2 Pre-test

Male and female subjects were recruited by advertisement in London morning and evening newspapers and by posters. All subjects completed a pre-test Medical Screening Questionnaire (Appendix 4) which was checked by researchers. Any volunteer whose completed Medical Screening Questionnaire indicated that they were not medically suitable to be involved in the research was not allowed to participate.

3.2.3 Anthropometric measurements

Subjects were weighed without shoes in indoor clothing on digital scales, measurement accuracy ± 0.1 kg, (Seca 780 231 7008, Gmbh & Co, Germany). Standing height was measured by stadiometer (Holtain Limited, Crymych, Dyfed, UK) and recorded to the nearest 0.1 cm. Waist and hip circumference were measured at the narrowest part of the torso and widest part of the buttocks and the waist-hip ratio (WHR) was calculated. Body Mass Index (BMI) was calculated using the equation weight (kg) / [height (m)]^2.

3.2.4 Resting metabolic rate

Subjects were fasted and instructed not to take exercise before attendance at the laboratory at 09.00am. Resting metabolic rate (RMR) was measured using an open system indirect calorimeter (Deltatrac II Metabolic Monitor, Datex Instrumentarium Corp., Helsinki). After standard calibration, subjects were required to lie prone on a bed for 40 minutes having a ventilated hood placed over head and shoulders to facilitate the measurement of pulmonary gas exchange. The ratio of oxygen consumed to carbon dioxide produced was used to calculate energy expenditure and hence metabolic rate. Once prone subjects were instructed to limit movement and to remain awake. The following conditions were observed:

- Noise in the laboratory was kept to a minimum and subjects listened to meditation music (Sleep, Nukarma) via headphones.
- Screens were placed around the beds in order to limit visual stimuli.
- Laboratory lights were turned off.

RMR expressed as kcal/day was calculated using the Weir equation (1949):

$((3.941 \times VO_2) + (1.11 \times VCO_2)) \times 1.44$

To allow for acclimatisation the first 20 minutes of the continuous measurements were excluded. Data collected in the last 20 minutes of the collection period were used to calculate RMR.

3.2.5 Body composition measurements

Total body fat mass and total body lean mass were measured by air displacement plethysmography (Bod Pod: Version 4.1, Body Composition System; Life Measurement Instruments, Concord, CA). After standard calibration subjects were weighed on the Bod Pod electronic scales wearing swim-wear or tight fitting underwear. Hair was covered in a close fitting cap and jewellery removed. Subjects were then required to sit in the Bod Pod chamber whilst 3 x 40 second tests were conducted during which subjects were instructed to limit movement and breathe normally. The Bod Pod calculates body fat percentage using the Siri equation (Siri, 1956). Body density is derived from body volume measured in the chamber and body weight in air.

3.2.6 Blood collection

All blood samples were taken by a trained phlebotomist. Venous blood samples were collected into 10 ml vacutainers™ primed with EDTA (unless otherwise stated), (Becton Dickinson Medical, UK) and immediately centrifuged for 12 minutes, 3000 rpm, at room temperature to obtain plasma. Each plasma sample was aliquotted into 3, decanted into 5ml sterilin tubes (Z5PE, Sterlin Ltd, UK) and frozen at -20°C. Fingerprick blood samples were obtained and immediately analysed for blood glucose concentrations using

the Hemocue Glucose 201+ Analyser (Hemocue AB, Sweden), measurement range 0 – 22.2 mmol/l. The intra-assay coefficient of variation (CV), calculated using EurotrolGlucoTrol-NG control solution was < 1.8% at a range of concentrations (Rajashekar, 2009).

3.2.7 Plasma hormone measurements

All plasma samples were frozen (-20ºC) at time of collection and were defrosted at room temperature for 10 – 15 minutes prior to assay.

3.2.7.1 MCH RIA

Where possible plasma samples were used which had not previously been thawed and refrozen. Plasma MCH concentrations were measured using the in-house competitive radioimmunoassay described in Section 3.1.2

3.2.7.2 Insulin RIA

Plasma insulin concentrations were measured using a commercially available insulin RIA kit (DSL-1600, Diagnostic Systems Inc, USA). The assay sensitivity was 1.3 µIU/ml, the intra-assay CV was 8.3% at 4.8 µIU/ml and 6.4% at 54.6 µIU/ml.

3.2.7.3 Leptin RIA

Plasma leptin concentrations were measured using a commercially available leptin RIA kit (HL-81HK, Millipore, USA). The assay sensitivity was 0.5 ng/ml, the intra-assay CV was 8.3% at 4.9 ng/ml and 3.4% at 25.6 ng/ml.

3.2.7.4 Progesterone RIA

Plasma progesterone concentrations were measured using a commercially available progesterone RIA kit (Progesterone double antibody ^{125}I kit, # 170102, MP Biomedicals, USA). The assay sensitivity was 0.2 ng/ml, the intra-assay CV was 11.9% at 0.79 ng/ml and 2.3% at 17.6 ng/ml.

The above commercially available assays i.e. leptin, insulin and progesterone RIAs were performed as per manufacturer's instructions except for the following modification: recommended volumes of all reagents and unknowns

were halved, e.g. where manufacturer's instructions indicated that 100 µl volume should be used, 50 µl volume was substituted.

4. Development and validation of a radioimmunoassay to measure MCH in plasma

4.1 Introduction

Although circulating MCH has been detected in both rodent (Stricker-Krongrad *et al.,* 2001) and human plasma (Gavrila *et al.,* 2005), the physiological relevance of circulating MCH remains largely unexplored at present. The requirement for further investigation into the role of circulating MCH has been publicly acknowledged (Pissios *et al.,* 2007). There has been published debate concerning the validity of detection methods in the only human study to describe circulating MCH (Mantzoros, C., 2005; Waters and Krause, 2005). Specifically a discrepancy in reported serum MCH concentrations between their cross-sectional (97.8 ± 22.8 pg/ml) and intervention studies (~ 20 pg/ml) and their failure to validate the commercially available RIA for plasma and serum samples were causes for concern (Takahashi, 2005; Waters and Krause, 2005). Therefore the first objective of this study was to develop and validate a competitive radioimmunoassay (RIA) for the detection of MCH in human plasma. A series of experiments were carried out to establish the optimal conditions for a RIA for MCH; including determining the optimal primary antibody concentration, range of sensitivity of the curve, specificity of detection, the effects of anti-coagulants in plasma collection containers and the effects of storage conditions on the stability of MCH in plasma. Once satisfactory results had been achieved a sample population was recruited in order to establish a reference range.

4.2 Material and Methods

Unless otherwise specified all reagents were described in Section 3.1.

4.2.1 Antibody concentration

Approximately 30% total binding affords the best competitive conditions for peptide hormones in a RIA. To determine the optimal antibody concentration, which yields ~ 30% total binding in the RIA, a series of antibody titre curves

were performed. The initial antibody titre curves were derived from antibody concentrations ranging from 1:100,000 to 1:320,000. After refinement antibody concentrations ranging from 1:20,000 to 1:50,000 were assessed (n=5). The titre curves were prepared in triplicate and included TC and NSB tubes. **Day 1**: As outlined in Table 4.1, a 1:300 NRS: Buffer 1 (v:v) solution was prepared. This solution was then used to prepare serial dilutions of the MCH antibody (ASB) to give a range of concentrations. Buffer 1 (200 µl) was added to all tubes except TCs. NRS:Buffer 1 solution (100 µl) was added to NSB tubes. Serially diluted ASB (100 µl) was then added to all tubes except TCs and NSBs. Radiolabelled MCH (1^{25}MCH) was diluted with assay Buffer 2 to produce 10,000 cpm in 100 µl. The prepared radiolabelled MCH was added to all tubes which were then vortexed and incubated overnight at 4ºC. **Day 2:** As Day 3 RIA Section 3.1.2. The resultant anti-body titre curves were plotted (software as per section 3.1.2) and were used to determine the optimal antibody concentration.

Table 4.1 Antibody Titre Protocol: Summary of volume of reagents added to each tube per day

Tube	**Day 1**				**Day 2**
	Buffer 1	**NRS:Buffer 1**	**ASB**	**I^{125} MCH**	**Antibody 2**
TC	-	-	-	100 µl	-
NSB	200 µl	100 µl	-	100 µl	100 µl
Serial dilutions	200 µl	-	100 µl	100 µl	100 µl

TC: total count, NSB: non-specific binding, NRS: normal rabbit serum, ASB: antisera buffer

4.2.2 Sensitivity of the RIA

A series of dilution curves of purified MCH in Buffer 1 were run to establish the minimal detection limit and range of MCH concentrations (n=15). The protocol and range of concentrations were as described in Section 3.1.2.

4.2.3. Specificity of detection

To determine possible cross-reactivity, a series of dilution curves (range 0.1 pg – 0.1 mg) of biomolecules reported to have a competitive or agonistic relationship with MCH were assayed: namely, human atrial natriuretic peptide (ANP: #A1663, Lot 066K4805, Sigma-Aldrich, UK) (Hervieu *et al.,* 1996); human α-melanocyte-stimulating hormone (α-MSH: #H1075, Lot 1008241, Bachem, Switzerland) (Barber *et al.,* 1987; Ludwig *et al.,* 1998); human adrenocorticotrophin (ACTH: #H1160, Lot 1003985, Bachem, Switzerland) (Baker *et al.,*1985) and Neuropeptide–E-I-MCH (NEI-MCH: #H4714, Lot 0561279, Bachem, Switzerland) (Maulon-Feraille *et al.,* 2002). A standard curve for MCH was run with the biomolecule dilutions treated as unknowns and plotted for comparison (n=2).

4.2.4 Dilution curve of plasma

Non-fasting human blood was collected from 2 sources and immediately centrifuged to obtain plasma. The plasma was added to a series of dilution curves (0.49 – 125 pg/ml) to determine whether the curves with plasma paralleled the standard curve (n=4). The dilution curves were obtained using the same method as for the RIA standard curves (Section 3.1.2) except 100 µl of plasma was substituted for Buffer 1 in the standard tubes and the NSB and B_0 tubes received 100 µl Buffer 1 + 100 µl plasma instead of 200 µl Buffer 1.

4.2.5 Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Mass Spectrometry (MS) (performed by Tania Murphy, PhD)

Cross–validation was conducted by RP-HPLC using a modified version of the method described by Maulon-Ferraille *et al.,* (2002). A series of plasma: 0.1 N HCl dilutions were subject to RP-HPLC to determine the optimum dilution for detection (n=1). The optimum dilution for detection was 1:9 plasma: 0.1 N HCl (v:v). The mixture was then centrifuged at 4°C and 1000 rpm for 10 minutes. The supernatant was removed and subjected to HPLC in a RP column (C18 Phenomenex, UK) with a gradient 20 – 60% (0.1%

trifluoroacetic acid in HPLC water: acentonitrile) for 60 minutes at a flow rate of 0.5 ml/min. Purified MCH was serially diluted and treated similarly for comparison. MCH was detected using UV absorbance at 230 nm. Protein fragments obtained by RP-HPLC were subject to MS for determination of analyte mass. Mass analysis was performed by single quadropole mass spectrometric detector (Dionex MSQ Plus, Dionex Corp., Massachusetts, USA) and MS data analysis was performed by Chromeleon LC/MS software (Dionex Corp., Massachusetts, USA).

4.2.6 RP-HPLC and RIA

To consolidate immunodetection of MCH in plasma by RIA, plasma collected in EDTA tubes and diluted in the ratio 1:9 plasma:0.1 N HCl (v:v), a 1:9 dilution of buffer:HCl in EDTA (v:v) and purified MCH were subject to RP-HPLC as described in Section 4.2.5. Fractions were collected at 1 minute intervals and the aliquots analysed by RIA as described in Section 3.1 (n=1).

4.2.7 Stability of MCH in plasma

Plasma was subjected to a number of storage conditions to test the stability of MCH in plasma determined by RP-HPLC. Briefly before plasma samples were processed by RP-HPLC as described in Section 4.2.5, they were left at: a) room temperature for 1 hour; b) 4°C for 1 hour; c) room temperature overnight; d) 4°C overnight; e) -20°C overnight; and f) -20°C before being thawed, refrozen and thawed again (n=1). Samples were checked against a freshly prepared sample.

4.2.8 Collection Methods

To determine the effects of different reagents customarily found in blood collection tubes on the detection of MCH in plasma by RP-HPLC, non-fasting blood samples from the same individuals were collected in vacutainers™ containing:

a) lithium heparin: anti-coagulant (green cap)

b) silica + gel: clot activator + gel for serum separation (orange cap)

c) fluoride oxalate: anti-coagulant and enzyme inhibitor (grey cap)

d) EDTA: anti-coagulant (lilac cap)

e) sodium citrate: reversible anti-coagulant (light blue cap)

The samples were processed as described in Section 4.2.5 (n=2).

4.2.9 Subjects

In order to establish a reference range for circulating MCH, fasting blood samples collected by other researchers at the University of Westminster were included in the present analyses. The researchers were Fawaz Alzaid, (MSc 2008/09), Barbora Doslikova (BSc (Hons) 2008/09) and Marcela Brito (BSc (Hons) 2009/10). Anthropometric measurements and fasting blood samples were collected from 197 volunteers in addition to those described in Chapter 6. Data from volunteers who participated in the studies described in Chapters 5 and 7 were not included. The recruitment methods for volunteers were similar to those described in Section 3.2.2. Blood collection, treatment and plasma storage methods were the same as those described in Section 3.2.6. Plasma MCH concentrations were measured using the in-house competitive radioimmunoassay described in Section 3.1.2. Anthropometric measurement methods were the same as those described in Sections 3.2.3 and 3.2.5. In total 242 male and female subjects were tested, of these 10 were excluded since it was not possible to obtain a fasting blood sample from them, 1 was excluded as his plasma MCH concentration was > 4 SD from the mean.

4.3 Statistical analyses

The inter-assay coefficient of variation was calculated using the following formula: CV = SD/[mean] x 100. Independent samples t-tests were used to establish inter-gender differences between anthropometric characteristics and circulating MCH concentrations. A one way between groups ANOVA was conducted to determine if there was an effect of body composition on

plasma MCH concentrations. The post-hoc test was performed using Tukey's adjustment for multiple comparisons. Associations between fasting plasma MCH concentrations and body composition parameters were determined by Pearson product-moment correlational analysis. Data were analysed using the Statistical Package for the Social Sciences (SPSS version 16.0 for Windows; Chicago, IL, US).

4.4 Results

4.4.1 Antibody concentration

The optimal antibody concentration which yielded ~30% binding was established as 1:30,000 (Figure 4.1). This concentration was used in the MCH RIAs described in Chapters 4, 5, 6 and 7.

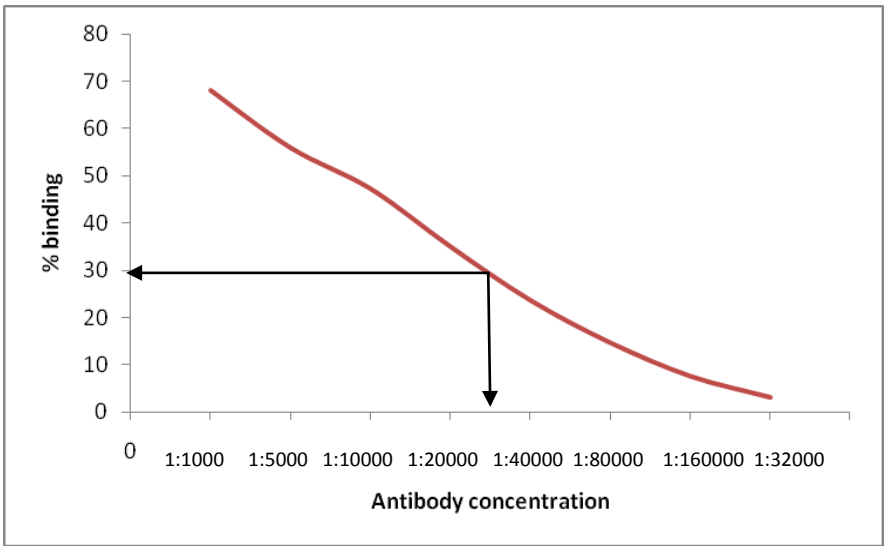

Figure 4.1: Example of antibody titre curve illustrating optimal antibody concentration

4.4.2 Sensitivity and repeatability of the RIA

The effective range was established as 19.5 – 1248 pg MCH/ml. This was taken as the level of detection in the assays that were used to measure MCH described in Chapters 4, 5, 6 and 7 (Figure 4.2). At 39 pg/ml and 156 pg/ml the inter-assay coefficient of variation was approximately 3.7%.

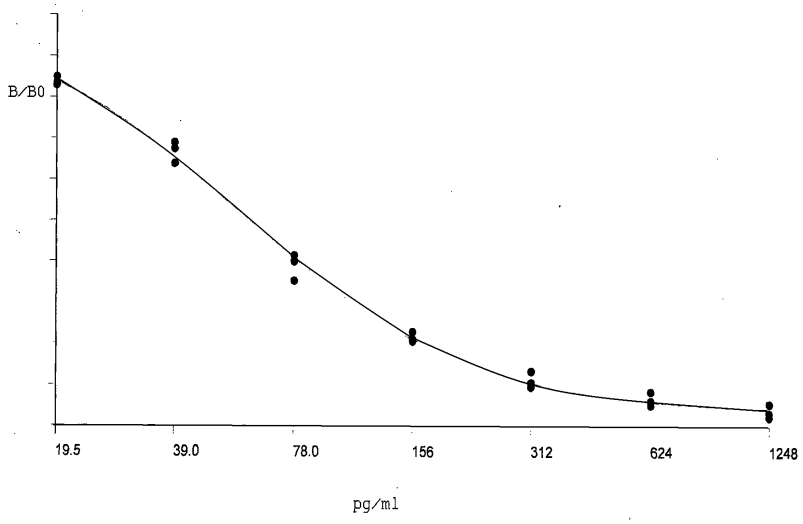

Figure 4.2: An example of a typical standard curve for MCH using 1:30000 antibody concentration: range 19.5 – 1248 pg/ml

4.4.3 Specificity of detection

Serial dilutions of ANP, α-MSH and ACTH failed to displace the MCH antibody. Only NEI-MCH at supraphysiological concentrations showed any potential for cross-reactivity with the MCH antibody (Figure 4.3).

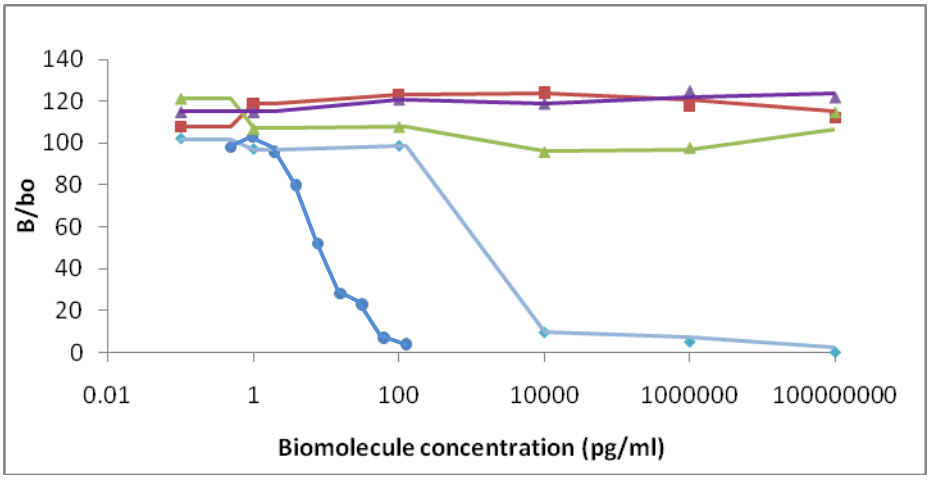

Figure 4.3: Standard curve for MCH ● + dilution curves for ANP ■ α-MSH ▲ ACTH ▲ NEI-MCH ◆

4.4.4 Dilution curve of plasma

Serial dilutions of two human plasma samples confirmed that the curves were parallel to the standard curve (Figure 4.4).

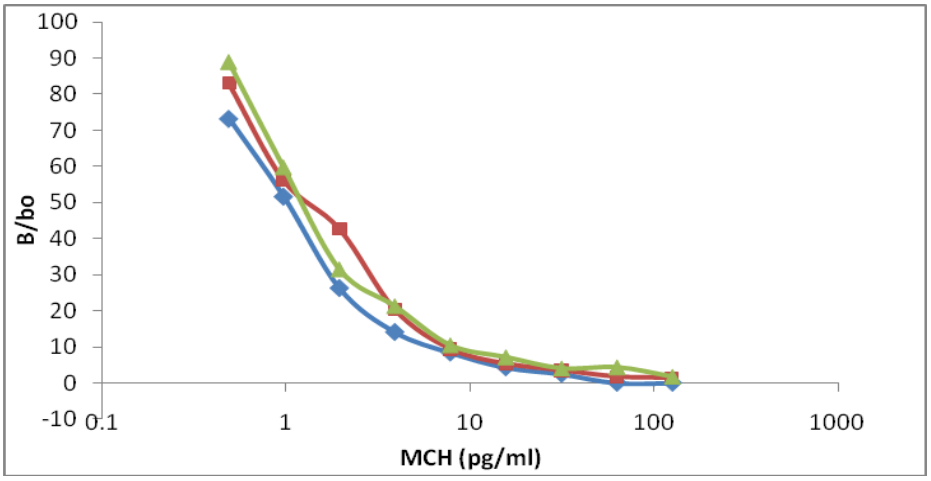

Figure 4.4: Standard curve for MCH + standard curve for MCH with plasma MCH ◆ Plasma A ■ Plasma B ▲

4.4.5 Reverse Phase-High Performance Liquid Chromatography and Mass Spectrometry

Purified MCH was detectable in the range 1 µg/ml - 1 mg/ml (see calibration curve, Figure 4.5). The retention time for MCH was 22.3 minutes on a RP C18 column, with a gradient of 20 - 60% (0.1% trifluoroacetic acid in HPLC water: acetonitrile) for 60 minutes, at a flow rate of 0.5 ml/min (Figure 4.6). In the example shown in Figure 4.6, the area under the curve for the peak eluted at 22.3 mins, was 6.4498 mAU (milli-absorption units) and from the calibration curve this corresponds to an MCH concentration of 14.87–15.32 µg/ml.

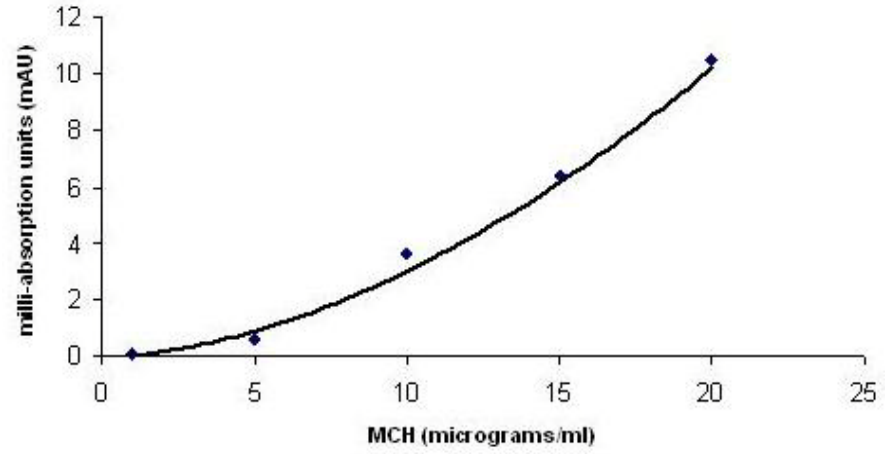

Figure 4.5: Calibration curve for MCH covering the range 1 - 20µg/ml

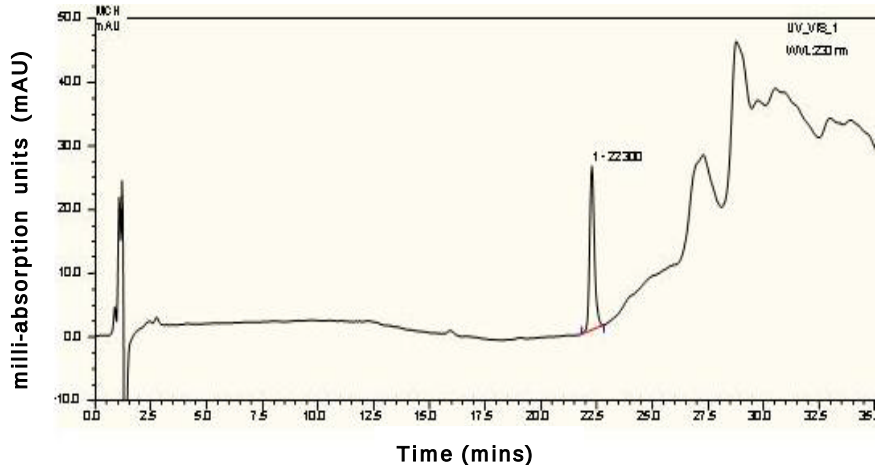

Figure 4.6: Chromatogram of purified MCH, retention time 22.3 minutes, area 6.4498 mAU,14.87–15.32 µg/ml. The peak labelled 1 – 22300 indicates the retention time at which the analyte (MCH) elutes from the column. The retention time is specific to MCH.

Mass spectrometry was used to detect and identify MCH in human plasma samples. An MCH standard calibration curve (1 µg/ml – 100 µg/ml) was generated by RP-HPLC/MS. It was predicted that product ions of m/z 796 and 2 of m/z 1194 would be generated specifically for MCH. Product ions at m/z 796 and m/z 1194 were detected at the correct elution time when purified MCH was analysed by MS (Figure 4.7a).

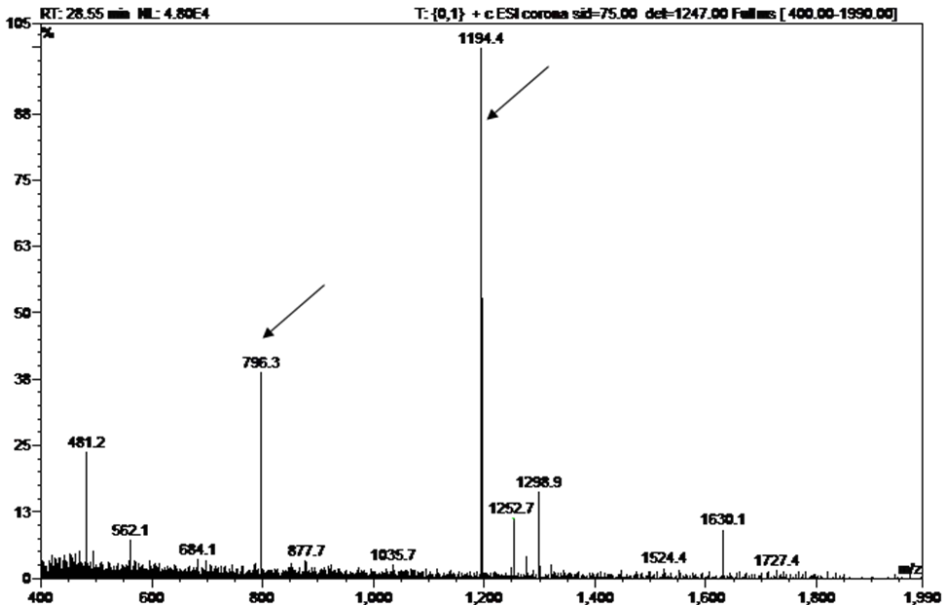

Figure 4.7a: Product ion mass spectrum obtained from purified MCH. The arrows indicate peaks at m/z 796 and 1194

Once purified MCH had been successfully analysed and detected by RP-HPLC and MS, human plasma samples were analysed for MCH content. Product ion mass spectra were generated from human plasma samples which displayed peaks with identical relative masses to those generated from purified MCH (Figures 4.7b & c).

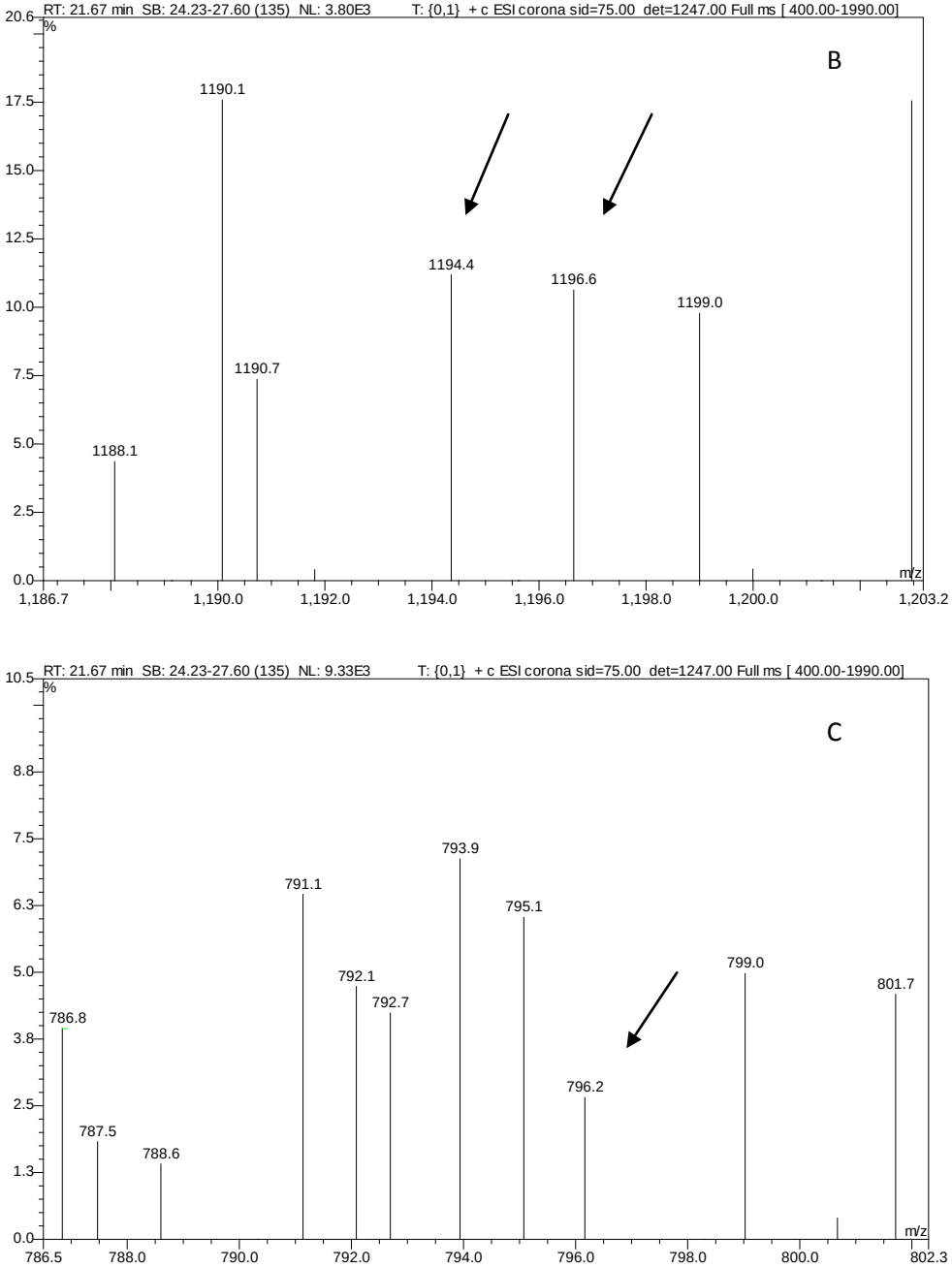

Figure 4.7b & c: Product ion mass spectrum obtained from the same plasma sample. The arrows indicate identical product ions to those presented in Figure 4.7a. Note that a different scale on the x axes has been used in Figures 4.7b & c compared to Figure 4.7a.

4.4.6 RP-HPLC and RIA

Purified MCH eluted from the column at 22.3 minutes. Immunoreactivity of purified MCH determined by RIA was detected between 20 – 24 minutes (Figure 4.8). MCH in a plasma sample was detected in the RP-HPLC fractions by RIA at 22 minutes at a concentration of 27.76 pg/ml which is within the established physiological reference ranges (Sections 4.4.9.2 and 5.4.2.1). An arbitrary value of 19.4 pg/ml was allocated to samples which were below the level of detection.

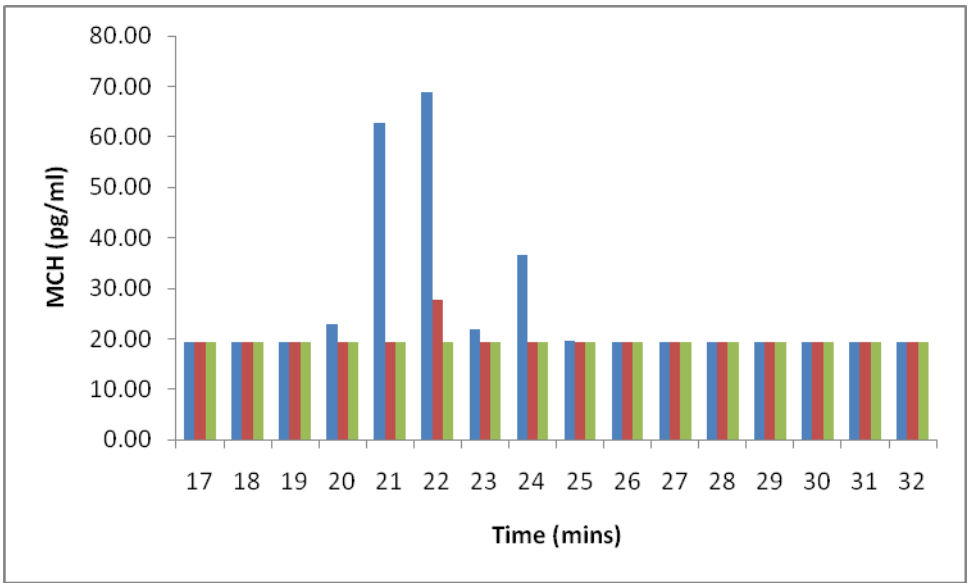

Figure 4.8: MCH immunoreactivity of samples fractionated by RP-HPLC. Purified MCH eluted at 22.3 minutes. Immunoreactivity of purified MCH determined by RIA was detected between 20 – 24 minutes

Plasma collected in EDTA tubes with HCl Purified MCH (µg/ml)

Buffer:HCl in EDTA

4.4.7 Stability of MCH in plasma

Results indicate that plasma MCH is not labile and that storage conditions do not affect recovery. There were no detectable differences between concentrations of MCH in plasma which had been subject to the storage conditions described in 4.2.7 and a freshly prepared sampled when measured by RP-HPLC. There was however a discrepancy between the

concentrations detected in plasma by RIA and by HPLC: this discrepancy is still being investigated.

4.4.8 Collection Methods

When plasma was run through the HPLC column a peak was detected at 22.3 mins. However MCH was only detected by HPLC in samples collected in the lithium heparin, EDTA and silica + gel vacutainers™. The area under the curve ranged from 0.0569-0.0859 mAU which is approximately 1.7 – 1.8 µg MCH/ml (see an example in Figure 4.9: sample collected with a silica + gel vacutainer™ (serum tube). Based on these results blood samples were only collected in lithium heparin or EDTA tubes in the studies described in Chapters 4, 5, 6 and 7.

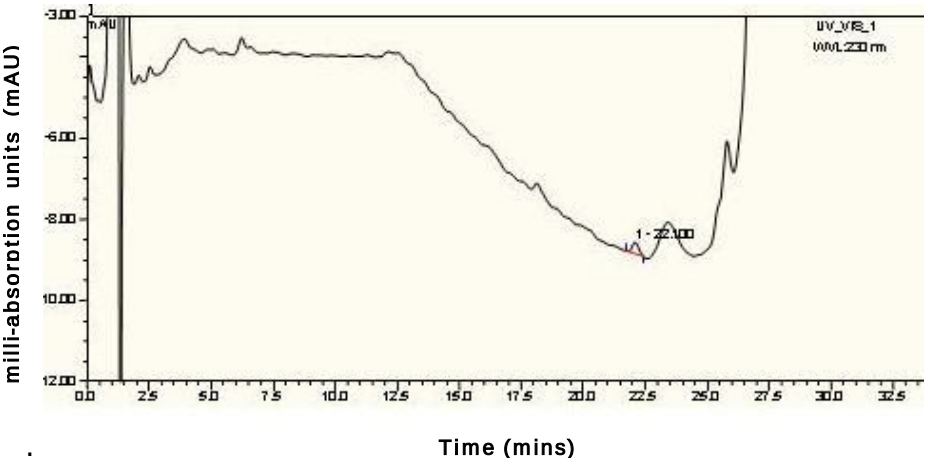

Figure 4.9: Chromatogram of plasma sample, retention time 22.3 minutes, area 0.0859 mAU, 1.7 – 1.8 µg/ml. The peak labelled 1 – 22300 indicates the retention time at which the analyte (MCH) elutes from the column.

4.4.9 Plasma MCH concentrations determined by in-house RIA

4.4.9.1 Demographic and anthropometric characteristics of study participants

Mean female and male ages were similar (p=0.150). The age range was from 18.0 – 76.0 years. Mean BMI was in the normal range for both males and females although males had significantly higher BMI than females (p<0.001). As expected males were significantly taller, (p<0.001), heavier

(p<0.001) and leaner (p<0.001) than females. Females had significantly greater percent fat mass (p<0.001) but not fat mass weight (kg) than males (Table 4.2; independent samples t-tests: p=0.521).

Table 4.2 Demographic, anthropometric and hormonal variables of study participants

	Female (n = 135)		**Male (n = 96)**	
	Mean ± SD	Range	Mean ± SD	Range
Age (yrs)	36.9±13.6	18.0–76.0	39.5±13.2	19.0–70.0
Height (m)	1.66±0.07	1.42–1.86	1.78±0.07***	1.56– 2.0
Weight (kg)	61.3± 9.8	42.1–98.5	80.1±12.7***	52.4–117.8
Fat mass (%)	27.7±8.9***	10.5–54.8	20.2±8.6	2.3–41.8
Lean mass (%)	72.2±8.9	45.2–89.5	79.8±8.6	58.2–97.7
Fat mass (kg)	17.6±8.3	6.1–51.2	16.8±9.2	1.3–45.4
Lean mass (kg)	43.7±5.1	28.4–55.6	79.8±8.6***	44.1–94.4
BMI (kg/m^2)	22.3±3.9	16.5–39.7	24.7±3.3***	19–35.1
Fasting MCH (pg/ml)	37.3±9.2	19.51–70.4	36.0±9.1	21.0–63.0

*** Significantly greater than the other gender (p<0.001)

4.4.9.2 Fasting plasma MCH concentrations

Fasting plasma MCH concentrations were detected in the range 19.51 – 70.4 pg/ml, though 1 subject had fasting MCH concentrations in excess of 150 pg/ml. This subject's sample was assayed several times and it was consistently high. Mean fasting plasma MCH concentrations were 36.7 ± 9.3 pg/ml (all individuals excluding the outlier). Within this assay 95% of the sample population would be expected to have fasting MCH concentrations between 19.4 and 55.4 pg/ml. There were no significant differences in mean fasting plasma MCH concentrations between males and females. When the sample population was grouped by gender and fat mass: male lean (ML):

body fat % < 21%; male excess fat (ME): body fat %≥ 21%; female lean (FL): body fat % < 31%; female excess fat (FE): body fat %≥ 31% (ACSM, 1996), there were no significant differences in plasma MCH concentrations between the groups (one-way between-groups ANOVA; p>0.05). Fasting plasma MCH concentrations were not significantly correlated with percent fat mass, percent lean mass, fat mass weight (kg), lean mass weight (kg), height or age. Circulating MCH concentrations did not change significantly with increasing age. There were however significant correlations between fasting plasma MCH concentrations and (a) body weight in females with excess fat, (b) male BMI, (c) BMI in females with excess fat, (d) BMI in males with excess fat (Table 4.3, Figure 4.10). Female correlations were inverse, that is higher plasma MCH concentrations were associated with lower body weight and BMI. Male correlations were in the positive direction.

Table 4.3: Associations between fasting MCH concentrations and body composition parameters

Gender/adiposity group	Body weight (kg)	BMI (kg/m^2)
Female EF (n=41)	r=-0.360, (p=0.021)*	-
Male all (n=90)	-	r=0.230, (p=0.030)*
Female EF (n=41)	-	r=-0.368, (p=0.018)*
Male EF (n=38)	-	r=0.472, (p=0.003)**

Values represent Pearson product-moment correlations. ** significant at 0.01 level (2-tailed), * significant at 0.05 level (2-tailed), EF: excess fat. Significant associations only reported.

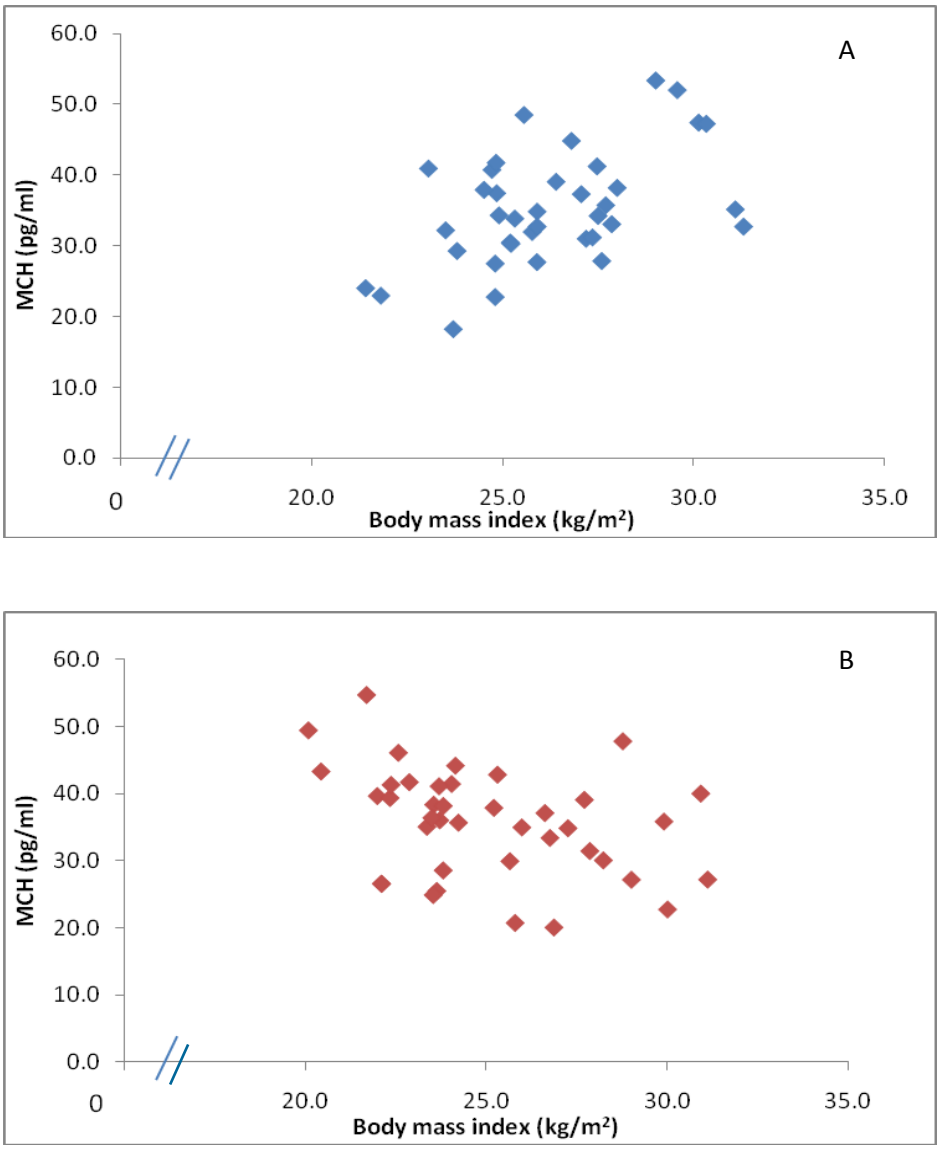

Figure 4.10: Plasma MCH concentrations and BMI in a) males with excess fat (n=38; r=0.472) and b) females with excess fat (n=41; r=-0.368), (Pearson product-moment correlations)

4.5 Discussion

A sensitive and specific RIA for the quantifiable measurement of MCH in human plasma has been successfully developed using antisera raised in rabbit. The only previous published attempt to detect circulating human MCH using a commercially available assay (Gavrila *et al.,* 2005) has been publicly criticised (Waters and Krause, 2005; Takahashi, 2005). Takahashi (2005) contends that MCH is not detectable in peripheral blood or tissue, due to the failure of his laboratory to detect circulating MCH in humans or rats with an in-house RIA using antisera against salmon MCH. Nevertheless results of

the current study confirm that MCH is detectable in human plasma by both RIA and RP-HPLC/MS. The effective range was established as 19.5 – 1248 pg/ml and the inter-assay coefficient of variation was 3.7% indicating this assay is highly reproducible.

The antisera's affinity for MCH and not other peptides reported to have a competitive or agonistic relationship with MCH was tested. Evidence of a competitive relationship between MCH and ANF (Hervieu *et al.,* 1996), α-MSH (Barber *et al.,* 1987; Ludwig *et al.,* 1998) and ACTH (Baker *et al.,*1985) has emerged from rodent and fish studies. Additionally there is evidence to suggest that a pro-MCH derived peptide (NEI-MCH) acts as a super-agonist to MCH (Maulon-Feraille *et al.,* 2002). Therefore to determine possible cross-reactivity, dilution curves substituting the above peptides for MCH standard were run. As expected the only molecule showing evidence of cross-reactivity was NEI-MCH though only at supraphysiological concentrations (Figure 4.3). Currently there is little evidence to suggest that NEI-MCH circulates therefore at physiological concentrations this assay is specific for MCH. Furthermore parallelism of the dilution curves of plasma to the standard curve confirms that other plasma components have no adverse effects on the curve. It should be noted here that Waters and Krause (2005) questioned the specificity of the Sigma antisera in a letter to an editor. However this comment was never followed up in a peer reviewed publication. Specifically the authors claim that MCH antisera obtained from Sigma (St Louis, MO) reacted with an immunoreactive substance in MCH knock-out mice. In response to this critique it was countered that leptin knock-out mice can carry a truncated leptin molecule which reacts with leptin antisera therefore even though the animal is leptin deficient specificity is not compromised (Mantzoros, 2005). In the current study the polyclonal MCH antisera (Sigma-Aldrich, UK) was raised in rabbit using synthetic human MCH as immunogen conjugated to keyhole limpet hemocyanin (KLH). The manufacturers state that at 50% binding, cross-reactivity with α-MSH, β-MSH, γ-MSH, β-endorphin, α-endorphin, ACTH (1-39), ACTH (1-24) is ≤0.01%. As discussed above the results of the current study are in agreement with the manufacturers.

To confirm that MCH is detectable and measurable in human plasma cross-validation was performed by RP-HPLC and MS. A peak was detected at 22.3 mins when plasma was run through the HPLC column which corresponds exactly to the elution time of purified MCH. Additionally when human plasma was subject to MS product ions of identical mass to those generated by purified MCH were observed. There was however a discrepancy between concentrations of plasma MCH detected by HPLC and those detected by RIA. The range of the standard curve for MCH generated by HPLC was 1 – 20 µg/ml. MCH was not detectable at lower concentrations whereas the MCH plasma samples measured by RIA mostly fell into the range 19.5 – 1249 pg/ml. Since these two analytical tools are entirely different this outcome is perhaps unsurprising. Whereas HPLC identifies the analyte based on hydrophobic and polar interactions, RIA measures immunoreactivity. RIAs are more sensitive than HPLC (Buice *et al.,* 1987; Granich *et al.,* 1989), as evidenced for instance by the results reported herein and the detection range of the aforementioned commercially available assay which has a similar lower detection limit (17 pg/ml *vs* 19.5 pg/ml). Concentration differences between plasma components measured by RIA and by HPLC are not uncommon (Carruthers *et al.,* 1983; Buice et al., 1987; Chen *et al.,* 2008). Discrepancies of this nature are not particularly pertinent so long as there is consistency between the results (Caruthers *et al.,* 1983). Furthermore results of RIA and HPLC measurements have been reported to correlate well (Wolf *et al.,* 1989; De-Remer *et al.,* 1997; Lips *et al.,* 1999). Therefore in order to consolidate the RIA results human plasma samples were fractionated by HPLC, collected at one minute intervals and quantified by RIA. Purified MCH eluted from the HPLC column at 22.3 minutes. In the RIA immunoreactivity in plasma samples was detected at 22 minutes. This combination of HPLC and RIA methods is recommended to improve the molecular specificity of the analysis (Desiderio, 1984). With regard to the discrepancy in the magnitude of the detected concentrations it could be that the RIA cannot recognise the molecular configuration of bound MCH whilst the HPLC acidification process may cleave bound MCH which would then be detectable in addition to free MCH accounting for the higher concentrations detected by HPLC.

HPLC was also used to examine the stability of MCH and the effects of collection methods. Plasma MCH retains stability under various conditions and even being left at room temperature overnight did not interfere with detection levels. Nor did freeze thaw cycles impact on detection. In contrast different collection methods produced variable results, MCH was detected in plasma collected in vacutainers primed with anti-coagulants lithium heparin or EDTA, or in serum tubes containing a clotting agent. Other anti-coagulants, namely sodium citrate and fluoride oxalate interfered with detection. These results indicate that collection methods for plasma MCH should be standardised. Also whilst it is desirable that storage conditions should be standardised, if on occasion it is not possible to adhere to standard practice the consequences should not be detrimental. Furthermore since it is impractical to recruit volunteers each time a plasma sample is required for analysis the finding that freeze/thaw does not affect detection levels permits optimal utilisation of resources.

Fasting blood samples from 135 female and 96 male volunteers were obtained in order to establish a reference range. It was the intention to obtain samples from a diverse population therefore volunteers were recruited from a range of ethnicities, ages and phenotype. The mean fasting plasma MCH concentration was 36.7 ± 9.3 pg/ml, therefore within this assay 95% of the population would be expected to have plasma MCH concentrations between 19.4 and 55.4 pg/ml though one subject had a fasting plasma MCH concentration of more than double the upper limit of the range. This was not an anomaly since his sample was assayed several times and, in a separate study a female sample also showed a consistently similarly high value. The physiological significance (if any) of these excessive values is not known though the male subject had a BMI and fat mass % greater than 2SD from the mean, and the female's height was more than 2SD from the mean. However neither physical attribute was unique within their own populations.

In rodents increased availability of hypothalamic MCH is associated with adiposity (Ludwig *et al.,* 2001; Gomori *et al.,* 2002) whilst decreased availability is associated with leanness (Marsh *et al.,* 2002; Kowalski *et al.,* 2006), therefore it was hypothesised that circulating MCH concentrations

would also be aligned to phenotype. In the current study whilst there were no associations between percent fat mass, percent lean mass, fat mass weight, lean mass weight, age or height there were significant correlations between circulating MCH concentrations and BMI in both males and females with excess fat (r=0.472, p=0.003; r=0.368, p=0.018 respectively). Also a modest correlation between circulating MCH concentrations and body weight in females with excess fat (r=0.360, p=0.021). These results indicate that circulating MCH may not be a marker of adiposity in humans since BMI and body weight measurements do not represent adiposity *per se.* Body weight and BMI, which is a product of the weight/height ratio are possessed of other components such as muscle, bone tissue, fluids *et cetera* therefore an effect of these constituents cannot be excluded. However the outcome that these relationships are only observed in those with excess fat is indicative of some altered signalling in the presence of adiposity. Throughout this series of studies associations between circulating MCH concentrations and the same body composition parameters were examined; the current results do not completely synchronise with results of the other studies in this series. Whilst there were associations between circulating MCH concentrations and certain body composition parameters the associations appeared to be gender and phenotype specific within the different populations examined. This subject is discussed more fully in Chapter 8. Nevertheless circulating MCH concentrations have consistently been associated with body composition parameters in all studies though only in sub-groups of the sample populations.

In agreement with the other studies in this series there were no differences between male and female fasting circulating MCH concentrations. In this respect current results agree with the only other study to date to measure circulating MCH in humans (Gavrila *et al.,* 2005); which found only a non-significant gender difference in serum MCH concentrations. Age-related changes in body composition did not appear to impact on circulating MCH concentrations since there were no differences in circulating MCH concentrations between those who were 40 +, 50 + or 60 + years and their younger co-subjects. Nor was there a significant correlation between age

and absolute circulating MCH concentrations. That said it is not known whether age-associated changes in health or nutritional status impact on circulating MCH concentrations and a longitudinal study would be required to further explore age-related changes in this respect.

4.6 Summary, limitations and future work

A sensitive and specific RIA for the measurement of circulating MCH in humans has been successfully developed and validated. By use of this assay a profile of normal circulating MCH in humans is emerging. In contrast to earlier published work where 1) circulating MCH was not detectable by RIA (Takahashi *et al.,* 2005) and 2) where MCH was detectable by RIA but the validity of the RIA was questioned (Gavrila *et al.,* 2005); results of the current study confirmed that MCH is detectable in human plasma by RP-HPLC/MS and by RIA, and that MCH concentrations in human plasma have been reliably measured by a radioimmunoassay.

Although not a cause for concern the discrepancy between concentrations of plasma MCH measured by HPLC and those measured by RIA should be investigated and there are ongoing plans for this work to be undertaken. Waters and Krause (2005) comments are noted. In conclusion current results indicate that this assay is sensitive, specific and sufficiently reliable for use in future research.

5. Circulating MCH concentrations and their association with resting metabolic rate, body composition and parameters of glucose homeostasis in young adults

5.1 Introduction

No reference ranges exist for concentrations of circulating human MCH and since it was confirmed that MCH is quantifiably measurable in human plasma (reported in Chapter 4), a cross-sectional study was undertaken in order to determine inter- and intra-subject variations of plasma MCH concentrations and their correlation with resting metabolic rate (RMR) and body composition, as empirical evidence from animal studies supports a strong role for MCH in appetite control and energy conservation (Shimada *et al.,* 1998; Ludwig *et al.,* 2001; Seigal-Liberman *et al.,* 2003). The pattern of release of circulating MCH has not been established therefore a further aim was to investigate whether circulating MCH concentrations were acutely responsive to food stimuli. Furthermore since both hypothalamic and peripheral MCH have been implicated in glucose homeostasis (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005; Pissios *et al.,* 2007) plasma MCH concentrations in both the fasted and fed states were examined in association with circulating concentrations of glucose, insulin and leptin. The sample population was tightly controlled for age and body mass since being the first study to characterise circulating MCH concentrations in humans, it was the intention to eliminate potential confounding factors such as age, increased fat mass and associated changes in health.

The objectives of this study were:

- To establish a reference range for circulating concentrations of MCH in young healthy humans.
- To describe associations between circulating MCH concentrations and a) RMR and b) body composition.
- To track the acute response of circulating concentrations of MCH in response to feeding in relation to glucose, insulin and leptin concentrations.

5.2. Subjects and experimental protocol

5.2.1 Participants

Thirty two male and female subjects were recruited as Section 3.2.2. The inclusion criteria for females were: pre-menopausal; non-oral contraceptive using; aged 18 – 30 years; and with a body mass index (BMI) of < 24.9. The inclusion criteria for males were: aged 18 – 30 years; BMI < 24.9. Those on medication for chronic illnesses, lactating females, recently lactating (within the last 12 months) and pregnant women were excluded.

5.2.2 Pre-test

Prior to attendance in the laboratory subjects were provided with and instructed how to complete a 3-day Food Diary. This diary was kept for two weekdays and one weekend day immediately prior to attendance in the laboratory to establish habitual food intake (Appendix 5). The Food Diaries were analysed using Dietplan 6 Personal P3 software (Forestfield Software Limited, Horsham, UK). Subjects also received a Subject Information Sheet (Appendix 3) and completed an Informed Consent Form and pre-test Medical Screening Questionnaire (Appendices 2 & 4).

5.2.3 Experimental protocol

Subjects arrived in the laboratory fasted at 09.00am and were tested in a four hour protocol. Upon arrival subjects were verbally familiarised with experimental procedures after which anthropometric measurements were taken as Section 3.2.3. Following this RMR was measured using the procedure described in Section 3.2.4. One fasting venous blood sample and 3 post-prandial blood samples were collected at designated intervals and nine fingerprick samples were also obtained at 15 minute intervals. See Figure 5.1 for timings. Venous blood samples were collected, treated and the plasma stored as Section 3.2.6. Plasma samples were subsequently assayed for MCH, leptin and insulin as described in Section 3.2.7. The

fingerprick blood samples were immediately quantified for tracking pre and post-prandial blood glucose concentrations. After RMR was measured subjects consumed a controlled meal of mixed macronutrient content. The calorific content of the meal was 510 k/cal for males and 388 k/cal for females. This represents the recommended percentage daily intake for breakfast (20%) of the Estimated Average Daily Requirements for Energy (Food Standards Agency, 1996; Department of Health, 1991). The macronutrient content of the meal was based on the Dietary Reference Values for protein, carbohydrate and fat for adult males and females (Department of Health, 1991). The glycaemic index for this meal was 59.45. After blood sampling was completed volunteers' total body fat and lean masses were measured by air displacement plethysmography (Bod Pod), as Section 3.2.5.

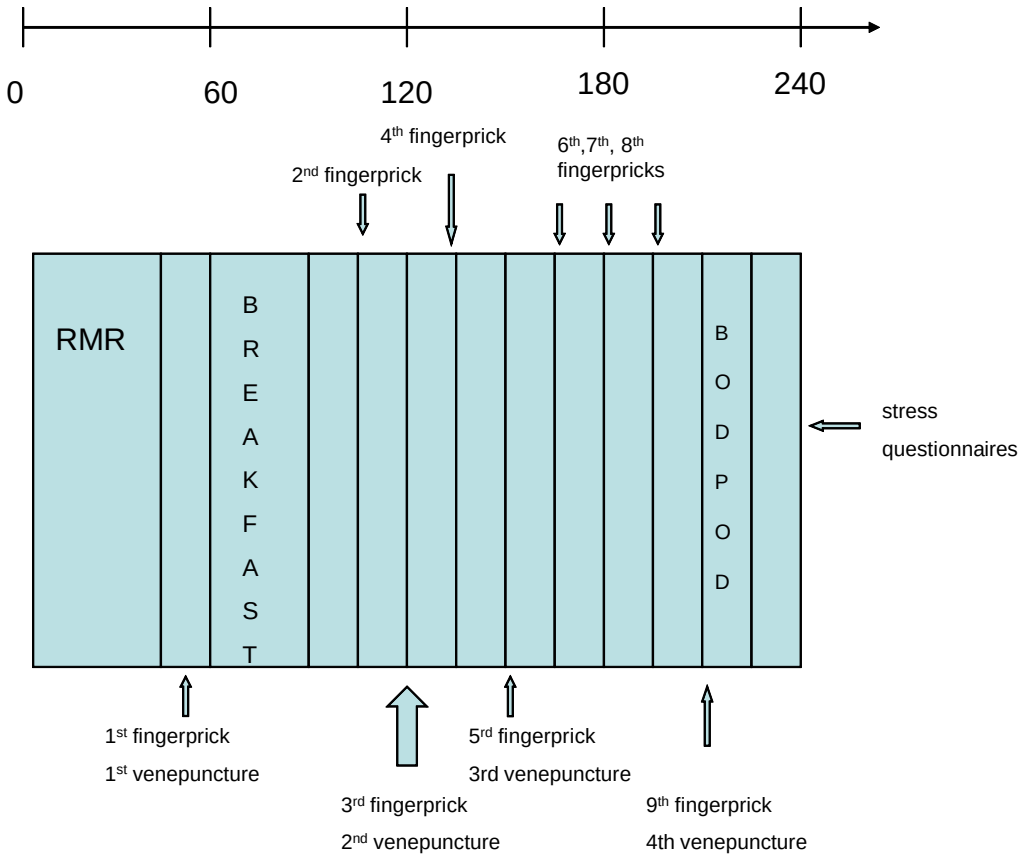

Figure 5.1: Schematic representation of experimental protocol

5.3 Statistical analyses

Normality of data was assessed by Shapiro-Wilk's W test and by examination of skewness and kurtosis values. Square root or log-transformed data was used where necessary. Homogeneity of variance was determined by Levene's Test for Equality of Variances. Independent samples t-tests were used to establish inter-gender differences between anthropometric characteristics and circulating hormone concentrations. Differences in pre- and post-prandial circulating hormone concentrations were assessed by paired samples t-tests. Comparisons between circulating hormone concentrations at the pre- and post-prandial sampling time-points were assessed by repeated measures design ANOVA. Post-hoc tests were performed using Bonferroni adjustment for multiple comparisons. One way between-groups ANOVAs were conducted to determine if there was an effect of body composition on plasma hormone concentrations. Post-hoc tests were performed using Tukey's adjustment for multiple comparisons. The group effect of size was calculated using partial eta squared or eta squared. The guidelines for interpretation were those proposed by Cohen (1988). Relationships between fasted and post-prandial plasma MCH concentrations, RMR and body composition were also determined by linear multiple regression. The incremental area under the curve (AUC) was calculated for MCH, leptin, insulin and glucose using the trapezoidal method (Matthews *et al.,* 1990). The strength of the associations between plasma MCH, leptin and insulin concentrations, RMR and body composition parameters were determined by Pearson product-moment correlational analysis. Partial correlation was used to control for compounding variables. Values are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Data were analysed using the Statistical Package for the Social Sciences (SPSS version 16.0 for Windows; Chicago, IL, US). Statistical significance was set at $p<0.05$.

5.4. Results

5.4.1. Subject characteristics

Demographic and anthropometric characteristics of the study participants are presented in Table 5.1. Males and females were of similar ages (p=0.130) and BMI (p=0.421). As expected males were significantly taller, (p<0.001), heavier (p<0.001) and had a greater lean mass % (p<0.001) than females. Females had significantly greater fat mass % than males (p<0.001).

Table 5.1 Demographic and anthropometric characteristics of study participants

	Female (n = 21)		**Male (n = 11)**	
	Mean ± SD	Range	Mean ± SD	Range
Age (yrs)	27.5±2.8	21.7–30.7	25.7±3.3	21.5–30.1
Height (m)	1.66±0.1	1.57–1.8	1.78±0.1***	1.65–1.9
Weight (kg)	61.4±6.3	46.0–72.5	72.4±9.2***	51.5–90.2
Fat Mass (%)	26.6±5.4***	16.7–37.9	15.5±6.6	7.9–29.6
Lean Mass (%)	73.3±5.4	62.1–83.3	84.4±6.6***	70.4–92.1
BMI (kg/m^2)	22.1±2.2	16.7–24.9	22.8±2.0	18.9–25.5

*** p<0.001 significantly greater than opposite sex

5.4.2 Plasma hormone concentrations

5.4.2.1 Pre- and post-prandial plasma hormone and blood glucose concentrations

The four sampling timepoints were designated F=fasted; PP1=30 minutes after feeding; PP2=60 minutes after feeding; and PP3=120 minutes after feeding.

MCH: In all individuals mean fasted plasma MCH concentrations (45.3 ± 11.0 pg/ml) were not different to mean post-prandial plasma MCH concentrations (45.2 ± 8.6 pg/ml). The ranges were 26.3 – 71.2 pg/ml and

33.0 – 74.2 pg/ml in fasted and post-prandial individuals respectively. There were no significant differences in plasma MCH concentrations at any of the four time-points (one-way repeated measures ANOVA; p=0.772). The intra-subject CV was 1.98% for fasted plasma MCH concentrations and 1.01% for post-prandial plasma MCH concentrations. The inter-subject CV for plasma MCH concentrations was 2.44%. The range in which 95% of plasma MCH concentrations fell was 28.02 – 62.38 pg/ml.

Male and female plasma MCH concentrations are presented in Table 5.2 and Figure 5.1a. There were no significant differences between male and female plasma MCH concentrations at any of the four sampling time-points (independent samples t-test; F: p=0.464; PP1: p=0.638; PP2: p=0.545; PP3: p=0.181). Similarly there was no effect of gender on mean MCH concentrations when fasted or after feeding (independent samples t-test; p=0.694 and p=0.716 respectively).

Leptin: Male and female plasma leptin concentrations are presented in Table 5.2 and Figure 5.1b. Circulating leptin concentrations decreased significantly in both males and females post-prandial. However there was a gender difference in the pattern of the response. Female circulating leptin concentrations had decreased significantly by the 1 hour sampling time-point but returned to near fasting by 2 hours post-prandial. Male circulating leptin concentrations did not decrease significantly until two hours post-prandial (one-way repeated measures ANOVA; p=0.037, p=0.028 respectively). Mean female leptin concentrations were significantly greater than mean male leptin concentrations at every sampling time-point (independent samples t-test; $p<0.05$).

Insulin: Male and female plasma insulin concentrations are presented in Table 5.3 and Figure 5.1c. Plasma insulin concentrations were significantly different at each sampling time-point: being lowest in the fasting samples and peaking at 30 mins post-prandial, they had not returned to fasting concentrations 2 hours post-prandial (all individuals, one-way repeated measures ANOVA: $p<0.005$; with a large effect of size, multivariate partial

eta squared=0.849). Male and female plasma insulin concentrations were not different at any sampling time-point.

Table 5.2: Fasted and mean post-prandial plasma MCH and leptin concentrations

MCH (pg/ml)				Leptin (ng/ml)			
Female (n=19)		Male (n=10)		Female (n=18)		Male (n=10)	
F	PP	F	PP	F	PP	F	PP
44.18 ±10.93	46.24 ±10.32	47.40 ±11.41	43.66 ±5.73	7.58 ±3.88[a]	6.84 ±3.46[b]	2.63 ±1.88[c]	2.53 ±1.91[c]

Mean ± SD, F: fasted, PP: mean post-prandial, values with different superscripts are significantly different from each other (paired samples t-test; $p<0.05$)

Table 5.3: Fasted and mean post-prandial blood glucose and plasma insulin concentrations

Glucose (mmol/l)				Insulin (ng/ml)			
Female (n=21)		Male (n=10)		Female (n=20)		Male (n=10)	
F	PP	F	PP	F	PP	F	PP
4.33 ±0.64	5.60 ±0.70	4.60 ±0.62	5.60 ±0.75	0.28 ±0.13[a]	1.80 ±0.78[b]	0.34 ±0.14[a]	1.66 ±0.71[b]

Mean ± SD, F: fasted, PP: mean post-prandial, values with different superscripts are significantly different from each other (paired samples t-test; $p<0.05$)

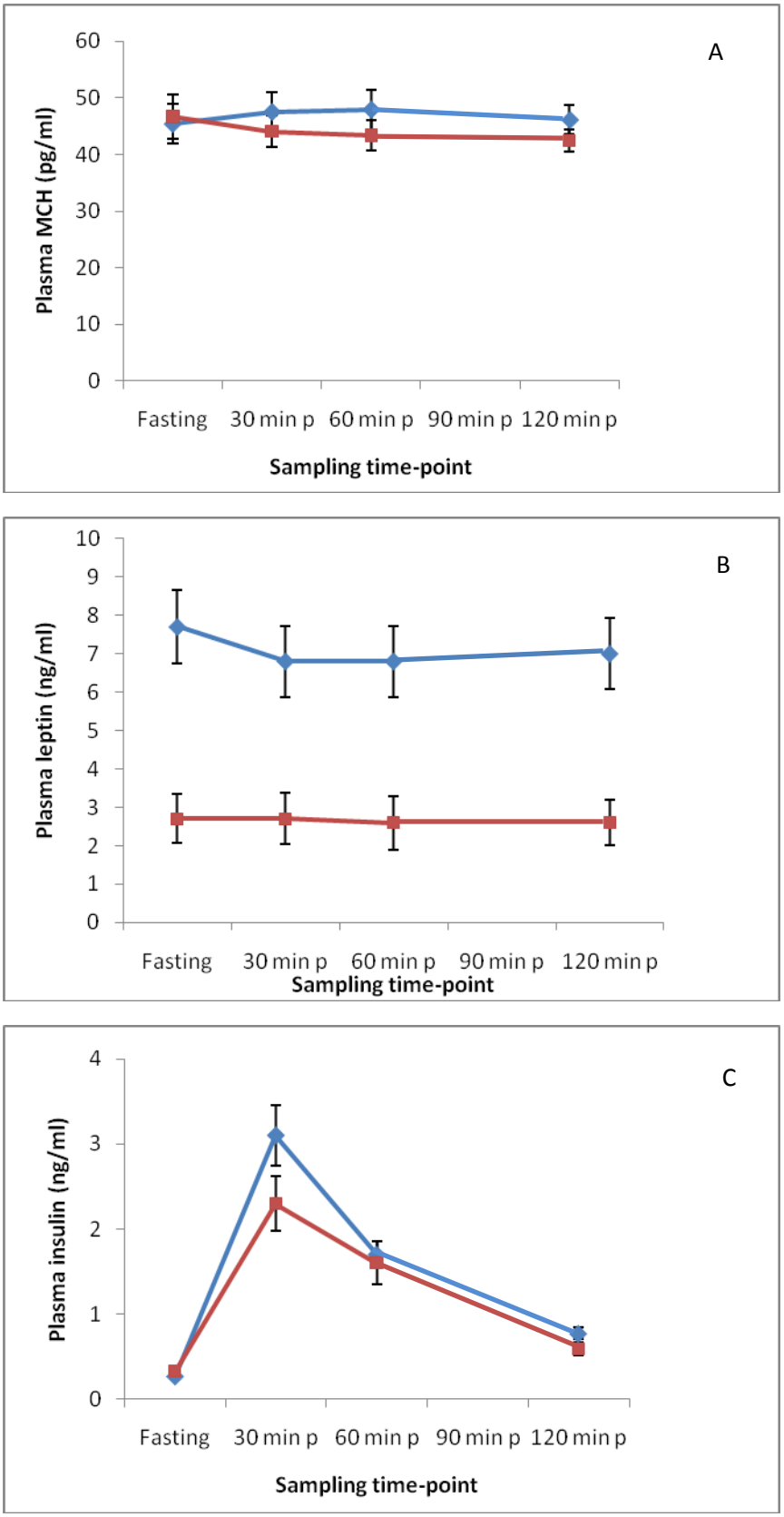

Figure 5.1: fasting and post-prandial a) MCH, b) leptin and c) insulin concentrations in young healthy males (n=10) and females (n=19, 18, 20 respectively) (mean ± SEM)
■ Male ◆ Female p: post-prandial

Glucose: Male and female blood glucose concentrations are presented in Table 5.3 and Figure 5.2. All subjects had fasted and 2 hr post-prandial blood glucose concentrations in the normal range, the customary peak 15 – 30 minutes post-prandial followed by a rapid return to near fasting values was observed. There were no differences between male and female blood glucose concentrations (independent samples t-test; p>0.05).

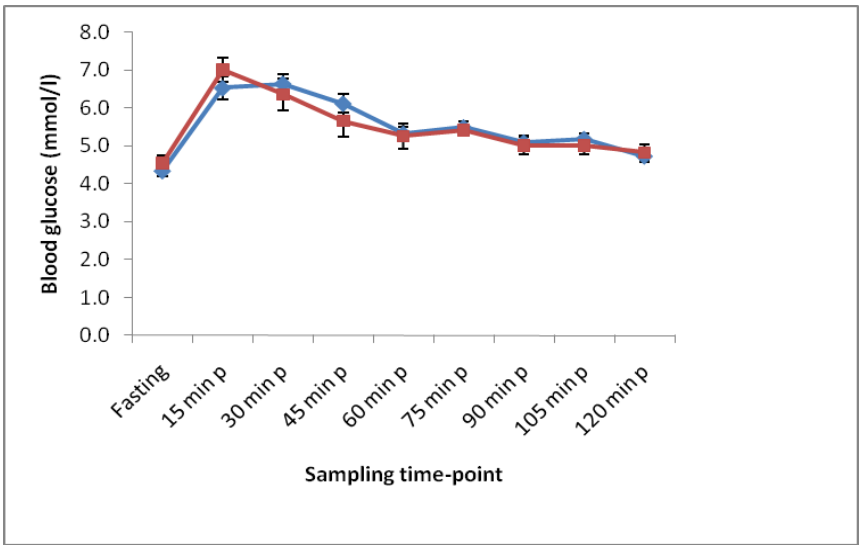

Figure 5.2: fasting and post-prandial blood glucose concentrations in young healthy males (n=10) and females (n=21) (mean ± SEM) ■ Male ◆ Female p: post-prandial

5.4.2.2 Associations between pre-and post-prandial plasma MCH, leptin, insulin and blood glucose concentrations

The MCH AUC was correlated with the insulin AUC in individuals with excess fat only (r=0.862, p=0.012, n=6). The insulin AUC was also correlated with mean plasma MCH concentrations in those with excess fat (r=0.891, p=0.009). There was a significant correlation between the MCH AUC and glucose AUC only in males (r=-0.836, p=0.019, n=7). At the one hour and two hour sampling time-points plasma leptin concentrations were correlated with plasma insulin concentrations in individuals with excess fat (r=0.818, p=0.047; r=0.894, p=0.016 respectively). Plasma leptin concentrations were correlated with blood glucose concentrations at the 2 hour sampling time-point in males only (r=-0.730, p=0.040).

5.4.3 Associations between plasma MCH and leptin concentrations and body composition parameters

In males, fasting plasma MCH concentrations were positively correlated with body lean mass weight (kg) and mean post-prandial plasma MCH concentrations were positively correlated with BMI. Mean concentrations of plasma leptin were strongly significantly correlated with fat mass percent, lean mass percent, fat mass weight (kg) and BMI in both males and females (Pearson product-moment correlation) (Table 5.4).

Table 5.4 Associations between mean plasma MCH and leptin concentrations and body composition parameters

	MCH (pg/ml)		**Leptin (ng/ml)**	
	F (n=19)	M (n=11)	F (n=19)	M (n=10)
Fat mass (%)	0.040	0.072	0.769**	0.856**
Lean mass (%)	0.238	0.005	-0.769**	-0.856**
Fat mass (kg)	0.004	0.497	0.766**	0.832**
Lean mass (kg)	-0.148	0.639# (p=0.034)*	0.072	0.022
BMI (kg/m^2)	0.030	0.662† (p=0.037)*	0.566*	0.842**
Body weight (kg)	-0.043	0.584	0.415	0.394

Values represent Pearson product-moment correlations, * *significant at 0.01 level (2 tailed), * significant at 0.05 level (2 tailed), # fasted MCH concentrations, † mean post-prandial concentrations. All other comparisons based on mean value of all four sampling time-points.

To explore the impact of body composition on circulating MCH and leptin concentrations subjects were sub-divided into four groups based on the American College of Sports Medicine's cut-off point between individuals assigned to a lean or excess fat category: male lean (ML)=body fat % < 21%; male excess fat (ME)=body fat %≥ 21%; female lean (FL)= body fat % < 31%; female excess fat (FE)=body fat % ≥ 31% (ACSM, 1996). **MCH:** There were no significant differences in mean concentrations of plasma MCH between any of the groups (p=0.928). **Leptin:** Circulating concentrations of

leptin were significantly different from each other for ML, FL and FE individuals (one-way between groups ANOVA with a large effect of size which was calculated as 0.599 using eta squared).

5.4.4 Associations between plasma MCH and leptin concentrations and resting metabolic rate

RMR was significantly higher in males than in females when expressed in absolute terms (kcal/day), though not when adjusted for lean body mass. RMR data presented immediately below were adjusted for lean body mass. RMR was negatively correlated with lean body mass weight (kg) in all individuals (r=-0.499, p=0.05) and in females only when split by gender (r=-0.450, p=0.046). Both male and female RMR were negatively correlated with body weight (r=-0.674, p=0.046; r=-0.479, p=0.002 respectively). The CV for RMR was 0.82% and 0.85% in males and females respectively. There were no significant correlations between fasted or mean plasma MCH concentrations and RMR in either males or females.

The group was further sub-divided based on % of predicted RMR. Predicted RMR (kcal/day) was calculated as the mean of the Fleisch and Harris-Benedict equations (Fleisch, 1951; Harris and Benedict, 1919). A RMR of ± 10% predicted is considered normal (McKardle *et al.,* 2001). Therefore the groups were N= ≥ 90% predicted RMR; L= < 90% predicted RMR. There were no significant correlations between RMR and fasted or post-prandial mean plasma MCH concentrations in either group. However when those with the highest metabolic rate were assigned to a separate category, that is, where RMR was ≥ 100% predicted, there was a significant correlation between fasted plasma MCH concentrations and females whose RMR was between 90 – 99.9% predicted (Table *5.5*: r=-0.798, p=0.018). RMR, per cent fat-free mass, fat-free mass (kg), per cent fat mass and gender did not contribute significantly to the variance in fasted or post-prandial plasma MCH concentrations (linear multiple regression; p>0.05). **Leptin:** Fasting and mean post-prandial plasma leptin concentrations were significantly correlated with RMR in all individuals, however there was no effect when leptin was controlled for fat mass.

Table 5.5 Associations between plasma MCH concentrations and RMR

	Fasted MCH concentrations	Post-prandial MCH concentrations
RMR mH (n=2)	NC	NC
RMR fH (n=5)	0.802 (p=0.102)	-0.645 (p=0.240)
RMR mM (n=5)	-0.185 (p=0.766)	-0.822 (p=0.088)
RMR fM (n=8)	-0.798 (p=0.018)*	-0.257 (p=0.538)
RMR mL (n=2)	NC	NC
RMR fL (n=5)	-0.335 (p=0.582)	-0.328 (p=0.525)

Values represent Pearsons product-moment correlations, * significant at 0.05 level (2-tailed), m: male, f: female, H: high, M: medium, L: low, NC: not computed

5.4.5 Associations between plasma MCH concentrations, energy intake and RMR

Eighteen 3-day food diaries were analysed (male n=9, female n=9). Male and females did not differ in total caloric intake per kg bodyweight or in the percentage of total daily energy intake provided by carbohydrate, protein or fat (independent samples t-test; $p>0.05$). Alcohol intake contributed 2.9% of total daily energy intake across the group. Total caloric intake was not associated with fasted or mean post-prandial plasma MCH concentrations. Neither were there significant associations between specific macronutrient intake and plasma MCH concentrations. RMR (per kg lean mass) was not associated with caloric intake in either males or females (Pearson product-moment correlation; $p>0.05$).

5.5 Discussion

5.5.1 Circulating MCH concentrations

A reference range for circulating MCH in humans has not been established. Therefore a primary objective of the current study was to determine normal values for circulating MCH in young, healthy individuals. To this end a tightly controlled population was chosen; age-group 18 – 30 yrs, BMI not in excess of 25 kg/m^2 and free of chronic health problems. The rationale for this constraint was to minimise confounding factors which may have been introduced by a more diverse sample population. Concurrently blood samples from a less stringently controlled sample population were collected in order to establish a broader-based reference range. The results of this concurrent study were reported in Chapter 4.

There were no differences between fasted and mean post-prandial plasma MCH concentrations. Male and female plasma MCH concentrations were not significantly different, although there was a trend for female concentrations to rise after eating whereas male concentrations decreased. The current findings are somewhat at odds with values reported in the only other study to measure circulating MCH in humans (Gavrila *et al.,* 2005). This cross-sectional study reported mean values which were significantly higher (97.8 ± 22.8 pg/ml) than those in the current study. Whilst their sample population was younger than the ones reported here, there were no direct associations between plasma MCH concentrations and age in the current study (Chapter 4). Confusingly in a separate intervention study the same authors reported baseline mean values which were inconsistent with the ones they reported in their cross-sectional study, therefore clearly it was important to establish a reference range with reproducible values. Results indicate that within this assay 95% of the population (young, healthy) would be expected to have plasma MCH concentration values falling into the range 19.5 - 62.4 pg/ml.

5.5.2 MCH and RMR

A putative role for MCH in energy conservation has been validated by findings from genetic studies which have consistently shown that ablation of functional hypothalamic MCH results in increased energy expenditure and in some cases increased locomotor activity (Shimada *et al.,* 1998; Marsh *et al.,* 2002; Segal Lieberman *et al.,* 2003; Jeon *et al.,* 2006). Furthermore some studies have shown this phenomena to be gender specific with males only demonstrating this trait (Chen *et al.,* 2002; Alon and Friedman, 2006). In one study locomotor activity was decreased following MCH administration (Santollo and Eckel, 2008). An interesting finding resulting from this rodent work is that increased energy expenditure resulting from decreased availability of physiological hypothalamic MCH may be accompanied either by hyper- or hypophagia. Whether these consummatory behaviours are compensation driven, that is, increased ingestion to compensate for increased energy output or a result of reduced MCH-induced food intake is not known. However in humans there is no evidence to support a role for circulating MCH in energy homeostasis, therefore in the first instance it was deemed important to describe associations between RMR and circulating MCH concentrations in young, healthy individuals. Fat-free mass has customarily been considered to be one of the strongest predictors of RMR in both male and females (Cunningham, 1991; Astrup *et al.,* 1992; Weinsier *et al.,* 1992), and whilst it is acknowledged that the practice of presenting RMR as a function of lean mass or fat-free mass is simplistic (Illner *et al.,* 2000, Wang *et al.,* 2000; Heymsfield *et al.,* 2002), it is nonetheless considered satisfactory to facilitate inter-gender comparisons in energy expenditure (McArdle *et al.,* 2001). In the current analyses RMR has been presented after adjusting for lean mass unless otherwise indicated.

As expected higher RMR was observed in males (absolute values) though there was no inter-gender difference in RMR when adjusted for lean mass. Neither were there differences in circulating MCH concentrations between males and females in this population. We found no evidence of a relationship between fasted or fed plasma MCH concentrations and RMR. In rodent studies manipulation of the availability of physiological MCH has a profound

effect on energy expenditure but the relevance of circulating MCH to metabolic rate in the absence of intervention has not been described. The results of this study indicate that circulating concentrations of MCH remain stable in the absence of energetic flux. However if up-regulation of MCH results in energy conservation (Santollo and Eckel, 2008), then it may be expected that individuals with lower metabolic rates be possessed of higher circulating concentrations of MCH. Therefore the sample population was sub-divided into 2 groups based on their RMR as a percentage of predicted (standard) RMR. Those with a measured RMR ≤ 89.9% predicted were designated L (low) and those with a measured RMR≥ 90% predicted were designated N (normal) since any value within ± 10% of standard is considered normal. There were no differences in fasted or post-prandial circulating MCH between the groups and RMR was not correlated with plasma MCH concentrations in either group. Curiously when the normal group was further sub-divided in an effort to determine whether there were any associations between circulating MCH and those with the highest metabolic rates, that is, ≥ 100% predicted RMR, a significant negative correlation was observed between females with normal but not high RMR (90 – 99.9% predicted) and fasted circulating MCH. Whilst this relationship is in the direction one would expect, that is higher RMR values associated with lower MCH concentrations, the small sample size (n=8) makes meaningful conclusions difficult. If circulating MCH concentrations are involved in a feedback mechanism conveying metabolic information it would be expected that those individuals with lower and higher metabolic rates would also display a relationship between RMR and circulating MCH concentrations.

To further explore the relationship between metabolic rate and circulating MCH regression analyses were performed. Results indicate that factors associated with variance in RMR, that is percent fat-free mass, percent fat-mass, fat-free mass (kg) and gender, aswell as RMR do not contribute significantly to the variance in fasted or post-prandial MCH concentrations. Furthermore in a separate model fasting MCH concentrations did not contribute significantly to the variance in RMR. These results suggest that

circulating MCH cannot be considered a biomarker of resting energy expenditure in young, healthy adults.

5.5.3 MCH and body composition

A further objective of this study was to determine whether there were any associations between body composition and circulating MCH concentrations since body composition undeniably impacts on metabolic rate and, as will also be discussed in Chapter 7, in rodents the MCH system appears to have the capacity to impact strongly on phenotype. Briefly a lean or obese phenotype can be induced by decreasing or increasing the availability of physiological hypothalamic MCH. The characteristic phenotype of an MCH knock-out model, whether lacking the peptide or the receptor, is one of leanness with increased energy expenditure, some studies have also reported resistance to diet-induced obesity (Chen *et al.,* 2002; Mashiko *et al.,* 2005; Kokkotou *et al.,* 2005); a trait which makes MCH antagonism attractive to pharmaceutical companies. In the current study mean circulating plasma MCH concentrations were not significantly correlated with percent body fat mass, percent body lean mass, body fat weight, lean mass weight, body weight or BMI in males or females. However in males only there were significant correlations between fasted MCH and BMI ($r=0.662$, $p=0.037$) and between mean post-prandial MCH and body lean mass weight (kg) ($r=0.639$, $p=0.034$). It is generally assumed that a higher BMI indicates a fatter individual but this is not true as a higher BMI can also be indicative of a greater lean mass. Therefore these results are not contradictory to each other but do not support the results of earlier rodent work where increased MCH has been associated with increased adiposity (Ludwig *et al.,* 2001, Gomori *et al.,* 2002).

If these results are reproducible and not anomalies then a further point arising from the current analysis is the question as to why the observed associations were so temporally specific, that is, only fasted MCH concentrations were correlated with BMI and only mean post-prandial MCH concentrations were correlated with lean body mass weight. Clearly body composition did not change measurably over the course of 2 hours and

fasted MCH concentrations were not significantly different to mean post-prandial concentrations in males or females. In the current study the sample size was modest (n=10) and further work is required. The studies described in Chapters 4, 6 and 7 examined the same parameters and since differing results were observed in each Chapter a full discussion in light of all the results is reserved for Chapter 8.

5.5.4 MCH response to feeding

Earlier work with rodents has consistently shown that administration or ablation of functional hypothalamic MCH impacts on feeding behaviours. This subject is discussed more fully in Section 5.5.5. In rodents central MCH expression is up-regulated after fasting (Qu *et al.,* 1996; Tritos *et al.,* 2001) though food restriction does not affect MCH mRNA or MCHR-1 mRNA (Elliott *et al.,* 2004). In humans serum MCH concentrations were increased to 127% of base-line after 2 days fasting (Gavrila *et al.,* 2005). However it has not been determined whether or not feeding acutely affects circulating MCH concentrations. Therefore circulating MCH concentrations both in the fasted state and in the acute period after feeding were measured; to this end blood samples were obtained at 30, 60 and 120 minutes post-prandial. Circulating leptin and insulin concentrations were also tracked since the post-prandial plasma leptin response is linked to insulin (Saad *et al.,* 1998; Romon *et al.,* 1999) though differences have been reported regarding the time-course for the leptin response (Dallongeville *et al.,* 1998; Romon *et al., 1999*; Teff *et al.,* 2004). Since it was the aim to examine the normal physiological response to feeding subjects were fed a combination of foods which may typically have been consumed for breakfast. The caloric and macronutrient contents were in line with Department of Health recommendations as described in Section 5.2.3.

Feeding did not appear to affect circulating MCH concentrations in the short term as there were no differences between fasted and fed MCH concentrations measured at any of the sampling time-points, nor was there a gender difference in the MCH response to feeding. In contrast circulating leptin concentrations had decreased significantly by the 1 hour post-prandial

sampling time-point in females only whilst males displayed a non-significant decrease in circulating leptin concentrations. The rapid female response is unlike that reported in previous studies in which the leptin response to feeding was not evident until 4hrs + post-prandial (Dallongeville *et al.,* 1998; Romon *et al.,* 1999; Romon *et al.,* 2003), though one study with both lean and obese men reported a response within 2 hours (Imbeault *et al.,* 2001). Differences in the leptin response seem to be influenced by gender, body composition and type of meal consumed. A difference in the magnitude of the leptin response has been observed following either high fat or high carbohydrate meals, the response being greater following the high carbohydrate meal (Romon *et al.,* 1999). Females displayed an elevated leptin response following a high fat meal whereas males did not (Romon *et al.,* 1999). In the current study the meal content was 60% carbohydrate/ 20% fat and this ratio has been designated high carbohydrate/low fat in other studies (Havel *et al.,* 1999) so a perceptible leptin response would have been expected. The direction of the leptin response also appears to be affected by body composition, Imbeault and colleagues (2001) reported an increase in post-prandial plasma leptin concentrations in lean men and a decrease in obese men which was thought to be related to a dysregulated leptin response to insulin in the obese. However most studies commonly report an increase in circulating leptin which commences several hours after eating. Since in this study the final sampling time-point was 2 hours post-prandial, it could be the lack of male response was entirely predictable, and what was observed may have been related to the morning nadir of leptin (Sinha *et al.,* 1996). Daily fluctuations in circulating MCH concentrations in humans have not yet been established, so at present it cannot be determined whether or not they impacted on current results.

A further objective of this study, aligned with the MCH response to feeding was to compare fasted and fed circulating MCH concentrations with blood glucose and plasma insulin concentrations since both hypothalamic and peripheral MCH has been implicated in glucose homeostasis and there is some evidence of local production of MCH in the endocrine pancreas (Pissios *et al.,* 2007). This was the major objective in the study described in

Chapter 6 with older adults. However by way of assessing the generalisability of the results across the studies albeit in different populations, the same parameters were examined here. The results of the current study are suggestive of an association between the post-prandial insulin response and the post-prandial MCH response since the AUC for insulin was positively correlated with the AUC for MCH and with mean plasma MCH concentrations in individuals with excess fat (r=0.862, p=0.012; r=0.891, p=0.009 respectively). Additionally the AUC's for MCH and glucose were negatively correlated in males only (r=-0.836, p=0.019). Two points arise from these results; first it appears that the circulating MCH and insulin response to food stimuli moves in the same direction but only in those with excess fat which could be a consequence of some insulin resistant state. In rodents there is evidence that MCH may be involved in insulin secretion and normal β-cell physiology (Pissios *et al.,* 2007). Hyperinsulinemia has been observed in a transgenic mouse model over-expressing MCH (Ludwig *et al.,* 2001). Pissios and colleagues (2007) suggest that this phenomena may be the result of MCH-driven insulin production. The results reported above are not incompatible with this framework. Secondly associations between the post-prandial MCH, glucose and insulin response are only evident in discrete sub-groups of the sample population and not across the group as a whole. However since the sample sizes were small robust conclusions cannot be drawn though the results are reported since they are in broad agreement with the results reported in Chapter 6.

5.5.5 MCH and energy intake

As noted above, in rodents the MCH system appears to have the capacity to impact strongly on feeding behaviours. Hyper- or hypophagia can be induced by manipulating the availability of physiological hypothalamic MCH concentrations. Whilst increased availability results in hyperphagia (Ludwig *et al.,* 2001, Gomori *et al.,* 2002, Santollo and Eckel, 2008), decreased availability can result in either hyperphagia (Chen *et al.,* 2002; Marsh *et al.,* 2002) or hypophagia (Mashiko *et al.,* 2005; Kowalaski *et al.,* 2006). This discrepancy may be attributed to whether a genetic or pharmacological

rodent model was used. Compensatory hyperphagia in response to reduced adiposity has been proposed as the cause of consummatory behaviours in MCHR-1 knockout models whilst antagonism of the MCHR-1 generally results in hypophagia (for review see Pissios, 2009). Accumulating evidence from behavioural and anatomical studies also supports a role for MCH activity in the hedonic aspect of feeding. Antagonism of the MCH receptor reduced consumption of highly palatable food (Morens *et al.,* 2005), whilst infusion of MCH resulted in increased alcohol, sucrose and saccharin intake (Duncan *et al.,* 2005; Sakamaki *et al.,* 2005; Furudono *et al.,* 2006). An apparent preference for fatty foods has also been observed following chronic ICV infusion of MCH in mice (Gomori *et al.,* 2002). MCH treatment also seems to be able to affect meal size, being increased following MCH administration (Santollo and Eckel, 2008), or decreased following antagonism of the MCH receptor (Kowalski *et al.,* 2004). Extra-hypothalamic sites are also involved in the MCH effect on ingestive behaviours. In a recent study food intake though not energy expenditure increased after injection of an MCH agonist into the nucleus accumbens shell (Guesdon *et al,* 2009), an area involved in motivation and reward (Grace *et al.,* 2007; Mahler *et al.,* 2007). Anatomically this area is densely populated by the MCH receptor, as are areas involved in olfaction and gustation (Kokkotou *et al.,* 2001, Saito *et al.,* 2001). Therefore it was hypothesised that circulating MCH concentrations may be associated with habitual food intake which was assessed by self-reported food diary. Nevertheless in the current study there were no associations between plasma MCH concentrations and total caloric intake or specific macronutrient intake. Needless to say ingestive behaviours are more complex in humans than in rodents, the cues and drives to eat being influenced by many non-biological factors. Although the results of interventional animal studies place MCH firmly in the neurocircuitry which regulates feeding behaviours, in free-living humans cognitive, emotional and environmental factors may over-ride pure biological input. Whilst these initial results do not endorse a role for circulating MCH in appetitive behaviours in humans there are many more aspects of these behaviours to be explored. For example in the current study it was not known whether the subjects were in positive, negative or eu-energy balance which may have impacted on

circulating MCH concentrations. Additionally the inherent limitations associated with self-reported food intake are acknowledged (Carriquiry, 2003), though for the purposes of this initial characterisation the self-reported food diary method was deemed to be adequate.

5.5.6 Summary, limitations and future work

A major outcome from this study was the establishment of a reference range for circulating MCH concentrations in young, healthy adults which is in broad agreement with the reference range derived from a larger and more diverse sample population described in Chapter 4. Secondly there may be an association between the post-prandial MCH response and the post-prandial insulin response which is currently tenuous in the light of the small sample size of the sub-groups. Thirdly despite evidence of a functional albeit possibly indirect link between the actions of central MCH and leptin, there appears to be no direct relationship between circulating MCH and leptin concentrations in the acute phase after feeding in this population. So it appears that circulating MCH does not fluctuate in concord with other primary metabolic mediators but it could be that it is only up or down-regulated in response to some perturbation in the system. Whereas leptin and insulin convey continuous metabolic information from the periphery to the CNS, MCH activity may only become apparent if a number of conditions are satisfied. A major limitation of the current study was the cessation of blood sampling 2 hours post-prandial. This combined with the absence of a 24-hour profile of circulating MCH concentrations hampers meaningful interpretation of these results and is a subject that should be addressed in future work; for example it could be that MCH behaves as leptin post-prandially with no real effect being observed until several hours after feeding. In terms of energy intake associations were not found between circulating MCH concentrations and total caloric intake or macronutrient preference, however the use of weighed rather than estimated food diaries would have improved confidence in this outcome.

A further finding was that RMR was not strongly associated with fasting MCH concentrations except in females whose RMR was in the normal category

though not in excess of 100% predicted (and in a separate study in older lean males whose RMR was in the same category). A larger sample population needs to be examined before more robust conclusions can be drawn with regard to a physiological role for circulating MCH in energy expenditure in humans.

An unexpected outcome of this study was that circulating MCH concentrations seem to be associated with lean mass rather than fat mass in younger adults and only in males. The results of this study and other studies undertaken in this series indicate that there is some sexual dimorphism associated with MCH activity. It may also be that, in young adults who are less likely to have a dysregulated metabolism circulating MCH may be better indexed to lean rather than fat mass.

6. The association between circulating concentrations of MCH with glucose and insulin in response to fasting and feeding in lean individuals and those with excess body fat

6.1 Introduction

In humans very little is known about the sources, targets and natural fluctuations of circulating and peripheral MCH. In rodents both central and peripheral MCH have been implicated in glucose homeostasis (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005). A recent study (Pissios *et al.,* 2007) detected MCH and its receptor in both human and mice islets and in clonal β-cell lines. Additionally MCH k/o mice exhibited reduced β-cell mass compared to controls in response to high fat feeding; and exogenous MCH stimulated insulin secretion in cultured human and mouse islets. In a previous study mice over-expressing MCH demonstrated islet hyperplasia which was out of proportion to their mildly obese phenotype (Ludwig *et al.,* 2001). If MCH stimulates β-cell activity it would be logical to investigate whether circulating concentrations of MCH change in the same direction as insulin. Furthermore since obesity is associated with insulin resistance (Kahn *et al.,* 2001) the hypothesis that subjects with normal amounts of body fat would exhibit a differential post-prandial MCH response to those with excess body fat was explored. Therefore the aims of this study were:

- To determine whether there is an association between circulating concentrations of MCH and circulating concentrations of glucose/insulin.
- To examine changes in circulating MCH concentrations in the fasted and fed states in lean individuals and those with excess body fat.

6.2. Subjects and experimental protocol

6.2.1 Participants

Two cohorts of subjects were recruited (n=50) by methods described in Section 3.2.2; a) those with excess body fat and b) lean individuals. Lean (L) was defined as ≤ 30.9% body fat in females and ≤ 20.9% body fat in males. Excess body fat (E) was defined as≥ 31% in females and ≥ 21% in males (ACSM, 1996). The inclusion criterion for both males and females was that they should be over 40 years of age. Those on medication for chronic illnesses were not automatically excluded though those on medication known to cause hypo- or hyperglycaemia or affect metabolic rate were precluded from participation. Exclusion criteria for females were pregnancy, lactating or recently lactating (within the last 12 months).

6.2.2. Pre-test

Subjects received a Subject Information Sheet (Appendix 6) and completed an Informed Consent form (Appendix 7). Otherwise as described in Section 5.2.2.

6.2.3 Experimental protocol

Subjects were tested in a 4 hour protocol described in Section 5.2.3. Venous and fingerprick blood samples were collected, treated and the plasma stored as described in Section 3.2.6. Plasma samples were subsequently assayed for MCH, leptin and insulin as described in Section 3.2.7.

6.3 Statistical analyses

Linear multiple regression analysis was not used to examine relationships between circulating MCH concentrations, RMR and body composition otherwise as section 5.3.

6.4 Results

6.4.1. Subject characteristics

Males and females were of similar ages (p=0.779) and BMI (p=0.423). Ages ranged from 40.4 to 69.9 years. Males were significantly taller (p<0.001), heavier (p<0.001) and leaner (p<0.001) than females. Females had significantly greater fat mass % than males (p<0.001). Females in the lean group did not differ in % fat mass from males in the excess fat group.

Demographic and anthropometric characteristics of the study participants are presented in Table 6.1 below.

Table 6.1 Demographic and anthropometric characteristics of study participants

	Lean		**Excess body fat**	
	Male (n = 10)	Female (n =11)	Male (n = 16)	Female (n = 13)
Age (yrs)	50.6±6.4[a]	51.4±9.3[a]	51.8±7.3[a]	52.5±5.9[a]
Height (m)	1.81±0.1[a]	1.64±0.1[b]	1.78±0.1[a]	1.66±0.1[b]
Weight (kg)	77.7±3.9[a]	59.5±8.2[b]	89.8±11.3[c]	76.7±11.4[a]
Fat mass (%)	12.7±3.6[a]	26.5±5.3[b]	26.5±5.3[b]	38.8 ± 5.4[c]
Lean mass (%)	87.2±3.8[a]	74.2±3.6[b]	74.3±6.2[b]	61.1±5.3[c]
BMI (kg/m^2)	23.5±1.5[a]	22.1±2.4[a]	28.0±3.5[b]	28.1±4.6[b]
Waist-Hip Ratio	0.83±0.1[a]	0.78±0.1[a]	0.91±0.1[b]	0.83±0.1[a]

Mean ± SD, within a category values with different superscripts are significantly different from each other (one-way between-groups ANOVA; p<0.05)

6.4.2 Plasma hormone concentrations

6.4.2.1 Pre and post-prandial plasma MCH concentrations

The four sampling timepoints were designated F=fasted; PP1=30 minutes after feeding; PP2=60 minutes after feeding; PP3=120 minutes after feeding. In all individuals plasma MCH concentrations increased after eating. Fasting plasma MCH concentrations were significantly lower ($p<0.005$) than those measured 1 hour and 2 hours postprandial with a large effect of size, multivariate partial eta squared=0.757. Female plasma MCH concentrations were significantly higher than fasting 2 hours post prandial ($p=0.023$), male plasma MCH concentrations were significantly higher than fasting at the 1 hour and 2 hour sampling time-points (Table 6.2: Figure 6.1 (a): one-way repeated measures ANOVA; $p=0.001$, $p=0.006$ respectively). Subjects were grouped by fat mass as Section 6.2.1. There was a significant increase from fasting at the 1 hour and 2 hour postprandial sampling time-points in both groups (Table 6.2: one-way repeated measures ANOVA; $p<0.05$). However there were no differences in plasma MCH concentrations between L and E at any of the sampling time-points. To further explore the effect of fat mass and gender on circulating MCH concentrations subjects were divided into four groups: male lean (ML): body fat % < 21%; male excess fat (ME): body fat % ≥ 21%; female lean (FL): body fat % < 31%; female excess fat (FE): body fat % ≥ 31% (ACSM, 1996). There was no effect of gender and adiposity though there was a trend for increased post-prandial plasma MCH concentrations compared to fasting (Figure 6.1 (b): one-way between groups ANOVA; $p>0.05$). Only subjects from whom blood samples were collected at each of the 4 time-points were included in the above analyses hence the change in subject numbers per group. The incremental area under the curve (AUC) for MCH was not different between the four groups nor when grouped only by gender or adiposity.

Table 6.2: Pre and post-prandial plasma MCH concentrations in lean males and females and those with excess body fat

	All (n=18)	Female (n=8)	Male (n=10)	L (n=9)	E (n=9)	FL (n=4)	FE (n=4)	ML (n=5)	ME (n=5)
MCH F	34.43 ±5.6[a]	33.08 ±4.5[a]	35.52 ±6.3[a]	35.17 ±7.3[a]	33.70 ±3.4[a]	33.07 ±5.3	33.09 ±4.5	36.85 ±8.8	34.19 ±2.6
MCH2 PP1	38.13 ±7.7	37.94 ±4.5	38.28 ±9.8	39.6 ±7.2	36.31 ±8.9	38.18 ±4.0	37.71 ±5.6	41.38 ±9.2	35.19 ±10.3
MCH3 PP2	40.13 ±5.7[b]	37.51 ±4.0	42.22 ±6.3[b]	41.45 ±6.5[b]	38.80 ±4.8[b]	38.17 ±3.7	36.85 ±4.8	44.07 ±7.5	40.36 ±4.8
MCH4 PP3	40.22 ±4.2[b]	38.87 ±2.7[b]	41.29 ±5.0[b]	41.7 ±4.3[b]	38.75 ±3.8[b]	39.93 ±2.5	37.82 ±2.7	43.09 ±5.2	39.49 ±4.7

Values with different superscripts are significantly different from each other within a group (one-way repeated measures ANOVA; p,0.05. Reduced n values are due to missing plasma samples.

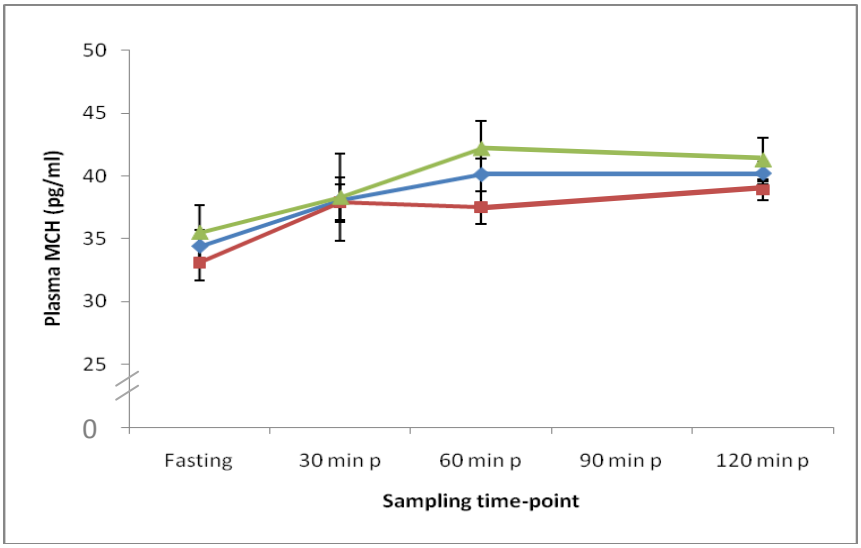

Figure 6.1 (a): Pre- and post-prandial plasma MCH concentrations in males (n=10) and females (n=8) (mean ± SEM) ● All ▲ Male ■ Female p: post-prandial

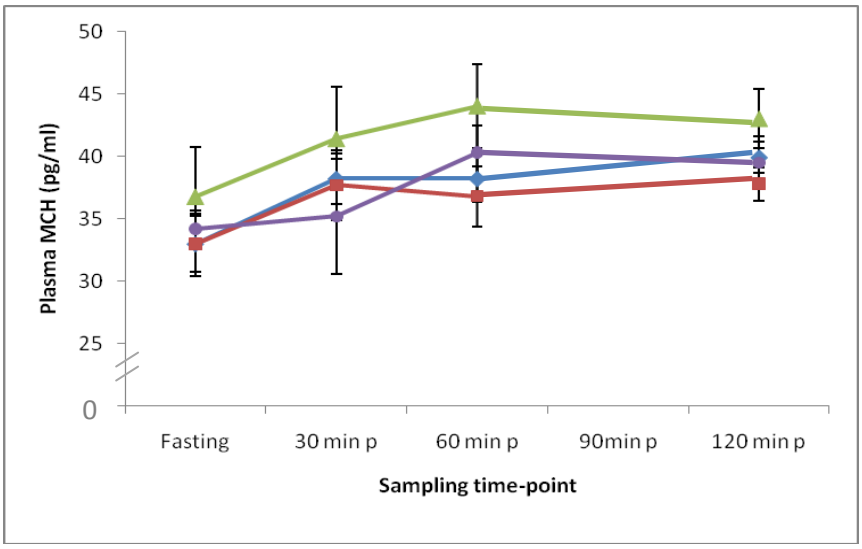

Figure 6.1 (b): Pre- and post-prandial plasma MCH concentrations in lean males and females and those with excess fat (mean ± SEM) ◆ Female lean (n=4) ▲ Male lean (n=5) ■ Female excess fat (n=4) ● Male excess fat (n=5) p: post-prandial

6.4.2.2. Pre- and post-prandial plasma leptin concentrations

In all individuals plasma leptin concentrations decreased in the acute period after feeding. However there was a gender difference in the pattern of the post-prandial leptin response. Female fasting plasma leptin concentrations were significantly higher than those measured 1 hour but not 2 hours post-prandial (p=0.037; partial eta squared=0.687). Male fasting plasma leptin concentrations had not decreased significantly by the 1 hour sampling time-point but were significantly lower than fasting by the 2 hour post-prandial sampling time-point. (Figure 6.2: one-way repeated measures ANOVA; p=0.028, partial eta squared=0.716).

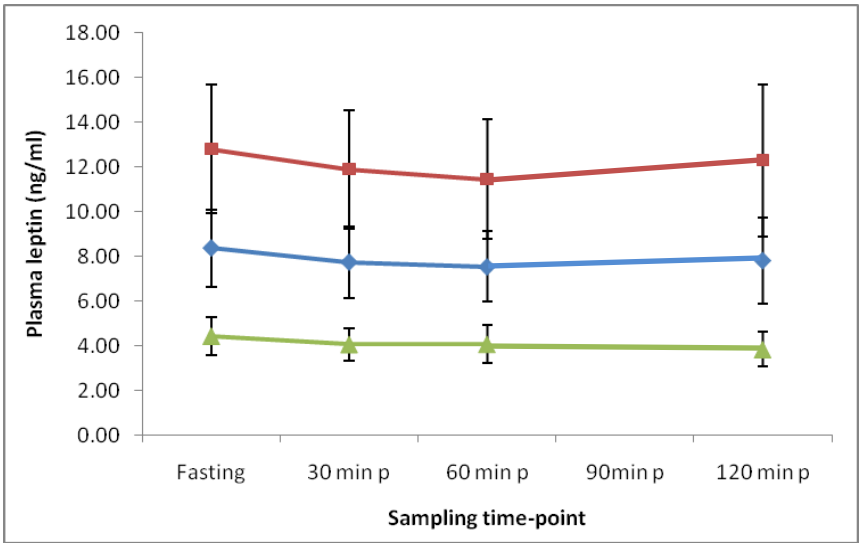

Figure 6.2: Pre- and post-prandial plasma leptin concentrations (mean ± SEM)
All (n=17) Male (n=9) Female (n=8) p: post-prandial

6.4.2.3 Pre- and post-prandial blood glucose and plasma insulin concentrations

All individuals responded as expected following break of fast, that is, there was a sharp significant increase in blood glucose and plasma insulin concentrations which then declined over the following 2 hour period. Mean post-prandial insulin concentrations were significantly higher in the excess fat group compared to the normal fat group (p=0.034). Subjects were grouped by fat-mass as Section 6.4.2.1. Plasma insulin concentrations were measured at the four sampling time-points, there were significant differences

between E and L only at 30 minutes post-prandial; E had significantly higher plasma insulin concentrations than L (Figure 6.3: p=0.033). This difference was not apparent when the groups were further sub-divided by gender as Section 6.4.2.1 (Figure 6.4: p>0.05). The AUC for insulin was not different between FL, FE, ML or ME (one-way between-groups ANOVA; p>0.05), nor when grouped by gender or adiposity only.

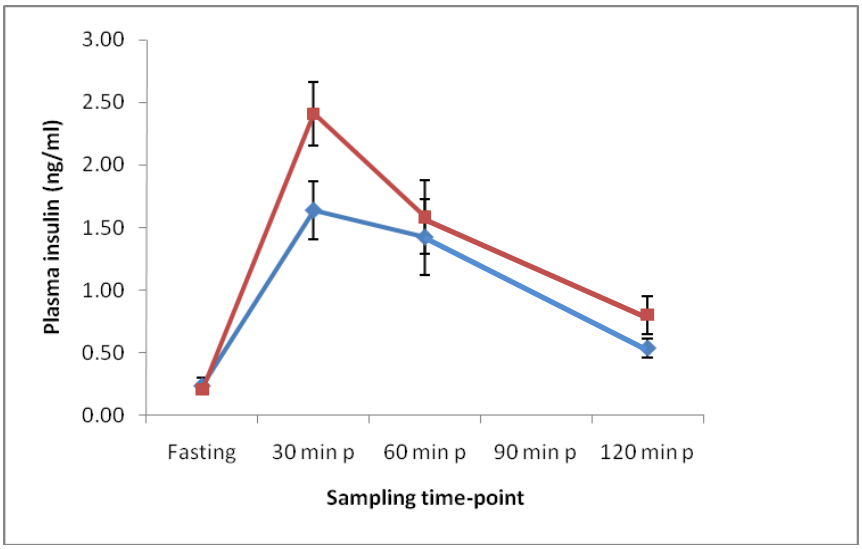

Figure 6.3: Pre- and post-prandial plasma insulin concentrations in lean individuals and those with excess fat (mean ± SEM) ◆ Lean (n=9) ■ Excess fat (n=10) p: post-prandial

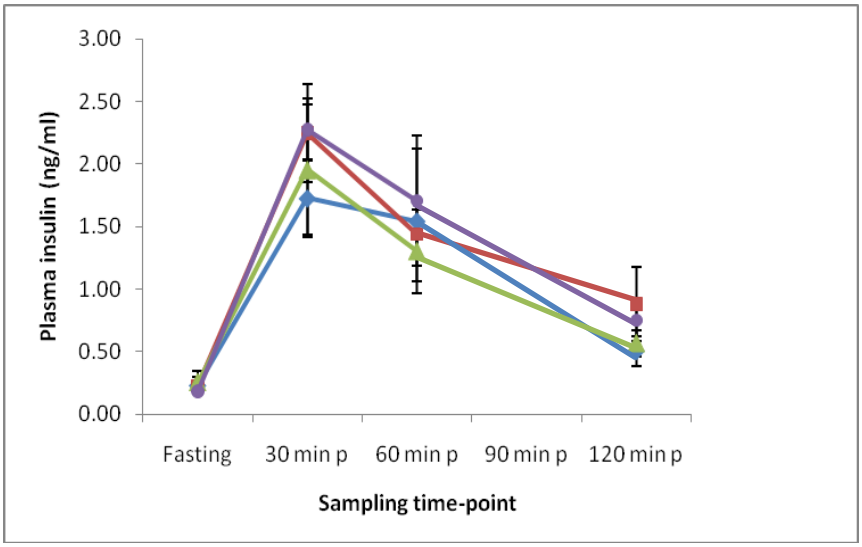

Figure 6.4: Pre- and post-prandial plasma insulin concentrations in lean males and females and those with excess fat (mean ± SEM) ◆ Female lean (n=4) ▲ Male lean (n=5) ■ Female excess fat (n=4) ● Male excess fat (n=6) p: post-prandial

The AUC for glucose was not different between those with excess fat and lean individuals of either gender. There were no significant differences

between male and female pre and post blood glucose concentrations at any sampling time-point. Nor were there significant differences when the data was grouped by fat mass or by fat mass and gender (Figure 6.5: one-way between-groups ANOVA; p>0.05).

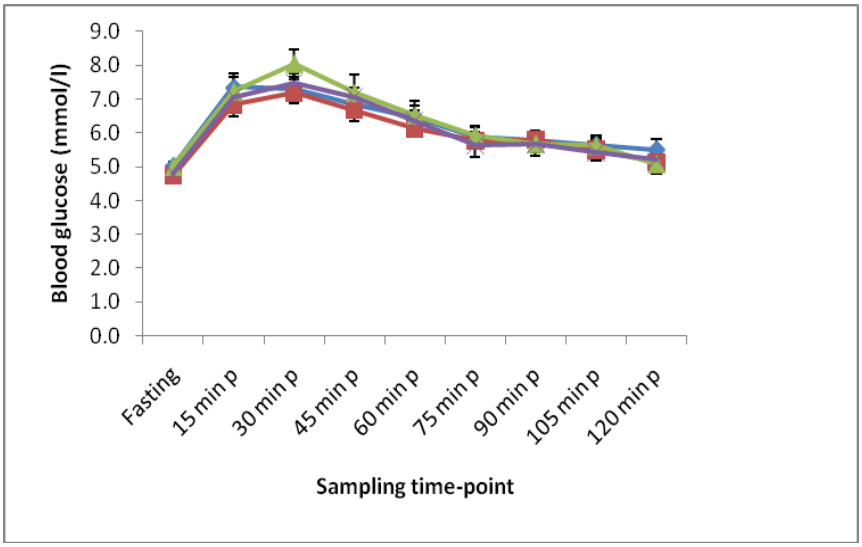

Figure 6.5: Pre- and post-prandial blood glucose concentrations in lean males and females and those with excess fat (mean ± SEM) ◆ Female lean (n=8) ▲ Male lean (n=9) ■ Female excess fat (n=11) ● Male excess fat (n=13) p: post-prandial

6.4.2.4 Associations between pre- and post-prandial plasma MCH and leptin concentrations

There were significant correlations at 2 sampling time-points 1) a negative correlation between fasted plasma MCH concentrations and fasted leptin concentrations in males with excess fat (r =-0.772, p=0.043, n=6) and 2) a positive correlation 30 minutes post-prandial between plasma MCH concentrations and plasma leptin concentrations in lean males (r =0.770, p=0.025, n=6). The MCH AUC was not significantly correlated with the leptin AUC.

6.4.2.5 Associations between pre- and post-prandial circulating MCH, glucose and insulin concentrations

For each subject the mean MCH plasma concentration of the four sampling time-points was calculated and used for the analyses in the following sections. The AUC for insulin was negatively correlated with mean MCH plasma concentrations (all individuals: r=-.485, p=0.014). In a model in which mean MCH plasma concentrations, percent fat mass and waist-hip ratio were the predictors for the insulin AUC in standard multiple regression analysis, 34% of the variance in the insulin AUC was explained by this model. Of the 3 independent variables only mean plasma MCH concentrations made a significant unique contribution (17.2%) to the variance in the insulin AUC (adjusted R square =0.340; part correlation co-efficient=-0.415; p=0.021). A moderate but significant association was observed between the glucose AUC and mean MCH plasma concentrations in those with excess fat only (r=-0.398, p=0.049). The AUC for glucose was not significantly correlated with the AUC for MCH or leptin.

6.4.2.6 Associations between pre- and post-prandial circulating leptin, glucose and insulin concentrations

As Section 6.4.2.5, for each subject the mean circulating leptin, glucose and insulin concentrations of the four sampling time-points were calculated and used for the analyses in the following sections. Thirty minutes after feeding there was a significant negative correlation between plasma leptin concentrations and blood glucose concentrations (all individuals: r=-0.345, p=0.049). When split by gender the association was not upheld in males though was in females (r=-0.852, p<0.001), this relationship was stronger in lean females (r=-0.989, p=<0.001).

Two hours after feeding there was a significant negative correlation in males with excess fat between plasma leptin concentrations and blood glucose concentrations (r=-0.714, p=0.031). All correlations for leptin are presented after controlling for fat mass.

6.4.3 Associations between mean plasma MCH concentrations and body composition parameters

There was a strong positive correlation between BMI, body weight and mean plasma MCH concentrations in lean females. There was also a strong positive correlation between mean post-prandial plasma MCH (that is, mean of the 3 sampling time-points post-feeding for each individual) and body fat weight in lean females (Table 6.3).

Table 6.3 Associations between mean plasma MCH concentrations with body composition parameters in lean males and females and those with excess body fat

	Female			**Male**		
	All (n=24)	L (n=11)	E (n=13)	All (n=26)	L (n=11)	E (n=15)
Fat mass (%)	-0.099	0.370	-0.155	-0.279	-0.472	0.071
Lean mass (%)	0.099	-0.370	0.155	0.279	0.472	-0.071
BMI (kg/m^2)	-0.074	0.613 (p=0.045)*	-0.204	-0.160	0.032	-0.034
Body weight (kg)	-0.143	0.602 (p=0.050)*	-0.408	-0.130	-0.058	0.013
Fat mass weight (kg)	-0.148	0.608# (p=0.047)*	-0.318	-0.241	-0.391	0.071
Lean mass weight (kg)	-0.081	0.545	-0.532	0.143	0.231	-0.042
Waist hip ratio	0.068	0.347	-0.163	-0.210	-0.375	0.134

Values represent Pearson product-moment correlations, * significant at 0.05 level (2 tailed);

L: lean, E: excess fat, # mean post-prandial MCH

6.4.4 Associations between plasma MCH concentrations and resting metabolic rate

Subjects were divided into 3 groups based on percentage of predicted RMR as in Section 5.6. The groups were Low (L)= ≤89.9% predicted RMR; Normal (N)=90 – 99.9% predicted RMR; High (H)= ≥ 100% predicted RMR as Section 5.4.4. There was a significant correlation between fasting plasma MCH concentrations and RMR in lean males in the N group only (r=0.781, p=0.038, n=7).

6.5 Discussion

6.5.1. MCH response to feeding in association with circulating leptin concentrations

Adiposity is associated with an impaired insulin response which is linked to leptin resistance and all 3 appear to be factors of aging though the cause-effect is currently unclear (Carrascosa *et al.,* 2009). Since both central and peripheral MCH have been implicated in glucose metabolism (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005; Pissios *et al.,* 2007), and there is ample evidence that MCH and leptin have the capacity to both inhibit and stimulate each other (Huang *et al.,* 1999; Bradley *et al.,* 2000; Kokkotou *et al.,* 2001; Bradley *et al.,* 2002) a primary objective of this study was to describe the post-prandial MCH, leptin and insulin response in older individuals characterised as having excess fat, and thereby being more likely to have some degree of insulin resistance, and to compare their response with their leaner counterparts.

In contrast to the study undertaken with 18 – 30 year olds reported in Chapter 5, there was a significant change in post-prandial MCH concentrations in both males and females. Circulating MCH concentrations had risen significantly by the 1 hour sampling time-point in males and by the 2 hour sampling time-point in females. Body composition did not appear to be a factor since this increase was evident in both lean individuals and those

with excess fat by the 1 hour post-prandial sampling time-point and remained elevated until the 2 hour post-prandial sampling time-point. Additionally, at least in the lean older individuals, their body fat mass, lean mass and BMI were similar to those of the younger group in both males and females ($p>0.05$). Furthermore both the younger and older groups were fed the same meal (Section 5.2.3) therefore meal composition does not appear to be relevant. Since this discrepancy does not appear to be related to body composition or meal content, the next question to be addressed is whether or not this increase could be driven by humoral factors. First MCH concentrations changed in the same direction as blood glucose and plasma insulin and there were correlations between the insulin AUC and mean plasma insulin concentrations discussed in Section 6.5.2. Secondly for the first time in this series of studies, a significant correlation was observed between plasma leptin and plasma MCH concentrations in males only. Specifically, after controlling for fat mass, fasted plasma MCH concentrations were inversely correlated with fasted plasma leptin concentrations in males with excess fat ($r=-0.722$, $p=0.043$), and 30 minutes post-prandial plasma MCH concentrations were positively correlated with plasma leptin concentrations in lean males only ($r=0.770$, $p=0.025$). In energy homeostasis hypothalamic leptin indirectly inhibits hypothalamic MCH via the NPY/AgRP metabolic pathway (for review see Schwartz *et al.,* 2000), therefore the inverse correlation between fasting MCH and leptin would be expected if this effect is reflected in the periphery. Few other studies have attempted to evaluate the association between circulating MCH and leptin. In rodents no direct correlation was found between these two metabolic markers in lean or obese animals (Stricker-Krongrad *et al.,* 2001). In humans fasting serum MCH concentrations were not associated with serum leptin concentrations in a younger sample population (17 ± 1.7 yrs; Gavrila *et al.,* 2005). Nor were there any relationships between plasma leptin and plasma MCH in 2 separate studies (Chapters 5 and 7). Therefore the question must be posed as to why a relationship was observed in the current study but only in 2 distinct cohorts; that is, older males with excess fat in the fasted state and older lean males post-prandial. Leptin action is altered with aging and is characterised by increased adiposity and the development of leptin

resistance, however it is not known which precedes which (Carrascosa *et al.,* 2009). It may be that the differential direction of the plasma MCH/plasma leptin relationship between those with excess fat and lean phenotypes (negative *versus* positive) is symptomatic of disruption between MCH and leptin signalling, in this context it is possible that MCH may be responding to some leptin resistant state. It must also be borne in mind that the n of the sub-groups was modest (males excess fat: n=9; males lean: n=9) therefore further substantive work is required, although the above observations support the sexual dimorphism which has already been reported in the MCH response in the current series of studies (Chapter 5) and by others (Chen *et al.,* 2002; Alon and Friedman, 2006).

At least in the acute phase after feeding plasma MCH concentrations and plasma leptin concentrations were moving in opposing directions, as leptin concentrations were decreasing, MCH concentrations were increasing though the leptin response was apparent earlier in females than males (1 hour *versus* 2 hours post-prandial). This is in agreement with the results reported in Chapter 5 but as noted there, this decrease may have been related to the morning nadir of leptin in circulation, and it may well be that plasma leptin concentrations subsequently rose which would have been in-line with current understanding regarding the acute leptin response to feeding (Boden *et al.,* 1996; Saad *et al.,* 1998; Attoub *et al.,*1999).

6.5.2. Post-prandial MCH response in association with the post-prandial glucose and insulin response

Currently the source(s) and targets of circulating and peripherally derived MCH are not known though there is evidence that it may be expressed in the pancreas in both rodents and humans. Results of a recent study suggest that MCH may be necessary for normal β-cell function and MCH has been shown to stimulate insulin release from βcells *in vitro* (Pissios *et al.,* 2007). Whether or not MCH acts in a paracrine or autocrine manner within the pancreas or is released into the circulation is not known. If MCH is active at the level of the endocrine pancreas it was hypothesised that the post-prandial

insulin response might be related to the post-prandial MCH response and could be altered in the presence of insulin resistance. Current results indicate that circulating MCH concentrations are associated with the peripheral post-prandial insulin response. First there was an inverse correlation between the insulin AUC and mean plasma MCH concentrations ($r=-.485$, $p=0.014$) with a shared variance of 23.5%. Secondly in a regression analysis where mean plasma MCH concentrations, percent fat mass and waist-hip ratio were predictors for the insulin AUC, mean plasma MCH concentrations made a significant unique contribution (17.2%) to the variance in the insulin AUC. However the nature of the association can only be speculated upon without further investigation. As previously noted the sources and targets of circulating and peripheral MCH are not currently known although both MCH and its receptor have been detected in human and mouse pancreatic islets and MCHR-1 has also been detected in a clonal β-cell line (Pissios *et al.,* 2007). It therefore seems likely that one of the sources of circulating MCH could be the endocrine pancreas and the inverse relationship between the insulin AUC and mean circulating MCH concentrations at least in the acute period after feeding could be indicative of increased MCH activity at the level of the pancreas. At least in rodents it would appear that MCH modulates normal islet physiology as evidenced by the failure of MCH k/o mice to display the customary islet hyperplasia associated with high-fat feeding (Pissios *et al.,* 2007) and the excessive islet hyperplasia observed in mice over-expressing MCH (Ludwig *et al.,* 2001). It is therefore entirely possible that MCH may be one of a number of neuropeptides which exert manifold influence at different levels of energy homeostasis though elucidation is ongoing regarding its specific interactions and pathways. In the current series of studies it was the aim to describe the relationship between circulating MCH and leptin in humans, given that leptin is a major peripheral adiposity signal which exerts its influence at the level of the hypothalamus. Apart from the aforementioned correlations between (a) fasting plasma MCH concentrations and leptin in males with excess fat and (b) post-prandial plasma MCH concentrations and leptin in lean males there were no other significant associations between circulating plasma MCH and leptin. Insulin increases leptin production (Bradley and Cheatham, 1999;

Lee *et al.,* 2006; Lee *et al.,* 2007). However in the current study circulating leptin concentrations were not significantly correlated with circulating insulin concentrations at any sampling time-point or between the respective AUCs. On the other hand there was a moderate but significant inverse association between the glucose AUC and mean plasma MCH concentrations in those with excess fat only (r=-.398, p=0.049). In terms of the discrete sampling time-points there was also a moderate but significant correlation between blood glucose and plasma leptin 30 minutes post-prandial (r=-.345, p=0.049). When split by gender this association was stronger in females only (r=-.852, p<0.001) and in lean females the correlation was (r=-.989, p<0.001). At the same sampling time-point plasma MCH concentrations were positively correlated with plasma leptin concentrations in lean males only (r=-770, p=0.025). Two hours post-prandial there was a significant inverse correlation between blood glucose and plasma leptin in males with excess fat only (r=-.714, p=0.031). One should exercise caution when interpreting results such as these in the absence of a more comprehensive depiction of the circulating hormonal milieu in the acute phase after feeding. The physiological relevance of such outcomes should be considered. Nonetheless these results suggest that circulating MCH is involved in nutrient related cross-talk between components of energy metabolism which is differentially regulated in the presence of adiposity.

At the outset of this study it was the intention to compare an older fatter cohort with an older leaner cohort reasoning that the more corpulent group would be more likely to have some degree of insulin resistance. However even those displaying morphological characteristics which would incline them towards insulin resistance; that is a BMI of > 30kg/m^2 and waist-hip ratio of > 1.0 for men and > 0.8 for women, had fasting and 2 hr post-prandial blood glucose concentrations within the normal range (< 6.1mmol/l fasting, < 7.8mmol/l 2 hrs post-prandial). Furthermore the individual Homeostatic Model Assessment (HOMA) scores (Matthews *et al.,* 1985), which is a mathematical model method for detecting insulin resistance, only exceeded 2.0 in 2 individuals. There appears to be no reference values for HOMA scores which represent insulin resistance, however scores in excess of 2.0

and 3.99 have been taken as definitive in other studies (Bakari and Onyemelukwe, 2005; Wahreneburg *et al.,* 2005). Although plasma insulin concentrations were significantly higher in those with excess fat than lean individuals 30 minutes post-prandial, the AUCs for insulin and glucose were not different between the excess fat and lean groups of either gender. Therefore it would seem that glucose homeostasis was still normal in both the lean group and the excess fat group, hence the hypothesis that an altered MCH response may have been observed in the presence of insulin resistance could not be further explored in the current study.

6.5.3 MCH and body composition

A further objective of this study, and one which has been attendant in the other studies in this series was to describe associations between circulating MCH and body composition in the discrete populations under scrutiny, since this association is differentially pertinent for each study. In the current study the fundamental enquiry was highly dependent on differences in body composition, specifically body fat and lean mass. As discussed in Chapters 5 and 7, up-regulation or central infusion of MCH is strongly associated with adiposity in rodents and conversely ablation of functional MCH results in a lean phenotype accompanied by increased energy expenditure. Conflicting results with regard to the association between circulating MCH and body composition have been found in this series of studies. In the current study, in contrast to the results reported in Chapter 5 a positive correlation between (a) BMI, body weight and mean plasma MCH concentrations and (b) body fat weight and mean post-prandial MCH was observed in lean females only ($r=0.613$, $p=0.045$; $r=0.602$, $p=0.05$; $r=0.608$, $r=0.047$ respectively). A high BMI in conjunction with a high body weight and a high body fat weight is indicative of adiposity. Based on results from rodent studies those with increased adiposity would be expected to have higher circulating MCH concentrations and the current results are not in complete accord with this. MCH was positively associated with markers of adiposity though not with fat mass percentage in lean women only. This is a further example of sexual dimorphism which has been previously noted in this context and curiously

based on the results reported here and in Chapter 5 (though not in Chapter 7) it seems that MCH signalling may be more acute in the lean rather than in the obese. Again the n was relatively modest (11) and larger studies are required to confirm or deny these results but the question is posed as to whether in some populations fat interferes with MCH signalling either directly or indirectly via leptin resistance or some other perturbation of the system.

With regard to the observed sexual dimorphism relating to circulating MCH concentrations in the energy homeostasis context it must be noted that results of previous work are suggestive of a gender-related component to MCH physiologic function. A body of evidence has accrued implicating an oestrogen-sensitive or oestrogen-dependent mechanism in the modulation of central MCH activity (Viale *et al.,* 1999; Murray *et al.,* 2000b; Mystkowski *et al.,* 2000) and in the current series of studies an association between plasma MCH concentrations and plasma progesterone concentrations in females has been reported (Chapter 7). Therefore a link between the MCH system and gonadal steroids is suggested . In males there is little published work in this context. In the current series of studies results of correlational analysis from a sub-sample of males (n=17, mean age: 38.7 ± 11.7 yrs) were consistent with a robust relationship between circulating MCH and circulating testosterone concentrations (r=0.537, p=0.026). An indirect mechanism may be operative since testosterone has been reported to modulate the activity of other anabolic central effectors, namely NPY (Sahu *et al.,* 1992; Urban *et al.,* 1993), which projects onto MCH neurones in the lateral hypothalamus.

Finally the other studies in this series (Chapters 5 & 7) have examined RMR in association with circulating MCH concentrations since the results of animal studies strongly suggest that the MCH system impacts on energy expenditure and anatomically there are several proposed mechanisms through which it may influence thermogenesis including suppression of the sympathetic nervous system (Astrand *et al.,* 2004; Messina and Overton, 2007) and reduction of uncoupling protein activity in brown adipose tissue (Ito *et al.,* 2003; Segal-Lieberman *et al.,* 2003). The conclusions derived from the aforementioned studies in this series are that in humans there is no strong association between RMR and circulating MCH concentrations. In the

current study an association was observed in a sub-section of the sample population, that is, in lean males with an RMR of 90 – 99.9% of predicted, which is considered normal (r=0.781, p=0.038). However in light of the small n (7), these results are reported but no inference is currently made.

6.5.4 Summary, limitations and future work

The main finding of this study is supportive of a role for circulating MCH in the acute post-prandial period in humans, as evidenced by the results of correlation and regression analyses between plasma MCH, leptin, insulin and blood glucose concentrations. However the strength and direction of these inter-relationships appears to be heavily influenced by combinations of gender and adiposity which is not entirely unexpected given the major roles of these circulating factors both as conveyors of metabolic information and effectors. Additionally in regression analysis mean plasma MCH concentrations made a significant contribution to the variance in the insulin AUC. Nevertheless further investigative work is required to explore certain inconsistencies; for example the AUC for MCH was not correlated with the AUC for insulin though mean plasma MCH concentrations were. A consistent observation in this population however was that plasma MCH concentrations rose in all groups post-prandial, the source of this increase is not known though the endocrine pancreas is a candidate. On the other hand fluctuations in circulating MCH concentrations may be responding to fluctuations in pancreatic insulin production. It should also be noted that MCH has many and varied physiologic functions and it is quite likely that circulating MCH concentrations may be responding to multiple input. It was also interesting to note in this and other studies in this series that circulating MCH concentrations seem to be associated with distinct body composition parameters in distinct populations and that it is associated with leaness as well as adiposity. It may also be that adiposity alters MCH signalling either directly or indirectly via insulin or leptin resistance.

A major limitation of the current study was the failure to recruit participants who were overtly insulin resistant as it was the intention to describe MCH

activity in the presence of both dysregulated and well-regulated glucose metabolism. Additionally, some problems were experienced obtaining four consecutive blood samples from subjects within a relatively short time period and this resulted in missing data which has reduced the power of the study. However since the missing data was missing completely at random (MCAR) the decision was taken to use the available data in the analyses rather than impute missing values. As little is known about the variance in circulating MCH concentrations it was thought that unnecessary bias and imprecision may have been introduced. In terms of methodology, the study would have been improved by the use of indwelling catheters to obtain blood samples, this would have enabled more frequent sampling and improved data collection. This option however was not available.

7. To investigate changes in concentrations of circulating MCH during the menstrual cycle and their associations with circulating progesterone, leptin and metabolic rate

7.1 Introduction

Evidence resulting from a number of recent studies is supportive of an emergent role for central MCH in the modulation of reproductive function. Based largely on interventional studies with rodents and *in vitro* work, several of those studies propose a role for MCH in the hypothalamic regulation of LH release (Tsukamara *et al.,* 2000; Chiocchio *et al.,* 2001; Gallardo *et al.,* 2004; Murray *et al.,* 2006). One recent study suggests that the MCH system is active at the level of GnRH; MCH has been shown to have a direct inhibitory effect on kisspeptin signalling which is essential for normal reproductive function, notably mediation of the preovulatory LH surge (Wu *et al.,* 2009). There is also neuroanatomical evidence supporting a mediatory role for centrally expressed MCH in reproductive activity (Williamson-Hughes *et al.,* 2005, Ward *et al.,* 2009). However a potential role for circulating MCH in the reproductive axis has not been investigated in any other species. Therefore in the first instance it was deemed important to determine if there were any cyclical changes in circulating MCH concentrations throughout the menstrual cycle in humans. In females intra-individual variations in energy expenditure during the menstrual cycle have been aligned to hormonal changes (Solomon *et al.,* 1982; Howe *et al.,* 1993). Whether or not phasic changes in consummatory behaviours contribute to altered energy expenditure has not been established (Webb, 1986; Pelkman *et al.,* 2001). Results of rodent studies suggest that hypothalamic MCH has the capacity to impact strongly on both energy expenditure (Segal-Lieberman *et al.,* 2006; Jeon *et al.,* 2006) and ingestive behaviours (Qu *et al.,* 1996; Ludwig *et al.,* 2001; Marsh *et al.,* 2002), though the activities of circulating MCH in this context are not known, therefore associations between resting metabolic rate, energy intake and circulating MCH concentrations during different phases of the menstrual cycle were examined. Since leptin is generally recognised as a key factor in both normal reproductive physiology and energy balance, circulating leptin

concentrations were also examined in relation to circulating MCH concentrations in an effort to determine if there was a quantifiably measurable relationship between these two circulating factors during the menstrual cycle. Therefore the aims of this study were:

- To track circulating MCH concentrations throughout the menstrual cycle in normally cycling women.
- To describe associations between circulating MCH concentrations, resting metabolic rate, energy intake and body composition at different stages of the menstrual cycle.
- To determine associations between circulating MCH, progesterone and leptin concentrations throughout the menstrual cycle.

7.2 Subjects and experimental protocol

7.2.1 Participants

Thirty one female subjects were recruited as Section 3.2.2. Inclusion criteria were that they should be pre-menopausal and having a regular menstrual cycle. Lactating females, recently lactating (within the last 12 months) and pregnant women were excluded. Subjects who were using oral contraceptives were allowed to participate and their data used as controls. Those on chronic medication were not routinely excluded though anyone taking superovulatory medication or medication known to affect metabolic rate were not allowed to participate. Data collected by another researcher at the University of Westminster has been included in the current analyses. The researcher was Faridah Shahab (BSc (Hons) 2008/09).

7.2.2 Pre-test

Subjects attended an initial laboratory visit during which they were verbally familiarised with all experimental procedures. Since subjects were expected to attend the laboratory on several occasions it was felt that that this

induction would limit drop-out rate. During the initial visit subjects were supplied with and instructed how to use a digital clinical thermometer (RJ95D, Maplin, UK). Prior to testing all subjects maintained a daily record of their waking oral temperature for 1 – 2 full menstrual cycles in order to establish when ovulation usually occurred within their monthly cycle. This temperature record was also kept during the month they attended the laboratory for testing. Subjects received a detailed Subject Information Sheet (Appendix 8) and completed an Informed Consent Form and pre-test Medical Screening Questionnaire as Sections (Appendices 9 & 4).

7.2.3. Experimental protocol

Subjects attended the laboratory four times over the course of one complete menstrual cycle. The visits were timed so that subjects attended twice during the follicular phase (early and late) and twice during the luteal phase (early and late). See Figure 7.1 for schematic representation of experimental protocol. Each visit subjects attended after an overnight fast. On the first visit anthropometric measurements were taken as described in Section 3.2.3. On all visits RMR and total body fat and lean mass were measured using the procedures described in Sections 3.2.4 and 3.2.5. A fasting venous blood sample was obtained on all visits which was collected, treated and the plasma samples stored as described in Section 3.2.6. Subsequently plasma samples were assayed for MCH, leptin and progesterone by methods described in Section 3.2.7. Subjects completed two 3-day Food Diaries (2 consecutive weekdays and 1 weekend day (Appendix 5), one during the late follicular phase and one during the late luteal phases of their monthly cycle. The Food Diaries were anaylsed using software described in Section 5.2.2.

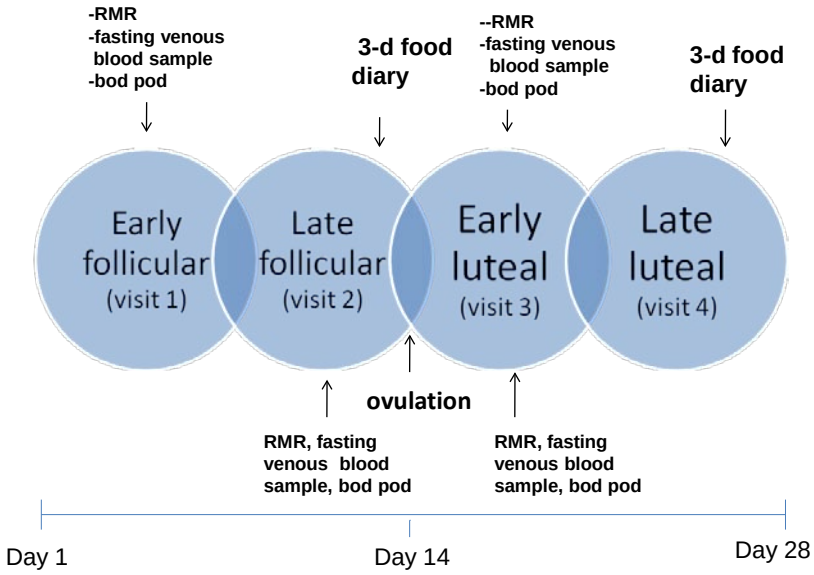

Figure 7.1: Schematic representation of the experimental protocol, in this example the length of the cycle is 28 days.

7.3 Statistical Analyses

Normality of each data set was assessed by Shapiro Wilks W test and by examination of Skewness and Kurtosis values. Log-transformed data were used where necessary. Homogeneity of variance was determined by Levene's Test for Equality of Variances. Differences between circulating hormone concentrations in the follicular and luteal phases of the monthly cycle were assessed by paired samples t-tests. Differences in circulating hormone concentrations between those subjects who had an ovulatory cycle or an anovulatory cycle were determined by independent samples t-tests and the non-parametric Mann-Whitney U test. Comparisons between circulating hormone concentrations during the early follicular, late follicular, early luteal and late luteal phases of the menstrual cycle were determined by repeated measures design ANOVA. Post-hoc tests were performed using Bonferroni adjustment for multiple comparisons. One-way between-groups ANOVAs

were conducted to determine if there was an effect of BMI and body fat % on plasma hormone concentrations. Post-hoc tests were performed using Tukey adjustment for multiple comparisons. The effect of size (group) was calculated using partial eta squared or eta squared. The guidelines for interpretation were those proposed by Cohen (1988). The strength of relationships between variables was determined by Pearson product-moment correlation coefficient. Partial correlation was used to control for compounding variables. Unless otherwise stated, values are presented as mean ± SD or mean ± SEM. Data were analysed using the Statistical Package for the Social Sciences (SPSS version 16.0 for Windows; Chicago, IL, US). Statistical significance was set at $p<0.05$.

7.4 Results

7.4.1 Subject characteristics

Of the 31 subjects data from 2 were excluded from the analyses since it was not possible to obtain blood samples from them. Another subject was excluded since her plasma MCH concentrations were greater than two standard deviations from the mean and two subjects were excluded due to irregularities in their menstrual cycles. Of the remaining 26 subjects 20 were classified as ovulators (ov) and 6 as non-ovulators (non-ov) based on whether their awakening body temperature changed mid-cycle and their plasma progesterone concentrations. Two of the non-ovulators were contraceptive pill users. Even though an anovulatory cycle will not have a true luteal phase (that is, a corpus luteum will not be formed and therefore there will be no luteal phase increase in circulating progesterone concentrations), for the purposes of these analyses plasma hormone concentrations measured during the last 16 days of the cycle have been designated as luteal phase for the non-ov group.

Demographic and anthropometric characteristics of the study participants are presented in Table 7.1. There were no differences in age, height, weight, BMI, percent fat mass and percent lean mass between the ovulatory and non-ovulatory women ($p>0.05$).

Table 7.1 Demographic and anthropometric characteristics of study participants

	Mean ± SD			Range		
	All (n=26)	**Ov** (n=20)	**Non-ov** (n=6)	**All** (n=26)	**Ov** (n=20)	**Non-ov** (n=6)
Age (yrs)	37.1±6.0	37.3±5.2	36.4±8.9	26.8-49.0	30.7-49.0	26.8-47.8
Height (m)	1.66±0.1	1.66±0.1	1.66±0.1	1.57-1.8	1.57-1.8	1.63-1.7
Weight (kg)	67.7±15.1	70.5±8.5	68.6±6.3	50.2-118.9	50.2-118.9	56.9-82.4
Fat mass (%)	29.9±8.0	29.4±8.5	31.4±6.3	16.3-49.2	16.3-49.2	23.2-38.4
Lean mass (%)	70.0±8.0	70.5±8.5	68.5±6.3	50.7-83.6	50.7-83.6	61.6-76.7
BMI (kg/m^2)	24.6±5.3	24.8±5.8	25.0±2.9	18.3-40.6	18.3-40.6	21.1-28.5

7.4.2 Plasma hormone concentrations

7.4.2.1 Plasma MCH concentrations during the menstrual cycle

Fasting plasma MCH concentrations measured during the early follicular, late follicular, early luteal and late luteal phases of the menstrual cycle were not significantly different at any of the four sampling time-points when data from the whole group were analysed (p=0.808); nor when the group was split between ovulatory (p=0.672) and non-ovulatory women (p=0.709). Mean plasma MCH concentrations in the follicular phase of the cycle were not significantly different to those in the luteal phase either in the group as a whole (p=0.130) or within women who did ovulate and those who did not (Table 7.2: ov: p=0.213; non-ov: p=0.459). There were no significant differences in plasma MCH concentrations between the ovulatory and non-ovulatory women in either phase of the menstrual cycle (Table 7.2: follicular: p=0.125; luteal: p=0.185). However there was a non-significant trend for ovulatory women to have higher plasma MCH concentrations in the follicular

and luteal phases than non-ovulatory women (Table 7.2). Both groups also appear to have higher MCH concentrations in the luteal phase than in the follicular phase though again this difference was not significant. Plasma MCH concentrations ranged from 29.2 - 61.8 pg/ml.

Table 7.2 Plasma hormone concentrations during the follicular and luteal phases of the menstrual cycle in ovulatory and anovulatory women

	Mean ± SD						**Range over the entire cycle**	
	All (n=26)		**Ov** (n=20)		**Non-ov** (n=6)		**Ov** (n=26)	**Non-ov** (n=6)
	F	L	F	L	F	L		
MCH (pg/ml)	41.7 ±6.9	43.2 ±7.3	42.7 ±6.8	43.9 ±7.9	37.8 ±5.9	39.4 ±6.6	32.6–61.8	29.2-48.5
Leptin (ng/ml)	11.4 ±10.9[a]	12.5 ±9.6[b]	11.5 ±11.8	12.6 ±10.2	11.3 ±8.6	12.3 ±8.4	1.8-50.3	3.2-27.9
Progesterone (ng/ml)	0.5 ±0.4[a]	8.1 ±5.2[b]	0.5 ±0.4[a]	10.0 ±3.8[b]	0.3 ±0.1[a]	0.5 ±0.3[a]	0.3-16.1	0.3-1.03

F: follicular; L: luteal; within a hormone values with different superscripts are significantly different from each other (paired samples t-test; $p<0.05$)

7.4.2.2 Plasma leptin concentrations during the menstrual cycle

Mean plasma leptin concentrations in the follicular phase of the cycle were significantly lower than those in the luteal phase in all individuals (11.4 ± 10.9 ng/ml *versus* 12.5 ± 9.6 ng/ml; paired samples t-test; p=0.044). There were no differences in plasma leptin concentrations between the follicular and luteal phases either within ovulatory and non-ovulatory women (ov: p=0.100; non-ov: p=0.226) or between ovulatory and non-ovulatory women (Table 7.2: independent samples t-test; follicular; p=0.978; luteal: p=0.914). The ovulatory womens' leptin concentrations were significantly higher in the early and late luteal phases of the cycle compared to the early follicular phase (Table 7.3: one-way repeated measures ANOVA; p=0.03; with a large

effect of size, multivariate partial eta squared =0.502). Only women from whom blood samples were collected at each of the four sampling time-points were included in this analysis hence the change in subject numbers per group.

Table 7.3 Plasma hormone concentrations during the menstrual cycle in ovulatory and anovulatory women

	Mean ± SD							
	Ov (n = 15)				**Non-ov (n = 3)**			
	EF	LF	EL	LL	EF	LF	EL	LL
MCH (pg/ml)	44.71 ±8.3	43.01 ±8.3	44.53 ±6.8	45.02 ±7.5	41.11 ±6.4	42.09 ±6.4	42.7 ±7.3	38.8 ±3.6
Leptin (ng/ml)	8.73 ±6.4[a]	9.84 ±7.2 [a,b]	10.62 ±7.0[b]	11.29 ±8.0[b]	14.19 ±9.3	18.10 ±10.6	17.07 ±10.9	15.4 ±8.4
Prog (ng/ml)	0.45 ±0.3[a]	0.83 ±1.6[a,b]	11.17 ±5.7[c]	8.63 ±5.3[c]	0.30 ±0	0.30 ±0	0.56 ±0.5	0.30 ±0.5

EF: early follicular, LF: late follicular, EL: early luteal, LL: late luteal, within a hormone values with different superscripts are significantly different from each other (one-way repeated measures ANOVA; p<0.05). Reduced n values are due to missing plasma samples.

7.4.2.3 Plasma progesterone concentrations during the menstrual cycle

Within ovulatory women plasma progesterone concentrations were significantly higher in the early and late luteal phases than in the early and late follicular phases of the cycle (p<0.005). Luteal phase plasma progesterone concentrations in ovulatory women were also significantly higher than follicular and luteal phase progesterone concentrations in non-ovulatory women (p<0.005). There were no significant changes in plasma progesterone concentrations in non-ovulatory women across the cycle (Table 7.3: p>0.005).

7.4.3 Associations between circulating MCH and leptin concentrations and BMI

Subjects were divided into 3 groups according to BMI to determine whether there was a difference in plasma MCH or plasma leptin concentrations between women who were in the normal, overweight or obese BMI categories. The groups were: B1= ≤ 24.9; B2= 25 – 29.9; B3= ≥ 30 kg/m^2. **MCH:** There were no significant differences in mean follicular or mean luteal MCH concentrations between the three categories of BMI for either ovulatory or non-ovulatory women (data not shown). **Leptin:** For both ovulatory and non-ovulatory women, as BMI increased circulating leptin concentrations increased. In ovulatory women B3 circulating leptin concentrations were significantly greater than B1 and B2 (Table 7.4: $p<0.005$). In non-ovulatory women circulating leptin concentrations were significantly greater in B2 than B1 (Table 7.4: $p<0.005$). Within the BMI categories there was a significant difference in the concentration of leptin between the follicular and luteal phase in ovulatory women in the B1 group only *(*Table 7.4: paired samples t-test; $p=0.002$)

7.4.4 Associations between circulating MCH and leptin concentrations and body fat and lean mass

Subjects were sub-divided into 3 groups according to body composition. The groups were: Lean (L)= 19 – 22% fat mass; Moderately Lean (ML)= 23 – 30% fat mass; Excess Fat (EF) ≥ 31% fat mass, (ACSM, 1996). **MCH:** There were no significant differences in the concentrations of MCH between the follicular and luteal phases both within and between a category in either ovulatory or non-ovulatory women (results not reported). **Leptin:** Ovulatory women in the EF group had significantly greater leptin concentrations than in ML and L groups (Table 7.4: $p<0.005$) There was a trend for greater leptin concentrations in EF than ML in non-ov women (Table 7.4: $p=0.643$). Within the per cent fat mass categories there was a significant difference in the concentrations of leptin between the follicular and luteal phases in the ML category in ovulatory women only (Table 7.4: $p=0.035$).

Table 7.4: Associations between circulating leptin concentrations in the follicular and luteal phases of the menstrual cycle with BMI and fat mass

Ov						Non-ov					
BMI											
Follicular			**Luteal**			**Follicular**			**Luteal**		
B1 (n=11)	B2 (n=5)	B3 (n=3)	B1 (n=11)	B2 (n=5)	B3 (n=3)	B1 (n=3)	B2 (n=3)	B3 (n=0)	B1 (n=3)	B2 (n=3)	B3 (n=0)
5.2± 3.3^{a*}	12.9± 6.7 ^{a,b}	32.0± 15.8^{c}	7.8± 4.9^{a*}	15.1± 5.5^{a,b}	28.8± 14.2^{c}	6.5± 3.0^{a}	18.1± 8.2^{b}	n/a	5.7± 3.1^{a}	16.9± 9.1^{b}	n/a
Fat Mass											
L (n=4)	ML (n=7)	EF (n=8)	L (n=4)	ML (n=8)	EF (n=8)	L (n=0)	ML (n=2)	EF (n=4)	L (n=0)	ML (n=2)	EF (n=4)
3.5± 1.2^{a}	4.9± 2.3^{a,b*}	21.2± 12.8^{c}	5.9± 3.0^{a}	8.0± 4.7^{a,b*}	21.1± 10.5^{c}	n/a	3.9± 1.06	15.0± 8.4	n/a	4.9± 1.5	16.0± 7.9

B1 : ≤ 24.9; B2: 25 – 29.9; B3: ≥ 30 kg/m$^{2;}$ L: lean, ML: moderately lean, EF: excess fat. Leptin concentrations are expressed as mean ± SD (ng/ml); within ov or non-ov groups values with different superscripts are significantly different from each other (one-way between-groups ANOVA; $p<0.05$); within ov or non-ov groups between follicular and luteal phases values with * are significantly different from each other (paired samples t-test; $p<0.05$). Reduced n values are due to missing plasma samples.

7.4.5 Associations between circulating MCH, leptin and progesterone concentrations during the menstrual cycle

There was a significant negative association between early luteal MCH concentrations and early luteal progesterone concentrations in ovulating women (Table 7.5: r =-0.533, p=0.023). There were no other significant associations between concentrations of any of the hormones examined at any of the sampling time-points in either the ov or the non-ov group. However there was a trend for an inverse relationship between MCH and

leptin, higher concentrations of MCH were associated with lower concentrations of leptin. There were no significant associations between plasma leptin and plasma progesterone at any of the sampling timepoints (data not shown).

Table 7.5: Bivariate analyses: Correlations between plasma MCH, leptin and progesterone during the menstrual cycle (ov women)

	MCH (EF)	MCH (LF)	MCH (EL)	MCH (LL)
LEP (EF) (n=19)	-0.103 (p=0.685)	-	-	-
LEP (LF) (n=17)	-	-0.175 (p=0.502)	-	-
LEP (EL) (n=18)	-	-	-0.155 (p=0.539)	-
LEP (LL) (n=19)	-	-	-	0.014 (p=0.955)
PROG (EF) (n=19)	0.030 (p=0.902)	-	-	-
PROG (LF) (n=18)	-	-0.334 (p=0.176)	-	-
PROG (EL) (n=18)	-	-	-0.533 (p=0.023)*	-
PROG (LL) (n=19)	-	-	-	0.057 (p=0.818)

Values represent Pearson product-moment correlations, *significant at 0.05 level, LEP: leptin, PROG: progesterone, EF: early follicular, LF: late follicular, EL: early luteal, LL: late luteal. Reduced n value is due to missing plasma samples.

7.4.6 Associations between circulating MCH and leptin concentrations and body composition parameters

For each woman the mean MCH and leptin concentrations over her menstrual cycle were calculated and used for the following analyses (Table 7.6: Figs 7.1(a) & 7.1(b). Whilst there were no significant associations between mean circulating MCH and body composition parameters there were trends for inverse relationships between % fat mass, BMI and body weight with circulating plasma MCH concentrations. By comparison there were strong significant positive relationships between circulating leptin concentrations and the same body composition parameters. However when associations between the body composition parameters and plasma MCH

concentrations measured at each of the four sampling time-points were examined there were significant correlations between % body fat mass, % body lean mass and late follicular MCH in women with excess fat only (r=-0.733, p=0.038; r=0.733, p=0.038 respectively; n=8).

Table 7.6 Associations between mean circulating MCH and leptin concentrations and body composition parameters

	MCH (n=26)	**Leptin (n=26)**
Fat mass (%)	-0.207 (p=0.310)	0.554 (p=0.003)**
Lean mass (%)	0.207 (p=0.310)	0-.554 (p=0.003)**
Fat mass (kg)	-0.224 (p=0.271)	0.530 (p=0.005)**
Lean mass (kg)	-0.114 (p=0.580)	0.145 (p=0.480)
BMI (kg/m^2)	-0.234 (p=0.250)	0.519 (p=0.007)**
Body weight (kg)	-0.201(p=0.325)	0.431 (p=0.028)*

Values represent Pearson product-moment correlations, * significant at 0.05 level (2 tailed)

** significant at 0.01 level (2 tailed)

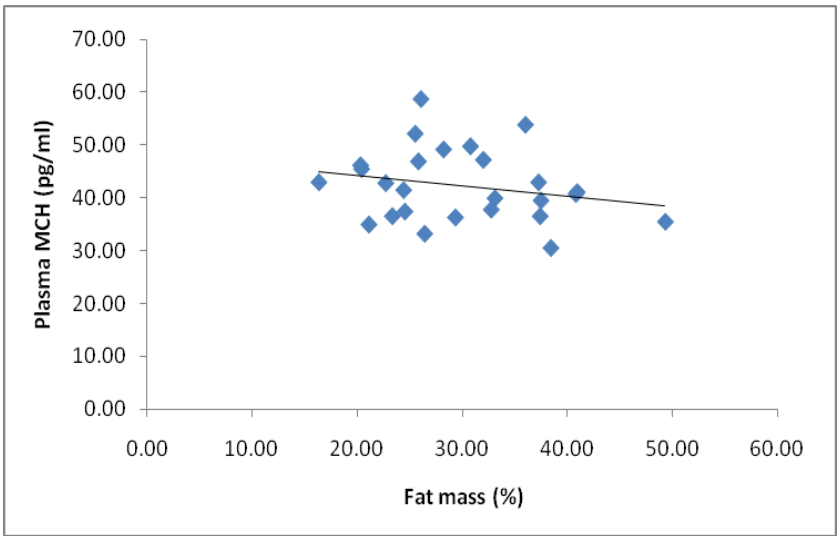

Figure 7.1(a): Correlation between menstrual cycle mean circulating MCH concentrations and body fat mass % (Pearson product-moment correlation, r=-0.270, p=0.310, n=26)

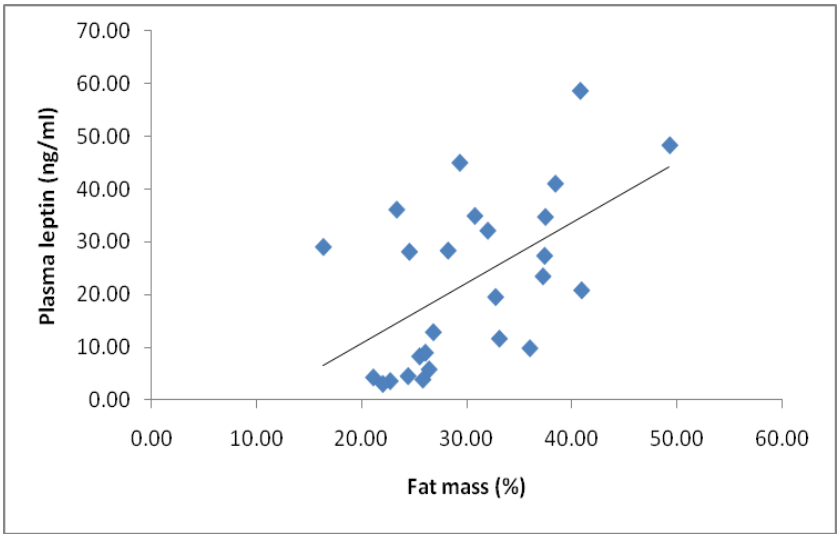

Figure 7.1(b): Correlation between menstrual cycle mean circulating leptin concentrations and body fat mass % (Pearson product-moment correlation, r=0.556, p=0.003, n=26)

7.4.7 Circulating MCH concentrations and resting metabolic rate

Resting metabolic rates (RMR) were compared during the early follicular, late follicular, early luteal and late luteal phases of the menstrual cycle. When the whole group was analysed RMR measured in the late follicular phase (absolute values kcal/day) was significantly lower (1274.9 kcal/day) than RMR measured in the late luteal phase (1328.1 kcal/day) (one-way repeated

measures ANOVA: p=0.001; with a large effect of size, multivariate partial eta squared=0.613). When ovulatory and non-ovulatory women were grouped separately there were no significant differences in RMR at any sampling time-point in non-ovulatory women however there were significant differences in RMR in the ovulatory women. Late follicular RMR was significantly lower than early though not late luteal RMR (Table 7.7: one-way repeated measures ANOVA; p=0.037; with a large effect of size, multivariate partial eta squared=0.603). There were no significant differences between ov RMR and non-ov RMR in any phase of the menstrual cycle.

Table 7.7: RMR (kcal/day) in ovulatory and anovulatory women during the menstrual cycle

EF		LF		EL		LL	
Ov (n=19)	Non-ov (n=5)	Ov (n=19)	Non-ov (n=5)	Ov (n=19)	Non-ov (n=5)	Ov (n=19)	Non-ov (n=5)
1283 ±143[a,b]	1348 ±214	1287 ±139[a]	1406 ±176	1320 ±141[b]	1382 ±185	1326 ±150[a,b]	1441 ±173

EF: early follicular, LF: late follicular, EL: early luteal, LL: late luteal. Within the ovulatory group values with different superscripts are significantly different from each other (one-way repeated measures ANOVA, p<0.05). Reduced n values are due to missing plasma samples.

There were no associations between plasma MCH concentrations and RMR when analyses were performed for the four sampling time-points during the menstrual cycle, neither in the group as a whole nor within ovulatory or non-ovulatory women.

7.4.7.1 Associations between circulating MCH, leptin, progesterone and RMR during the menstrual cycle in ovulatory women (n = 18)

Ovulatory women were divided into 2 groups based on their % predicted RMR. Predicted RMR (kcal/day) was calculated as the mean of the Harris-Benedict and Fleisch equations as Section 5.4.4. A RMR of ± 10% predicted is considered normal (McKardle *et al.,* 2001). Therefore the groups were N (normal)= ≥ 90% predicted; L (low)= ≤ 89.9% predicted. **MCH:** There were no strong associations between plasma MCH concentrations and RMR in either

group at any of the four sampling time-points in the menstrual cycle **Leptin:** There were no significant correlations between plasma leptin concentrations and RMR after controlling for fat mass. **Progesterone:** There were no significant correlations between RMR and plasma progesterone concentrations in either group in any phase of the menstrual cycle

7.4.8 Associations between plasma MCH concentrations, energy intake and RMR

Twenty-four 3-day food diaries kept by 12 ovulatory women were analysed. Each woman kept one diary in the follicular phase and one in the luteal phase of her cycle. There were no differences in caloric (kcal/day or kcal per kg/bodyweight/day) or percent macronutrient intake between the follicular and luteal phases of the cycle ($p>0.05$). Neither were there significant correlations between fasting plasma MCH concentrations and caloric or percent macronutrient intake in either phase of the cycle (Pearson product-moment correlation; $p>0.05$). Energy intake was not associated with RMR (absolute values) in either phase of the cycle.

7.5 Discussion

7.5.1 Circulating MCH concentrations during the menstrual cycle

Although all subjects had regular menstrual cycles both ovulatory and anovulatory females were included in the study in order to compare the MCH response in the presence/absence of progesterone. This was deemed an important distinction since if MCH plays a role in normal reproductive function it would be expected that circulating concentrations of the hormone would differ between ovulatory and anovulatory cycles. If fluctuations of peripheral MCH conform to those of central MCH in the rodent (Gallardo *et al.,* 2004), then it would be expected that there would be a difference in circulating MCH concentrations between the follicular and luteal phases of the menstrual cycle.

Plasma MCH concentrations at four phases of the menstrual cycle, namely: early follicular, late follicular, early luteal and late luteal were measured in both ovulatory and anovulatory women and there appears to be no significant changes in circulating MCH which may be demarcated by phases of the menstrual cycle. There was however a non-significant trend for plasma MCH concentrations to be higher in ovulatory women compared to anovulatory women and for luteal concentrations to be higher than follicular concentrations in both. This is in contrast to the behaviour of other metabolic markers of energy homeostasis such as leptin. Considerable disparity in circulating leptin concentrations in different phases of the menstrual cycle have been reported; commonly luteal phase concentrations being higher than follicular phase concentrations (Hardie *et al.,* 1997; Paolisso *et al.,* 1999; Ludwig *et al.,* 2000; Geisthovel *et al.,* 2004). The findings reported herein are in broad agreement with these results. Although plasma leptin concentrations were not significantly different in any phase of the menstrual cycle between ovulatory and anovulatory women, plasma leptin concentrations were significantly lower in the early follicular phase compared to the early and late luteal phases in ovulatory women only. This discrepancy between ovulatory and anovulatory women should be treated with caution since a full data set for only 3 subjects in the anovulatory group was available for analysis. In energy homeostasis central leptin antagonises the actions of MCH via the anabolic NPY/AgRP metabolic pathway (for review see Schwartz *et al.,* 2000). In the reproductive axis there is some evidence that MCH may act collaboratively with leptin in mediating the LH surge (Murray *et al.,* 2000c). However few studies have elucidated the nature of the relationship between leptin and MCH in the periphery. The only other study to date to measure circulating MCH concentrations in humans found no significant associations between fasting plasma leptin and MCH concentrations (Gavrila *et al.,* 2005). In the current study there were no significant correlations between fasting plasma MCH concentrations and plasma leptin concentrations in any phase of the menstrual cycle. However there was a trend for inverse correlations between circulating MCH and leptin which is in line with the reported suppression of hypothalamic MCH activity by leptin.

7.5.2 MCH and RMR

As discussed in Chapter 5 central MCH appears have a profound influence on energy expenditure at least in rodents. Variations in energy expenditure have been observed during the human menstrual cycle (Solomon *et al.,* 1982; Webb, 1986; Bisdee *et al.,* 1989; Meijer *et al.,* 1992; Henry *et al.,* 2003). Elevated RMR in the luteal phase has been attributed to the thermogenic effect of progesterone. Therefore a key objective of the current enquiry was to establish whether or not there were significant associations between cyclic RMR and circulating concentrations of MCH in healthy women. In agreement with previous work there were significantly higher RMR values in the luteal phase of the cycle compared to the follicular phase (kcal/day). When ovulatory and anovulatory women were grouped together late follicular RMR was significantly lower than late luteal though not early luteal RMR. When analysed separately anovulatory women did not display significant fluctuations in RMR over the cycle but in ovulatory women RMR was significantly higher in the early luteal phase than in the late follicular phase. The apparent delayed rise in luteal RMR in the whole group compared to the earlier elevated luteal RMR in the ovulatory women was likely due to the masking effects of decreased early luteal RMR in non-ovulatory women.

Circulating MCH concentrations do not appear to be aligned to RMR since there were no significant associations between RMR and plasma MCH concentrations when analyses were performed at the four sampling time-points in the whole group or within ovulatory and anovulatory women. Nor were there significant associations between RMR and plasma MCH concentrations at any sampling time-point in those with either a normal or low metabolic rate. However whilst there was no direct association between RMR and plasma MCH concentrations in the luteal phase, there was a significant inverse correlation ($r=-0.533$, $p=0.023$) between plasma MCH and plasma progesterone concentrations in the early luteal phase of the cycle in ovulatory women only. Given the acknowledged thermoregulatory actions of both MCH and progesterone, which are in opposing directions this finding is

unsurprising, though it is suggestive of a signalling pathway between MCH and gonadal steroids.

7.5.3 MCH, body composition and BMI

There is a wealth of evidence documenting the contribution of MCH to the obese phenotype and conversely the phenomena of the lean phenotype characteristic of MCH or MCHR-1 k/o rodents. Reduced adiposity, increased lean mass and reduced body weight accompanied by either hyper- or hypophagia have been reported in genetically modified animals or those to whom an MCHR-1 antagonist has been administered (Chen *et al.,* 2002; Marsh *et al.,* 2002; Mashiko *et al.,* 2005; Bjursell *et al.,* 2006; Kowalski *et al.,* 2006). Increased body mass and adiposity have been observed following MCH infusion or up-regulation of MCH expression (Ludwig *et al.,* 2001; Gomori *et al,.* 2002). Interestingly MCH induced food consumption appears to be related to the hedonic aspects of feeding which was discussed in Chapter 5. In humans a phasic change in food intake has been observed, specifically a reduction in food intake in the ovulatory period followed by a rise in the luteal phase (Lyons *et al.,* 1989; Paolisso *et al.,* 1999). Therefore since MCH plays a key role in systemic energy balance it would seem logical to investigate whether or not circulating MCH concentrations were associated with anthropomorphic dimensions or caloric intake.

There were no significant correlations between percent body fat mass, percent body lean mass, fat mass weight (kg), lean mass weight (kg) BMI or body weight (kg) and mean plasma MCH over one entire cycle. This was in contrast to plasma leptin which was strongly associated with all of the above parameters with the exception of lean body mass weight (Table 7.6). There was however an inverse association between percent fat mass and circulating MCH concentrations measured in the late follicular phase of the cycle in women with excess fat only ($r=-0.733$, $p=0.038$, $n=8$). The association between circulating MCH concentrations and certain body composition parameters in discrete sub-groups of the sample population has been a recurrent phenomenon throughout this series of studies. It seems

that circulating MCH concentrations are altered in the presence of adiposity or leanness which is gender specific in a given population. However by definition the sample sizes of the sub-groups were small therefore inferential caution must be exercised. It is tempting to speculate that there may be a relationship with oestrogenic activities. In rodents endogenous oestradiol appears to suppress MCH activity during the oestrous phase of the reproductive cycle which could be said to correspond to the follicular phase in humans since it is a period of high circulating oestrogen. In the current study there was a trend, though non-significant, for follicular phase circulating MCH concentrations to be lower than those in the luteal phase. Whether or not this was partly due to oestrogenic inhibition is a subject for further investigation. Furthermore although not significant, MCH correlations were in the inverse direction to leptin, that is, correlations between percent fat mass, BMI, body weight and plasma MCH were negative, whilst correlations between percent lean mass and plasma MCH were positive.

The impact of BMI and body composition on circulating MCH concentrations was explored. Subjects were divided into 3 groups based on BMI categories which are widely accepted as representing normal weight, overweight or obese individuals. There were no differences in circulating MCH concentrations between women in any of the categories, in either the follicular or luteal phases, and no differences between ovulatory or anovulatory women. Since BMI does not represent adiposity *per se,* women were also grouped into 3 body composition categories based on the ASCM's classification of a lean, moderately lean and excess fat phenotype. In this paradigm body composition does not appear to impact on circulating MCH concentrations, no differences being found between any category in either phase of the cycle and no differences between ovulatory and anovulatory women. As expected circulating leptin concentrations increased in line with increased adiposity and BMI in both ovulatory and anovulatory women; the increases were not statistically significant in the anovulatory women but the small sample size was likely a factor. Leptin concentrations were also significantly higher in the luteal phase compared to the follicular phase in women in the normal BMI category and in the moderately lean category,

which did not appear to be related to fluctuations in percent fat mass or body weight.

7.5.4 MCH, energy intake and RMR

In rodents hypothalamic MCH has the capacity to impact strongly on feeding behaviours, this subject was also discussed in Chapter 5. Briefly pharmacologically or genetically altered availability of hypothalamic MCH induces significant changes in consummatory behaviour. Generally increased availability of hypothalamic MCH resulted in hyper-phagia (Qu *et al.,* 1996; Ludwig *et al.,* 2001) whilst hyper- or hypophagia may be induced by decreased availability of hypothalamic MCH depending on the rodent model (Chen *et al.,* 2002; Kowalski *et al.,* 2006). In normally cycling pre-menopausal women evidence suggests that energy intake is strongly influenced by ovarian hormones. In an analysis of 37 groups of women, 27 of the groups exhibited increased energy intake in the luteal compared to the follicular phase of the cycle. A non-significant tendency for increased luteal phase energy intake was observed in a further 7 groups (Dye and Blundell, 1997). It has been hypothesised that an interaction between circulating oestrogen and progesterone post-ovulation is responsible for the altered ingestive behaviours (for review see Davidsen *et al.,* 2007). An apparent increased preference for carbohydrates (Bowen *et al.,* 1990; Cross *et al.,* 2001; Martini *et al.,* 2004) or fats (Tarasuk *et al.,* 1991; Johnson *et al.,* 1995) in the luteal phase has been reported in a number of studies. However in the current study although data from ovulatory women exclusively were analysed, no differences were observed either in energy intake or macronutrient composition of the diet between the follicular and luteal phases. Nor were there any associations between energy intake and RMR (absolute values). There are several factors which are thought to contribute to the variation in phasic energy expenditure in women including hormonal (Solomon *et al.,* 1982; Howe *et al.,* 1993) and sympathetic nervous system activity (Day *et al.,* 2005). The contribution of energy intake though a feasible candidate has not been well defined. There appear to be few studies in which energy intake and expenditure have been measured in the same

women (Davidsen *et al.,* 2007). A previous study reported increased luteal phase energy intake and energy expenditure which were not related (Pelkman *et al.,* 2001*).* In the current study energy intake and expenditure were not aligned in either study. Curiously though mean caloric intake (absolute and per kg bodyweight) were almost identical between the 2 phases. This brings into question the validity of self-reported estimated food diaries; difficulties associated with this type of nutritional tool are acknowledged (Kaczowski *et al.,* 2000).

7.5.5 Summary, limitations and future work

The main finding of this study was that fasting plasma MCH concentrations remain relatively stable over the course of a normal menstrual cycle. There was very little difference in circulating MCH concentrations between women who ovulated and those who did not apart from in the early luteal phase of the cycle when plasma MCH concentrations were inversely correlated with plasma progesterone concentrations in ovulatory women. Superficially this finding may be interpreted as a disassociation between MCH and female reproductive function in the periphery. However it must be said that in this study plasma MCH was measured at only 4 time-points over an entire cycle, nothing is known about secretion or clearance rates, or whether MCH is secreted in a pulsatile or continuous manner. Additionally all samples were obtained from fasted subjects between 08.00 – 10.00am. In a separate study in this series plasma MCH concentrations increased post-prandial, therefore it is entirely possible that, since only fasting samples were obtained, temporal fluctuations may have been missed. In rodents MCH expression follows a diurnal pattern, nothing is known in this regard in humans and would be a subject for future work.

With regard to metabolic rate it would appear there are no strong associations between RMR and plasma MCH concentrations in either phase of the cycle either in ovulatory or anovulatory women. Therefore it would

seem that plasma MCH does not strongly reflect energy expenditure in the periphery.

Throughout this study the actions of circulating MCH have been compared to those of circulating leptin, which having a dominant role in both energy homeostasis and reproductive function might be considered a yardstick for MCH activity in the periphery. However in the current study there were no significant associations between plasma MCH and plasma leptin during the menstrual cycle. Fluctuations in circulating MCH were not reflected in fluctuations in circulating leptin although there was a consistent trend for negative MCH correlations to be matched by positive leptin correlations both within the contexts of body composition and RMR. With regard to body composition it seems that circulating MCH does not convey information relating to fuel stores and availability in the same manner that leptin does, though again perhaps the use of fasting plasma samples was in some way restrictive since in a separate study we found significant associations between certain body composition parameters and post-prandial MCH concentrations.

A principal limitation of this study was the imbalance in sample size between ovulatory and anovulatory women. The small sample size of the anovulatory group (n=6) made comparisons and conclusions regarding disparities between the groups difficult. Except in the case of the 2 women who were contraceptive pill users, it was not possible to confirm whether women were having ovulatory or anovulatory cycles until testing was complete. Although the sample size was comparable to and in some cases larger than previous work in this area, it was disappointing to have to exclude 5 subjects (on the grounds of various anomalies) from what was initially an adequate though relatively modest sample size. Further work with larger sample sizes is required. Additionally as noted above, the use of fasting plasma samples only may have limited the scope of the study.

Clearly the capacity for further work with circulating MCH is enormous, this initial characterisation of MCH activity in the menstrual cycle instigates many

questions. Based on the results of this study it would seem that a potential relationship between circulating MCH and oestrogen in human females would be worthy of investigation. The current results indicate that one cannot make assumptions regarding the activities of circulating MCH based on knowledge of hypothalamic MCH activity. Whilst circulating MCH does not appear to be overtly reactive in the menstrual cycle, it could be that circulating MCH behaves in a manner more subtle than this initial investigation has been able to detect.

8. Synthesis of Findings

This series of studies was designed to describe normal patterns and fluctuations of circulating MCH as a prelude towards exploring the physiological relevance of circulating MCH in humans. To this end it was necessary to develop and validate a specific, reliable and sensitive means of measuring circulating MCH concentrations since previous work by others has failed to realise this objective. The enhanced sensitivity of the RIA permits measurement of minute quantities of product in blood and is commonly used for the quantification of hormone and non-hormone proteins, drugs, metabolites, viral agents and other substances found in very low concentrations. Before exploratory work with human subjects could commence the assay had to be satisfactorily validated. As described in Chapter 4 the validation procedure was undertaken to identify optimal conditions for the operation of the RIA and potential sources of variation in the results. Once the validation procedure was satisfactorily completed sample populations were recruited.

Chapter 5 describes the first study with human subjects although concomitantly data was being collected which is presented in Chapter 4. During data collection for Chapter 5 all procedures which were subsequently used in Chapters 6 and 7 and concomitantly in Chapter 4 were refined and standardised. There were common parameters which were examined in Chapters 5, 6 and 7; specifically 1) the association between circulating MCH concentrations and body composition parameters; 2) the association between circulating MCH and leptin concentrations 3) the association between circulating MCH concentrations and RMR. Although subjects were drawn from 3 entirely different populations these factors were relevant to the aims of each study. Additionally in Chapters 5 and 6 the MCH post-prandial response to feeding was explored therefore associations between circulating MCH, glucose and insulin were examined. Energy intake in association with MCH was examined in Chapters 5 & 7. This approach was helpful in detecting emergent trends or patterns.

Once all the data had been reconciled there were both consistencies and inconsistences throughout this series of studies.

8.1 Consistent trends:

8.1 1 Circulating MCH

Although there were slight differences in the calculated reference ranges of circulating MCH (± 2 SD from mean) between the four groups examined the lower and upper limits were 19.4 – 62.38 pg/ml, therefore 95% of all subjects tested would be expected to have had circulating MCH concentrations within this range although there were 2 subjects with excessively high values. There were no differences between male and female circulating MCH concentrations in any sample population. This was in broad agreement with the results of the only other study to measure circulating MCH in humans who reported a gender difference which lost significance after adjusting for certain variables (Gavrila *et al.,* 2005). However although there were no gender differences in absolute MCH concentrations there were sexually dimorphic patterns of association throughout the studies.

8.1.2 Post-prandial MCH response in association with circulating glucose and insulin concentrations

The results of Chapters 5 and 6 are supportive of a post-prandial relationship between circulating MCH, glucose and insulin. In the older cohort there was a negative relationship between the incremental area under the curve (AUC) for insulin and mean circulating MCH concentrations (all individuals). In regression analysis mean circulating MCH concentrations made a significant unique contribution to the variance in the insulin AUC. In the younger cohort there was a positive relationship between the respective AUC's for insulin and MCH as well as with the insulin AUC and mean circulating MCH concentrations but only in individuals with excess fat. There was also a negative association between the respective AUC's for glucose and MCH but in males only. As discussed in Chapter 6 there is evidence to suggest that

MCH may be active at the level of the endocrine pancreas and may be involved in insulin production (Pissios *et al.,* 2007). The results presented here are the first to suggest that circulating MCH may have a role in this paradigm in humans. The differential direction of the relationships between circulating MCH and insulin between the different cohorts could be the result of well regulated or dysregulated glucose metabolism in younger/older leaner/fatter individuals.

8.1.3 MCH and RMR

The results of the RMR analyses are in broad agreement across Chapters 5, 6 and 7 in that there were no robust relationships between circulating MCH concentrations and RMR in any of the sample populations. In rodents hypothalamic MCH has the capacity to strongly impact on energy expenditure and there are several proposed mechanisms through which it may act including modulation of autonomic nervous system activity (Astrand *et al.,* 2004; Messina and Overton, 2007) and uncoupling protein expression (Ito *et al.,* 2003; Segal-Lieberman *et al.,* 2003). Hence it was hypothesised that this effect would be reflected in the periphery, however no such associations were observed except in 2 small cohorts. In Chapter 5 circulating MCH concentrations were negatively associated with RMR in females with normal but not ≥ 100% predicted RMR and in Chapter 6 the same parameters were positively associated in lean males. However the sample sizes were small (n = 8 & 7 respectively) and larger sample sizes are required in order to verify or deny whether circulating MCH concentrations are aligned to energy expenditure within a specific range of RMR. In Chapter 7 there were no associations between RMR and circulating MCH at any of the sampling time-points during the menstrual cycle. Although RMR was significantly higher in the luteal phase than in the follicular phase circulating MCH did not change significantly over the cycle. So it would appear that in humans any association between energy expenditure and MCH is not readily detectable by plasma content.

8.1.4 MCH, energy intake and RMR

In the studies reported in Chapters 5 and 7 subjects kept food diaries immediately prior to testing (Chapter 5) and during the test period (Chapter 7). Intervention studies with rodents clearly demonstrate that increased or decreased availability of hypothalamic MCH can alter consummatory behaviours. Since to date the best substantiated role for MCH is in energy homeostasis it was hypothesised that differences in energy or specific macronutrient intake may be reflected in circulating MCH concentrations in humans. However there were no associations between circulating MCH concentrations and energy or specific macronutrient intake in either study. Nor was there a phasic change in energy intake in the study reported in Chapter 7; this is contrary to the results of a sizeable proportion of earlier work where a luteal phase increase in energy intake has been reported. In the current study the nutritional tool was the estimated self-reported food diary which although has inherent limitations was deemed to be sensitive enough to detect trends or patterns. However in this instance there were none. In free-living humans, consummatory behaviours are far more complex than in rodents and the results herein demonstrate that more sophisticated techniques would be required in order to pursue this line of enquiry. Energy intake was not aligned to RMR in either study.

8.2 Inconsistencies between studies:

8.2.1 Circulating MCH and leptin concentrations

Throughout this series of studies circulating MCH and leptin concentrations have been compared. In the widely subscribed to lipostatic model of energy homeostasis (for review see Woods, 2005), leptin inhibits the anabolic pathway through which MCH operates. There is also evidence that leptin and MCH may interact in the periphery; for example the MCH receptor has been detected on rodent adipocytes (Bradley and Mansfield, 2002). Leptin's dual roles in energy homeostasis and the reproductive axis have been well-researched and are well-defined, and since emerging evidence suggests that

MCH has similar dual roles, a common objective of this series of studies was to detect any relationships between circulating MCH and leptin in different human populations. There were no consistent significant associations between circulating MCH and leptin concentrations in the studies described in Chapters 5 and 7. In Chapter 6 there was an association at two discrete sampling time-points, that is fasting and 30 minutes post-prandial. However these associations were only observed in sub-groups of the population. Fasting plasma MCH and leptin concentrations were negatively associated in males with excess fat only and 30 minutes post-prandial the association was positive in lean males only. Throughout this series of studies some sexual dimorphism has been observed in the MCH response though the question is raised as to why this outcome was not observed in males in Chapter 5. As discussed in Chapter 6 some disruption between MCH and leptin signalling in the older cohort may be responsible since the observed correlations were in the opposite direction between lean individuals and those with excess fat Again the sample sizes were small (n=7) and larger scale enquiry is required. However there was a trend for circulating MCH and leptin concentrations to be inversely correlated which, whilst non-significant was consistent across all the studies.

8.2.2 Circulating MCH concentrations and body composition parameters

This element of the enquiry produced significant associations between circulating MCH concentrations and body composition parameters in all four human studies. However these associations were between different sub-groups of the populations and different body composition parameters in each study (Table 8.1).

Table 8.1 Summary of correlational analysis between circulating MCH concentrations and body composition parameters reported in Chapters 4, 5 6 and 7

	BMI (kg/m^2)	Body weight (kg)	Fat mass (%)	Lean mass (%)	Fat mass (kg)	Lean mass (kg)
Female (EF)	r=-0.368 p=0.018* (n=41)	r=-0.360 p=0.021* (n=41)	r=-0.733 p=0.038* (n=8)	r=0.733 p=0.038* (n=8)		
Male (EF)	r=0.472 p=0.003** (n=38)					
Female (L)	r=0.613 p=0.045* (n=11)	r=0.602 p=0.050* (n=11)			r=0.608 p=0.047* (n=11)	
Male (A)	r=0.230 p=0.030* (n=90) r=0.662 p=0.037* (n=11)					r=0.639 p=0.034* (n=11)

Values represent Pearson-product moment correlations, ** significant at 0.01 level (2 tailed) * significant at 0.05 level (2 tailed) Chapter 4 Chapter 5 Chapter 6 Chapter 7 (late follicular phase only) EF: Excess fat; L: Lean; A: All
Significant results only reported.

The association between circulating MCH concentrations and BMI in four sub-groups and also between MCH and body weight in 2 sub-groups is somewhat curious since, as discussed in Chapter 4, although superficially BMI and body weight may be taken as markers of adiposity, there are substantial non-adipose components to both measurements. However in Chapters 5 and 6 there are corroborative associations between MCH and lean mass or fat mass (percent or absolute) which indicate circulating MCH may be considered a marker of adiposity or leanness in different populations. For example in Chapter 6 a positive correlation between mean plasma MCH concentrations and BMI, body weight and fat mass weight in females designated as lean was observed. There were no such associations in Chapter 5, although mean fat mass percentage, lean mass percentage and BMI were almost identical between the females in Chapter 5 and lean

females in Chapter 6, the difference in mean age was approximately 20 years. In Chapter 5 the associations between circulating MCH concentrations and body composition parameters were in males and appeared to be indexed to leanness rather than adiposity. Therefore it would seem that MCH activity in the periphery has both age and gender-related components. The two major peripheral adiposity signals; leptin and insulin are processed differently in males and females, female brains being more sensitive to leptin and male brains being more sensitive to insulin. In terms of body fat, leptin correlates better with total body fat in females and insulin correlates better with total body fat in males (Clegg *et al.,* 2003, Woods *et al.,* 2003). It could be that MCH also displays a sexually dimorphic sensitivity which correlates better with lean mass in males or fat mass in females.

8.2.3 Circulating MCH and leptin post-prandial response

In the studies reported in Chapters 5 and 6 subjects consumed identical breakfasts (Section 5.2.3). Blood samples were then collected at 30, 60 and 120 mins post-prandial. In Chapter 6 both males and females demonstrated a significant increase from baseline in circulating MCH concentrations. This was accompanied by a significant decline in circulating leptin concentrations. In Chapter 5 there was a non-significant increase in circulating MCH concentrations post-prandial in females though in males there was a non-significant dip. In agreement with Chapter 6 circulating leptin concentrations declined significantly. Whether or not the post-ingestive leptin response was related to the meal, MCH concentrations or the morning nadir of leptin requires qualification. There were no associations between circulating MCH and leptin concentrations at any of the four sampling time-points and the respective AUC's were not related. The discrepancy between the MCH response to feeding between the two groups may be the result of an exaggerated MCH response in the older group which could have become apparent in the younger group over a longer time-course. The final sampling time-point was 2 hours post-prandial. Currently a 24 hour profile for circulating MCH has not been established and is a subject which invites further enquiry.

8.3 Circulating MCH concentrations during the menstrual cycle

The study reported in Chapter 7 was somewhat distinct from those reported in previous chapters since, although common parameters were measured, subjects attended the laboratory on four separate occasions and circulating MCH concentrations were tracked over the course of one menstrual cycle. Comparisons of the common parameters are reported above. The results of this chapter indicate that circulating MCH concentrations remain relatively stable over the course of the menstrual cycle though an association between circulating MCH concentrations and circulating progesterone concentrations in the luteal phase are suggestive of MCH input. However it must be admitted that the data derived from four distinct sampling time-points can be limited. If as is suspected, MCH acts in a subtle manner it may be prone to fluctuations that were missed by the time-course of these observations. Based on these results it is therefore recommended that future investigation should utilise more frequent sampling which is tightly aligned to the individual's menstrual cycle.

9. Conclusion

The results reported herein are the first to examine normal circulating MCH concentrations in humans. An important outcome of this study was the demonstration that circulating MCH can be reliably and quantifiably measured in humans by means of a relatively inexpensive, simple radioimmunoassay. The pharmaceutical industry is currently very interested in developing MCHR-1 antagonists not only for obesity pharmacotherapy but as a co-therapeutic for anxiety and mood related disorders; however much of the evidence supporting the efficacy of MCH antagonism in such paradigms is derived from animal models. Nothing is known about the role of circulating MCH in humans hence the quantification of MCH in human plasma will be important in assessing the systemic consequences of deletion of functional MCH. Moreover the RIA will be constructive in establishing whether or not MCH may be used as a specific biomarker. Reference ranges for circulating MCH concentrations have also been established, the upper and lower limits across all four groups were 19.4 – 62.4 pg/ml. Although no robust physiological effect of MCH was observed some trends have emerged which are worthy of further investigation. First the MCH post-prandial response particularly in relation to insulin; current results are supportive of a role for circulating MCH in glucose homeostasis in humans, which is in agreement with the findings of research with animal models. Secondly these results indicate that circulating MCH concentrations are aligned in some way to body composition and further investigation would serve to delineate that relationship. Thirdly although no strong associations were observed between energy expenditure, energy intake and circulating MCH concentrations, other researchers should not be dissuaded from pursuing this line of enquiry since it is suspected that there are subtle mechanisms involved which may become apparent under further investigation. Overall it would seem that circulating MCH concentrations are not overtly reactive but there appears to be some differential regulation in the presence of a combination of gender and adiposity which is variable depending on the population under examination. Since one of the key roles of MCH is in energy homeostasis it would be

expected that its expression is in a permanent state of flux and this initial characterisation may be used as an index for future investigators.

10. Recommendations for future work

Based on the outcomes of the studies described in this thesis in addition to the points raised above, the following recommendations are made:

1) It is important that a 24 hour profile of circulating MCH is established to lend contextual relevance to results such as those reported herein.

2) An association was observed between circulating MCH concentrations and circulating progesterone concentrations. It seems likely that circulating MCH concentrations may also interact with oestrogen and this line of enquiry should be pursued in order to further delineate the physiological relevance of MCH in human reproductive function.

3) Evidence derived from rodent studies indicates that MCH may be involved in peripheral lipid metabolism; this subject has never been explored in humans and should logically be addressed given its emergent role in peripheral glucose homeostasis. Associations between circulating MCH, lipoproteins and triglycerides would serve to further understanding of the metabolic repertoire of circulating MCH.

4) The MCH RIA described herein requires minimal sample preparation, is simple to perform and has the capacity for high through-put. Its utilisation will facilitate further research into the physiological role of circulating MCH by other laboratories.

5) Future investigators should consider the use of indwelling catheters rather than venepuncture for the collection of blood samples. This would increase points of observation and enable a more temporally flexible method of data collection.

6) Finally an interesting new area which would lend itself to this type of investigation would be to examine circulating MCH concentrations in association with circulating cortisol concentrations since there is an accruing body of evidence which implicates MCH in the stress axis which is also aligned to nutritional status and reproductive function.

References

Alon, T., Friedman, J.M., (2006). Late-onset leanness in mice with targeted ablation of melanin-concentrating hormone neurons. *Journal of Neuroscience,* 26/2: 389 – 397

Ahima, R.S., (2006) Adipose tissue as an endocrine organ. *Obesity,* 14: 242S – 249S

American College of Sports Medicine. Based on information from American College of Sports Medicine, The American Council on Exercise, McArdle, W.D., Katch, F.I., Katch, V.L., (1996). Exercise Physiology (4th Ed), *Lippincott, Williams and Wilkins*, USA

Astrand, A., Bohlooly-Y, M., Larsdotter, S., Mahlapuu, M., Andersen, H., Tornell, J., Ohlsson, C., Smith, M., Morgan, D.G.A., (2004). Mice lacking melanin concentrating hormone receptor 1 demonstrate increased heart rate associated with altered autonomic activity. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 287: R749 – R758

Astrup, A., Buemann, B., Christensen, N.J., Madsen, J., Gluud, C., Bennett, P., Svenstrup, B., (1992). The contribution of body composition, substrates and hormones to the variability in energy expenditure and substrate utilisation in premenopausal women. *Journal of Clinical Endocrinology and Metabolism*, 74: 279 - 286

Attademo, A,M., Rondini, T.A., Rodrigues, B.C., Bittencourt, J.C., Celis, M.E., Elias. C.F., (2006). Neuropeptide Glutamic Acid-Isoleucine may induce luteinising hormone secretion via multiple pathways. *Neuroendocrinology*, 83: 313 – 324

Attoub, S., Levasseur, M., Buyse, M., Goiot, H., Laigneau, J.P., Moizo, L., Hervatin, F., Le Marchand-Brustel, Y., Lewin, J.M., Bado, A., (1999). Physiological role of cholecystokinin B/gastrin receptor in leptin secretion. *Endocrinology,* 140: 4406 – 4410

Bakari, A.G., Onyemelukwe, G.C., (2005). Insulin resistance in type 2 diabetic Nigerians. *International Journal of Diabetes and Metabolism*, 13: 24 - 27

Baird, J-P., Rios, C., Loveland, J.L., Beck, J., Tran, A., Mahoney, C.E., (2008). Effects of hindbrain melanin-concentrating hormone and neuropeptide Y administration on licking for water, saccharin and sucrose solutions. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 294: R329 – R343

Baker, B.I., Bird, D.J., Buckingham, J.C., (1985). Salmonid melanin concentrating hormone inhibits corticotrophin release. *Journal of Endocrinology*, 106: R5 – R8

Barber, L.D., Baker, B.I., Penny, J.C., Eberle, A.N., (1987). Melanin concentrating hormone inhibits the release of αMSH from teleost pituitary glands. *General and Comparative Endocrinology,* 65 (1): 79 – 86

Bayer, L., Effermann, E., Serafin, M., Grivel, J., Machard, D., Muhlethaler, M., Jones, B.E., (2005). Opposite effects of noradrenaline and acetylcholine upon hypocretin/orexin versus melanin concentrating hormone neurons in rat hypothalamic slices. *Neuroscience,* 130: 807 – 811

Bisdee, J.T., James, W.P.T., Shaw, M.A., (1989). Changes in energy expenditure during the menstrual cycle. *British Journal of Nutrition*, 61: 187 - 199

Bittencourt, J.C., Presse, F., Arias, C., Peto, C., Vaughan, J., Nahon, J.L., Vale, W., Sawchenko, P.E., (1992). The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *Journal of Comparative Neurology*, 319: 218-245

Bjursell, M., Gerdin, A-K., Ploj, K., Svensson, D., Svennson, L., Oscarsson, J., Snaith, M., Tornell, J., Bohlooly-Y, M., (2006). Melanin-concentrating hormone receptor 1 deficiency increases insulin sensitivity in obese leptin-deficient mice without affecting body weight. *Diabetes,* 55: 725 – 733

Boden, G., Chen, X., Mozzoli, M., Ryan, I., (1996). Effect of fasting on serum leptin in normal human subjects. *Journal of Clinical Endocrinology and Metabolism,* 81: 3419 – 3423

Bowen , D.J., Grunberg, N.E., (1990). Variations in food preference and consumption across the menstrual cycle. *Physiology and Behaviour*, 47: 287 - 291

Bradley, R.L., Cheatham, B., (1999). Regulation of ob gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes,* 48: 272 – 278

Bradley R.L., Cleveland, K.A., Cheatham, B., (2001) The adipocyte as a secretory organ: mechanisms of vesicle transport and secretory pathways. *Research Progress in Human Research,* 56: 329 - 358

Bradley, R.L., Kokkotou, E.G., Maratos-Flier, E., Cheatam, B., (2000). Melanin-concentrating hormone regulates leptin synthesis and secretion in rat adipocytes. *Diabetes,* 49: 1073 – 1077

Bradley, R.L., Mansfield, J.P.R., Maratos-Flier, E., Cheatam, B., (2002). Melanin-concentrating hormone activates signalling pathways in 3T3-L1 adipocytes. *American Journal of Physiology, Endocrinology and Metabolism,* 283: E584 – E592

Breton, C., Schorrp, M., Nahon J.L., (1993). Isolation and characterization of the human melanin concentrating hormone gene and a variant gene. *Brain Research and Molecular Brain Research,*18: 297 – 310

Brown, S.N., Chitravanshi, V.C., Kawabe, K., Sapru, H.N., (2007). Microinjections of melanin concentrating hormone into the nucleus tractus solitaries of the rat elicit depressor and bradycardic responses. *Neuroscience,* 150: 796 – 806

Buice, R.G., Stentz, F.B., Gurley, B.J., (1987). Analytical methodologies for cyclosporine phramacokinetics: a comparison of radioimmunoassay with High Performance Liquid Chromatography. *Journal of Liquid Chromatography and Related Technologies,* 10: 421 – 438

Carrascosa, J.M., Ros, M.,Andres, A., Fernandez-Agullo, T., Arribas, C., (2009). Changes in the neuroendocrine control of energy homeostasis by adiposity signals during aging. *Experimental Gerontology*, 44: 20 - 25

Carriquiry AL., (2003). Estimation of usual intake distributions of nutrients and foods. *Journal of Nutrition*, 133: 601S - 608S

Carruthers, S.G., Freeman, D.J., Koegler, J.C., Howson, W., Keown, P.A., Laupacis, A., Stiller, C.R., (1983). Simplified liquid-chromotographic analysis for cyclosporine A, and comparison with radioimmunoassay. *Clinical Chemistry,* 29: 181 - 183

Chen, H., McCoy, L.F., Schleicher, R.L., Pfeiffer, C.M., (2008). Measurement of 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmuassay method. *Clinica Chimica Acta*, 391: 6 -12

Chen, Y., Hu, C., Hsu, C.K., Zhang, Q., Bi, C., Asnicar, M., Hsuing, H.M., Fox, N., Slieker, L.J., Yang, D.D., Heinman, M.L., Shi, Y., (2002). Targeted disruption of the melanin-

concentrating hormone receptor-1 results in hyperphagia and resistance to diet-induced obesity. *Endocrinology,* 143: 2469 - 2477

Chiocchio, S.R., Gallardo, M.G.P., Louzan, P., Gutnisky, V., Tramezzani, J.H., (2001). Melanin-concentrating hormone stimulates the release of luteinizing hormone-releasing hormone and gonadotropins in the female rat acting at both median eminence and pituitary levels. *Biology of Reproduction,* 64: 1466 – 1472

Clegg, D.J., Riedy, C.A., Smith, K.A.,Benoit, S.C., Woods, S.C., (2003). Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes,* 682 - 687

Cohen, J.W., (1988) Statistical power analysis for the behavioral sciences (2nd ed). Hillsdale, NJ: *Lawrence Erlbaum Associates*, pp 284 - 287

Cross, G.B., Marley, J., Miles, H., Willson, K., (2001). Changes in nutrient intake during the menstrual cycle of overweight women with premenstrual syndrome. *British Journal of Nutrition,* 85: 475 - 482

Cunningham, J.C., (1991). Body composition as a determinant of energy expenditure: a synthetic review and proposed general prediction equation. *American Journal of Clinical Nutrition*, 54: 963 - 969

Dallongeville, J.B., Hecquet, B., Lebel, J., Edme, L., Le Fur, C., Fruchart, J.C., Auwerx, J., Romon, M., (1998). Short term response of circulating leptin to feeding and fasting in man: influence of circadian cycle. *International Journal of Obesity and Related Metabolic Disorders,* 22: 728 – 733

Davidsen, L., Vistisen, B., Astrup, A., (2007). Impact of the menstrual cycle on determinants of energy balance: a putative role in weight loss attempts. *International Journal of Obesity, 31:* 1777 – 1785

Day, D.S., Gozansky, W.S., Van Pelt, R.E., Schwarz, R.S., Kohrt, W.M., (2005). Sex hormone suppression reduces resting energy expenditure and β-adrenergic support of resting energy expenditure. *Journal of Clinical Endocrinology and Metabolism,* 90: 3312 – 3317

De Remer, M., D'Ambrosio, R., Bartos, L., Cousins, S., Morse, G.D., (1997). Radioimmunoassay of zidovudine: extended use and potential application. *Therapeutic drug monitoring,* 19: 195 – 200

Department of Health (1991). Report on Health and Social Subjects No.41.Dietary Reference Values for Food Energy and Nutrients for the United Kingdom*. HMSO,* London.

Desiderio, D.M., (1984) Analysis of neuropeptides by liquid chromatography and mass spectrometry. Elselvier Science, Amsterdam, Netherlands, p90

Dewey, K.G., (1998). Effects of Maternal Caloric Restriction and Exercise during Lactation. *Journal of Nutrition,* 128 (2): 386S – 389S

Duncan, E.A., Proulx, K., Woods, S.C., (2005). Central administration of melanin-concentrating hormone increases alcohol and sucrose/quinine intake in rats. *Alcoholism: Clinical and Experimental Research*, 29: 958 - 964

Dyck, B., (2005). Small molecule melanin-concentrating hormone receptor 1 (MCH1R) antagonists as anxiolytic and antidepressive agents. *Drug Development Research,* 65: 291 – 300

Dye, L., Blundell, J.E., (1997). Menstrual cycle and appetite control: implications for weight regulation. *Human Reproduction,* 12: 1142 - 1151

Eckel, L.A., (2004). Estradiol: an indirect control of meal size. *Physiology and Behaviour,* 82: 35 – 41

Elliott, J.C., Harrold, J.A., Brodin, P., Enquist, K., Backman, A., Bystom, M., Lindgren, K., King, P., Williams, G., (2004). Increases in melanin-concentrating hormone and MCH receptor levels in the hypothalamus of dietary-obese rats. *Molecular Brain Research,* 128: 150 – 159

ESHERE Capri Workshop Group (2006) Nutrition and reproduction in women. *Human reproduction in women,* 12 : 193 - 207

Fleisch, A., (1951). Le metabolisme basal standard et sa determination au moyen du "Metabocalculator". *Helv Med Acta*, 1: 23 - 44

Flier, J.S., (2006). AgRP in energy balance. Will the real AgRP please stand up? *Cell Metabolism*, 3: 83 – 85

Food Standards Agency (2006). *Nutrient and Food Based Guidelines for UK Institutions*

Furudono, Y., Ando, C., Yamamoto, C., Kobashi, M., Yamamoto, T., (2006). Involvement of specific orexigenic neuropeptides in sweetener–induced overconsumption in rats. *Behavioural Brain Research*, 175 (2): 241 - 248

Gallardo, M.G.P., Chiocchio, S.R., Tramezzani, J.H., (2004). Changes of melanin-concentrating hormone related to LHRH release in the median eminence of rats. *Brain Research,* 1030: 152 – 158

Garcia, M.C., Lopez, M., Gualillo, O., Seoane, L.M., Dieguez, C., Senaris, R.M., (2003). Hypothalamic levels of NPY, MCH and prepro-orexin mRNA during pregnancy and lacation in the rat: role of prolactin. *FASEBJ,* 17: 1392 – 1400

Gavrila, A., Chan, J.L., Miller, L.C., Heist, K., Yiannakouris, N., Mantzoros, C.S., (2005). Circulating melanin-concentrating hormone, agouti-related protein, and alpha-melanocyte-stimulating hormone levels in relation to body composition: alterations in response to food deprivation and recombinant human leptin administration. *Journal of Clinical Endocrinology and Metabolism,* 90: 1047 - 1054

Geary, N., (2000). Estradiol and appetite. *Appetite,* 35: 273 – 274

Geisthovel, F., Jochman, N., Widjaja, A., Horn, R., Brabant, G., (2004). Serum pattern of circulating free leptin, bound leptin, and soluble leptin receptor in the physiological menstrual cycle. *Fertility and Sterility*, 81 (2): 398 - 402

Gerendai, I., Toth, I.E., Boldogkoi, Z., Medveczly, I., Halasz, B., (1998). Neuronal labeling in the rat brain and spinal cord from the ovary using viral transneuronal tracing technique. *Neuroendocrinology,* 68 (4): 244 - 256

Gomori, A., Ishihara, A., Ito, M., Mashiko, S., Matsushita, H., Yumoto, M., Ito, M., Tanaka, T., Tokita, S., Moriya, M., Iwaasa, H., Kanatani, A., (2002). Chronic introcerebroventricular infusion of MCH cause obesity in mice. *American Journal of Physiology, Endocrinology and Metabolism,* 284: E583 – E588

Gonzalez, M.I., Baker, B.I., Wilson, C.A., (1997). Stimulatory effect of melanin-concentrating hormone on luteinising hormone release. *Neuroendocrinology,* 66: 254 – 262

Gosman, G.F., Katcher, H.L., Legro, R.S., (2006). Obesity and the role of gut and adipose hormones in female reproduction. *Human Reproduction Update*, 12 (5) : 585 – 601

Grace, A.A., Floresco, S.B., Goto, Y., Lodge, D.J., (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviours. *Trends in Neuroscience,* 30; 220 – 227

Granich, G.G., Krogstad, D.J., Connor, J.D., Kelly, Desrochers, K.L., Sherwood, C., (1989). High-performance liquid chromatography (HPLC) assay for rivavirin and comparison of the HPLC assay with radioimmunoassay. *Antimicrobial Agents and Chemotherapy*, 33: 311 - 315

Guesdon, B., Paradis, E., Samson, P., Richard, D., (2009). Effects of intracerebroventricular and intra-accumbens melanin-concentrating hormone agonism on food intake and energy expenditure. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 296: R469 – R478

Hamman, A., Matthaei, S., (1996). Regulation of energy balance by leptin. *Experimental and Clinical Endocrinology & Diabetes*,104, 293–300.

Hara. J., Yanagisawa M, Sakurai T., (2005). Difference in obesity phenotype between orexin knockout mice and neuron-deficient mice with same genetic background and environmental conditions. *Neuroscience Letters*, 380: 239 - 242

Hardie L., Trayhurn, P., Abramovich, D., Fowler, P., (1997). Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy. *Clinical Endocrinology*, 47: 101 – 106

Harris, J.A., Benedict, F.G., (1919). A biometric study of basal metabolism in man. *Carnegie Institute of Washington,* Washington, DC: Publication no 279

Havel, P.J., Townsend, R., Chaump, L., Teff, K., (1999). High-fat meals reduce 24-h circulating leptin concentrations in women. *Diabetes,* 48: 334 - 341

Henry, C.J.K., (2003). Intra-individual variation in resting metabolic rate during the menstrual cycle. *British Journal of Nutrition*, 89: 811 – 817

Hervieu, G.J., Nahon, J-L., (1995). Pro-melanin-concentrating hormone messenger ribonucleic acid and peptides expression in peripheral tissues of the rat. *Neuroendocrinology,* 61: 348 – 364

Hervieu, G.J., Segretain, D., Nahon, J-L., (1996). Development and stage-dependent expression of melanin concentrating hormone in mammalian germ cells. *Biology of Reproduction,* 54: 1161 - 1172

Heymsfield, S.B., Gallagher, D., Kotler, D.P., Wang, Z., Allison, D.B., Heshka, S., (2002). Body-size dependence of resting energy expenditure can be attributed to nonenergetic homogeneity of fat-free mass. *American Journal of Physiology, Endocrinology and Metabolism,* 282: E132 – E138

Hoogduijn, M.J., Ancans, J., Suzuki, I., Estdale, S., Thody, A.J., (2002). Melanin-concentrating hormone and its receptor are expressed and functional in human skin. *Biochemistry and Biophysical Research Communications*, 296: 698 – 701

Howe, J.C., Rumpler, W.V., Seale,J.L., (1993). Energy expenditure by indirect calorimetry in premenopausal women: variation within one menstrual cycle. *Journal of Nutritional Biochemistry*, 4: 268 - 273

Huang, Q., Viale, A., Picard, F., Nahon, J-L., Richard, D., (1999). Effects of leptin on melanin-concentrating hormone expression in the brain of lean and obese lep^{ob}/lep^{ob} mice. *Neuroendocrinology,* 69: 145 -153

Illner, K., Brinkmann, G., Heller, M., Bosy-Westphal, A., Muller, M.J., (2000). Metabolically active components of fat free mass and resting energy expenditure in non-obese adults. *American Journal of Physiology, Endocrinology and Metabolism, 278: E308 – E315*

Imbeault, P., Doucet, E., Mauriege, P., St-Pierre, S., Couillard, C., Almeras, N., Despres, J.P., Tremblay, A., (2001). Difference in leptin response to a high-fat meal between lean and obese men. *Clinical Science*, 101: 359 - 365

Ito M., Gomori, A., Ishihara, A., Oda, A., Mashiko, S., Matsushita, H., Yumoto, M., Ito, M., Sano, H., Tokita, S., Moriya, M., Iswaasa, H., Kanatani, A., (2003). Characterisation of MCH-mediated obesity in mice. *American Journal of Physiology, Endocrinology and Metabolism,* 284: E940 – E945

Jeon, J.Y., Bradley, R.L., Kokkotou, E.G., Marino, F.E., Wang, X., Pissios, P., Maratos-Flier, E., (2006). $MCH^{-/-}$ mice are resistant to aging-associated increases in body weight and insulin resistance. *Diabetes,* 56: 428 – 434

Johnson, W.C.,Carr-Nangle, R.E.,Bergeron, K.C., (1995). Macronutrient intake, eating habits and exercise as moderators of menstrual distress in healthy women. *Psychosomatic Medicine,* 57: 324 - 330

Kaczkowski CH., Jones P.J.H., Feng J., Bayley H.S., (2000). Four-day multi-media diet records under-estimate energy needs in middle-aged and elderly women as determined by doubly-labelled water. *Journal of Nutrition,* 130: 802-805

Kahn, S.E., Prigeon, R.L., Schwartz, R.S., Fujimoto, W.Y., Knopp, R.H., Brunzell, J.D., Porte, D., (2001). Obesity, body fat distribution, insulin sensitivity and islet β-cell function as explanations for metabolic diversity. *Journal of Nutrition*, 131: 354S-360S

Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M., Baker, B.I., (1983). Characterisation of melanin-concentrating hormone in chum salmon pituitaries. *Nature,* 305: 321 – 323

Kennedy, A.R., Todd, J.F., Stanley, S.A., Abbot, C.R., Small, C.J., Ghatei, M.A, Bloom, S.R., (2001). Melanin-concentrating hormone (MCH) suppresses thyroid stimulating hormone (TSH), *in vivo* and *in vitro*, via the hypothalamus and the pituitary. *Endocrinology,* 142 (7): 3265 – 3268

Kerman, I.., Bernard, R., Rosenthal, D., Beals, J., Akil, H., Watson, S.J., (2007). Distinct populations of presympathetic-premotor neurons express orexin or melanin concentrating hormone in the rat hypothalamus. *Journal of Comparative Neurology,* 505: 586 - 601

Knollema, S., Brown, E.R., Vale, W., Sawchenko, P.E., (1992). Novel hypothalamic and preoptic sites of prepro-melanin-concentrating hormone messenger ribonucleic acid and peptide expression in lactating rats. *Journal Neuroendocrinology*, 4: 709 – 717

Kokkotou, E., Jeon, J.Y., Wang, X., Marino, F.E., Carlson, M., Trombly, D.J., Maratos-Flier, E., (2005). Mice with MCH ablation resist diet-induced obesity through strain-specific mechanisms. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* 298: R117 – R124

Kokkotou, E., Tritos, N.A., Mastaitis, J. W., Slieker, L., Maratos-Flier, E., (2001). Melanin-concentrating hormone receptor is a target of leptin action in the mouse brain. *Endocrinology,* 142: 680 – 686

Kowalski, T.J., Farley, C., Cohen-Williams, M.E., Varty, G.,Spar, B.D., (2004). Melanin-concentrating hormone-1 receptor antagonism decreases feeding by reducing meal size. *European Journal of Pharmacology*, 497: 41 -47

Kowalski, T.J., Spar, B.D., Weig, B., Farley, C., Cook J., Ghibaudi, L., Fried, S., O'Neill, K., Del Vecchio, R.A.,McBriar, M., Guzik, H., Clader, J., Hawes, B.E., Hwa, J., (2006). Effects of a selective melanin concentrating hormone 1 receptor antagonist on food intake and energy homeostasis in diet-induced obese mice. *European Journal of Pharmacology,* 535 (1-3): 182 - 191

Lashen, H., Fear, K., Sturdee, D.W., (2004). Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. *Human Reproduction*, 19: 1644 – 1646

Lee, M.J., Fried, S.K., (2006). Multilevel regulation of leptin storage, turnover and secretion by feeding and insulin in rat adipose tissue. *Journal of Lipid Research*, 47: 1984 – 1993

Lee, M.J., Yang, R.Z., Gong, D.W., Fried,, SK., (2007). Feeding and insulin increase leptin translation. Importance of the leptin mRNA untranslated regions. *Journal of Biological Chemistry,* 282: 72 - 80

Lin, S., Boey. D., Herzog, H., (2004). NPY and Y receptors: lessons from transgenic and knockout models. *Neuropeptides* 2004; 38: 189 - 200

Lips, P., Chapuy, M.C., Dawson-Hughes, B., Pols, H.A.P., Holicks, M.F., (1999). An international comparison of serum 25-Hydroxyvitamin D measurements. *Osteoporosis International*, 9: 394 - 397

Loveland, J.B., McClamrock, H.D., Malinow, A.M., Shahara, F.I., (2001). Increased body mass index has a deleterious effect on *in vitro* fertilization outcome. *Journal of Assisted Reproduction and Genetics,* 18: 382 – 386

Ludwig, D.S., Mountjoy, K.G., Tatro, J.B., Gillette, J.A., Frederich, R.C., Flier, J.S., Maratos-Flier, E., (1998). Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *American Journal of Physiology,* 274: E627 – E633

Ludwig, D.S., Tritos, N.A., Mastaitis, J.W., Kulkarni, R., Kokkotou, E., Elmquist, J., Lowell, B., Flier, J.S., Maratos-Flier, E., (2001). Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. *Journal of Clinical Investigation,* 107: 379 – 386

Ludwig M., Klein, H.k., Diedrich, K., Ortmann, O., (2000). Serum leptin concentrations throughout the menstrual cycle. *Archives of Gynecology and Obstetrics,* 263: 99 – 101

Lyons, M.P., Truswell, A.S., Miro, M.,Vizzard, J., Abraham, S.F., (1989). Reduction of food intake in the ovulatory phase of the menstrual cycle. *American Journal of Clinical Nutrition,* 49: 1164 -1168

Mackenzie, F.J., Hunter, A.J., Daly, C., Wilson, C.A., (1984). Evidence that the dopaminergic incerto-hypothalamic tract has a stimulatory effect on ovulation and gonadotrophin release. *Neuroendocrinology,* 39: 289 – 295

Mahler, S.V., Smith, K.S., Berridge, K.C., (2007). Endocannabinoid hedonic hotspot for sensory pleasure: anandamide in nucleus accumbens shell enhances “liking” of a sweet reward. *Neuropyschopharmacology,* 32: 2267 – 2278

Martini, M.C.,Lampe, J.W., Kurzer, M.S., (1994). Effect of the menstrual cycle on energy and nutrient intake. *American Journal of Clinical Nutrition*, 60: 895 - 899

Mantzoros,, C., (2005). Authors' response: Melanin-concentrating hormone and energy balance. *Journal of Clinical Endocrinology and Metabolism,* 90: 6337

Marsh, D.J., Weingarth, D.T., Novi, D.E., Chen, H.Y., Trumbauer, M.E., Chen, A.S., Guan, X.M., Jiang, M.M., Feng, Y., Camacho, R.E., Shen, Z., Frazier, E.G., Yu, H., Metzger, J.M., Kuca, S.J., Shearman, L.P., Gopal-Truter, S., MacNeil, D.J., Strack, A.M., MacIntyre, D.E., Van der Ploeg, L.H.T., Qian, S., (2002). Melanin-concentrating hormone-1 deficient mice are lean, hyperactive and hyperphagic and have altered metabolism. *Proceedings of the National Acadamy of Science, USA,* 99 (5): 3240 – 3245

Mashiko, S., Ishihara, A., Gomori, A., Moriya, M., Ito, Makoto, Iwaasa, H., Matsuda, M., Feng, Y., Shen, Z., Marsh, D.J., Bednarek, M.A., MacNeil, D.J., Kantani, A., (2005). Antiobesity effect of a melanin-concentrating hormone 1 receptor antagonist in diet-induced obese mice. *Endocrinology,* 146: 3080 – 3086

Matthews, J.N.S., Altman, D.G., Campbell, M.J., Royston, P., (1990). Analysis of serial measurements in medical research. *British Medical Journal,* 300: 230 – 235

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., Turner, R.C., (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologica,* 28: 412 - 419

Maulon-Feraille, L.M., Zuana, O.D., Suply, T., Rovere-Jovene, C., Audinot, V., Levens, N., Boutin,J.A., Duhault, J., Nahon, J-L., (2002). Appetite-boosting property of pro-melanin-concentrating hormone (131-165) (neuropeptide-glutamic acid-isoleucine) is associated with proteolytic resistance*. Journal of Pharmacology and Experimental Therapeutics,* 302 (2):766-73

McArdle, W.D., Katch, F.I., Katch, V.L., (2001). Exercise Physiology (5th ed), *Lippincott, Williams and Wilkins,* USA, pp 188 - 200

Meijer, G.A., Westerterp, K.R., Saris, W.H., ten Hoor, F., (1992). Sleeping metabolic rate in relation to body composition and the menstrual cycle*. American Journal of Clinical Nutrition*, 55: 637 - 640

Messager, S., Chatzidaki, E.E.,Ma, D.,Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R., Malinge, I., Lomet, D., Carlton, M.B., College, W.H., Caraty, A., Aparicio, S.A., (2005). Kisspeptin directly stimulates gonadatropin-releasing hormone release via G-protein-coupled receptor 54. *Proceedings of the National Academy of Sciences of USA*, 102 (5): 1761 – 1766

Messina, M.A., Boersma, G., Overton, J.M., Eckel, L.A., (2006). Estradiol decreases the orexigenic effect of melanin-concentrating hormone in ovariectomized rats. *Physiology and Behaviour,* 88: 523 – 528

Messina, M.A., Overton, (2007). Cardiovascular effects of melanin-concentrating hormone. *Regulatory Peptides,* 139: 23 – 30

Morens, C., Norregaard, P., Receveur, J.M., van Dijk, G., Scheurink, A.J., (2005). Effects of MCH and MCH1-receptor antagonist on (palatable) food and water intake. *Brain Research,* 1062 (1-2): 32 – 38

Mori, M., Harada,M., Terao, Y., Sugo, T., Watanabe, T.,Shimomura, Y.,Abe, M., Shintani,Y., Onda,H.,Nishimura,O.,Fujino, M., (2001). Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor. *Biochemical and Biophysical Research Communications 283***:** 1013–1018

Morton, G.J., Mystkowski, P., Matsumoto, A.M., Schwartz, M.W., (2004). Increased hypothalamic melanin-concentrating hormone gene expression during energy restriction

involves a melanocortin-independent estrogen-sensitive mechanism. *Peptides,* 25: 667 - 674

Moschos, S., Chan, J.L., Mantzoros, C.S., (2002). Leptin and reproduction: a review. *Fertility and Sterility,* 77 (3): 433 – 444

Murray, J.F., Adan, R.A.H., Walker, R., Baker, B.I., Thody, A.J., Nijenhuis, W.A.J., Yukitake, J., Wilson, C.A., (2000a). Melanin-concentrating hormone, melanocortin receptors and regulation of luteinising hormone release. *Journal of Neuroendocrinology,* 12: 217 – 223

Murray, J.F., Baker, B.I., Levy, A., Wilson, C.A., (2000b). The influence of gonadal steroids on pre-pro melanin-concentrating hormone mRNA in female rats. *Journal of Neuroendocrinology,* 12: 53 -59

Murray, J.F., Mercer, J.G., Adan, R.A.H., Datta, J., Aldairy, C., Moar, K.M., Baker, B.I., Stock, M.J., Wilson, C.A., (2000c). The effect of leptin on luteining hormone release is exerted in the zona incerta and mediated by melanin-concentrating hormone. *Journal of Neuroendocrinology,* 12: 1133 - 1139

Murray, J.F., Hahn, J.D., Kennedy, A.R., Small, C.J., Bloom, S.R., Haskell-Luevano, C., Coen, C.W., Wilson, C.A., (2006). Evidence for a stimulatory action of melanin-concentrating hormone on luteinizing hormone release involving MCH1 and Melanocortin-5 receptors. *Journal of Neuroendocrinology,* 18: 157-167

Muschamp, J.W., Hull, E.M., (2007). Melanin concentrating hormone and estrogen receptor-α are coextensive but not co-expressed in cells of male rat hypothalamus. *Neuroscience Letters,* 427: 123 - 126

Mystkowski, P., Seeley, R.J., Hahn, T.M., Baskin, D.G., Havel, P.J., Matsumoto, A.M., Wilkinson, C.W., Peacock-Kinzig, K., Blake, K, A., Schwartz, M.W., (2000). Hypothalamic Melanin**-**Concentrating Hormone and Estrogen-Induced Weight Loss. *Journal of Neuroscience,* 20 (22):8637-8642

Nahon, J.L., Presse, F., Bittencourt, J.C., Sawchenko, PE., Vale, W., (1989). The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. *Endocrinology,* 125: 2056 – 2065

NHS Information Centre, Lifestyles Statistics (2010) Statistics on obesity, physical activity and diet: England 2010, *The Health and Social Care Information Centre*

Nicholls, D.G., Locke, R.M., (1984). Thermogenic mechanisms in brown fat. *Physiological Reviews,* 64 (1): 1 – 64

Oftedal, O.T., (2004) Lactation, Land Mammals, Species Comparisons. In: Pond WG, Bell AW (eds). Encyclopaedia of Animal Science*. Marcel Dekker Inc*: New York, pp 562 - 564

Oldfield, B.J., Giles, M.E., Watson, A., Anderson, C., Colville, L.M., McKinley, M.J., (2002). The neurochemical characterization of hypothalamic pathways projecting polysynaptically to brown adipose tissue in the rat. *Neuroscience,* 110: 515 – 526

Paolisso, G., Rizzo, M.R., Mazziotti, G., Rotondi, M., Tagliamonte, M.R., Varricchio, G., Carella, C., Varricchio, M., (1999). Lack of association between changes in plasma leptin concentration and in food intake during the menstrual cycle. *European Journal of Clinical Investigation,* 29: 490 - 495

Parkes, D.G., (1996). Diuretic and natriuretic actions of melanin-concentrating hormone in conscious sheep. *Journal of Neuroendocrinology,* 8: 57 – 63

Pasquali, R., Pelusi, C., Genghini, S., Cacciari, M., Gambineri, A., (2003). Obesity and reproductive disorders in women. *Human Reproduction Update,* 9: 359 – 372

Pelkman, C.L., Chow, M., Heinback, R.A., Rolls, B.J., (2001). Short-term effects of a progestational contraceptive drug on food intake, resting energy expenditure, and body weight in young women. *American Journal of Clinical Nutrition,* 73: 19 - 26

Pereira-da-Silva, M., Torsoni, M.A., Nourani, H.V., Augusto, V.D., Souza, C.T., Gasparetti, A.L., Carvalheira, J.B., Ventrucci, G., Cristini, M.,Marcondes, G., Cruz-Netto, A, P., Saad, M.J.A., Boschero, A.C., Carneiro, E.M., Vellosos, L.A (2003). Hypothalamic melanin-concentrating hormone is induced by cold exposure and participates in the control of energy expenditure in rats. *Endocrinology,* 144 (11):4831 – 4840

Pereira-da-Silva, M., De Souza, C. T., Gasparetti, A, L., Saad, M.J.A., Velloso, L.A., (2005). Melanin-concentrating hormone induces insulin resistance through a mechanism independent of body weight gain. *Journal of Endocrinology,* 186: 193 - 201

Pissios, P., (2009). Animal models of MCH function and what they can tell us about energy balance. *Peptides,* 30: 2040 - 2044

Pissios, P., Maratos-Flier, E., (2003). Melanin-concentrating hormone: from fish skin to skinny mammals. *Trends in Endocrinology and Metabolism*, 14 (5): 243 – 248

Pissios, P., Ozcan, U., Kokkotou, E., Okada, T., Liew, C.W., Liu, S., Peters, J.N., Dahlgren, G., Karamchandani, J., Kidva, Y.C., Kurpad, A.J., Kennedy, R.T., Maratos-Flier, E., Kulkarni, R.N., (2007). Melanin-concentrating hormone is a novel regulator of islet function and growth. *Diabetes,* 56: 311 – 319

Presse, F., Nahon, J.L., Fischer, W.H., Vale, W., (1990). Structure of the human melanin concentrating hormone mRNA. *Molecular Endocrinology,* 4: 637 - 645

Qu, D., Ludwig, D.S., Gammeltoft, S., Piper, M., Pelleymounter, M.A., Cullen, M.J., Mathes, W.F., Przypek, R., Kanarek, R., Maratos-Flier, E., (1996). A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature,* 380: 243 – 247

Rajashekar, V., (2009). Novel nutritional intervention in manipulation of energy expenditure and blood glucose regulation in humans. University of Westminster, PhD

Romon, M.,Lebel, P., Fruchart, J.C., Dallongeville, J., (2003). Postprandial leptin response to carbohydrate and fat meals in obese women. *Journal of the American College of Nutrition*, 22: 247 - 251

Romon, M., Lebel, P., Velly, C., Marecaux, N., Fruchart, J.C., Dallongeville, J., (1999). Leptin response to carbohydrate or fat meal and association with subsequent satiety and energy intake. *American Journal of Physiology,* 277: E855 – E861

Saad, M.F., Khan, A., Sharma, A., Michael, R., Riad-Gabriel, M.G., Boyadjian, R., Jinagouda, S.D., Steil, G.M., Kamdar, V. (1998). Physiological insulinemia acutely modulates plasma leptin. *Diabetes,* 47: 544 – 549

Sahu, A., Phelps, C.P., White, J.D., Crowley, W.R., Kalra, S.P., Kalra, P.S., (1992). Steroidal regulation of hypothalamic neuropeptide Y release and gene expressions. *Endocrinology*, 130: **3331**

Saito, Y., Cheng, M., Leslie, F.M., Civelli, O., (2001). Expression of the melanin-concentrating hormone (MCH) receptor mRNA in rat brain. *Journal of Comparative Neurology,* 435: 26 – 40

Saito, Y., Nothacker, H.P., Wang, Z., Lin, S.H., Leslie, F.M., Civelli, O., (1999). Molecular characterisation of the melanin-concentrating hormone receptor. *Nature,* 400: 265 - 269

Sakamaki, R., Uemoto, M., Inui, A., Asakawa, A., Ueno, N., Ishibashi, C., Hirono, S., Yukioka, H., Kato, A., Shinfuku, N., Kasuga, M., Katsuura, G., (2005). Melanin-concentrating hormone enhances sucrose intake. *International Journal of Molecular Medicine,* 15 (6): 1033 – 1039

Sandig, H., McDonald, J., Gilmour, J., Arno, M., Lee, T.H., Cousins, D.J., (2007). Human Th2 cells selectively express the orexigenic peptide, pro-melanin-concentrating hormone. *Proceedings of the National Academy of Sciences, USA,* 104 (30): 12440 - 12445

Santollo, J., Eckel, L.A., (2008). The orexigenic effect of melanin-concentrating hormone (MCH) is influenced by sex and stage of estrous cycle. *Physiology and Behaviour*, 93: 842 - 850

Saper, C.B., Chou, C.H., Elmquist, J.K., (2002). The need to feed: homeostatic and hedonic controls of eating, *Neuron,* 36: 199 - 211

Schneider, J.E., (2004). Energy balance and reproduction. *Physiology and Behaviour,* 81 : 289 - 317

Schwartz, M.W., Woods, S.C., Porte, D., Seeley, R.J., Baskin, D.G., (2000). Central nervous system control of food intake. *Nature,* 404: 661 – 671

Schlumberger, S.E., Talke-Messerer, C., Zumsteg, U., Eberle, A.N., (2002). Expression of receptors for melanin-concentrating hormone (MCH) in different tissues and cell lines. *Journal of Receptors and Signal Transduction.* 22: 509 – 531

Segal-Lieberman, G., Bradley, R.L., Kokkotou, E., Carlson, M., Trombly, D.J., Wang, X., Bates, S., Myers, M.G., Flier, J.S., Maratos-Flier, E., (2003). Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype. *Proceedings of the National Academy of Science,* 100 (17) 10085 - 10090

Segal-Lieberman, G., Rubinfeld, H., Glick, M., Kronfeld-Shaw, N., Shimon, I., (2006). Melanin concentrating hormone stimulates human growth hormone secretion: a novel effect of MCH on the hypothalamic-pituitary axis. *American Journal of Physiology, Endocrinology and Metabolism*, 290: E982 – E988

Shimada, M., Tritos, N.A., Lowell, B.B., Flier, J.S., Maratos-Flier, E., (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature,* 396 : 670 – 674

Sinha, M.K., Ohannesian, J.P., Heiman, M.L., Kriaucuinas, A., Stephens, T.W., Magosin, S., Marco, C., Caro, J.F., (1996). Nocturnal rise of leptin in lean, obese and non-insulin dependent diabetes mellitus subjects. *Journal of Clinical Investigation,* 97: 134 - 1347

Siri, W.E., (1956) The gross composition of the body. *Advances in Biological Medical and Physics*, 4: 239 - 280

Sita, L.V., Elias, C.F., Bittencourt, J.C., (2007). Connectivity pattern suggests that incerto-hypothalamic area belongs to the medial hypothalamic system. *Neuroscience,* 148: 949 - 969

Smith, G.P., (1996). The direct and indirect controls of meal size. *Neuroscience and Biobehavioural Reviews,* 20: 41 - 46

Smith, D.G., Davis, R.J., Rorick-Kehn, L., Morin, M., Witkin, J.M., McKinzie, D.L., Nomikos, G.G., Gehlert, D.R., (2006). Melanin-concentrating hormone receptor modulates neuroendocrine, behavioural and corticolimbic neurochemical stress responses in mice. *Neuropyschopharmacology,* 31 (6) : 1135 - 1145

Smith, M.S., Grove, K.L., (2002). Integration of the regulation of reproductive function and energy balance: lactation as a model. *Frontiers in Neuroendocrinology,* 23 : 225 – 256

Solomon, S.J., Kurzer, M.S., Calloway, D.H., (1982). Menstrual cycle and basal metabolic rate in women. *American Journal of Clinical Nutrition*, 36: 611 - 616

Stellar, E., (1954). The physiology of motivation. *Psychology Review,* 61: 5 - 22

Stricker-Krongrad, A., Dimitrov, T., Beck,B., (2001). Central and peripheral dysregulation of melanin-concentrating hormone in obese zucker rats. *Brain Research and Molecular Brain Research,* 92 : 43 – 48

Sun, G., Tian, Z., Murata, T., Narita, K., Honda, K.,Higuchi, T., (2004). Central and peripheral immunoreactivity of melanin-concentrating hormone in hypothalamic obese and lactating rats. *Journal of Neuroendocrinology,*16: 79 – 83

Takahashi, K., (2005). Letter re: Melanin-concentrating hormone and an RIA kit. *Journal of Clinical Endocrinology and Metabolism,* 19 December, 2005

Takahashi, K., Totsune, K., Murakami, O., Sone, M., Satoh, F., Kitamuro, T., Noshiro, T., Hayashi, Y., Sasono, H., Shibaharas, S., (2001). Expression of melanin concentrating hormone receptor messenger ribonucleic acid in tumour tissues of pheochromocytoma, ganglioneuroblastoma and neuroblastoma. *Journal of Clinical Endocrinology & Metabolism*, 86: 369 – 374

Tan, C.P.,Sano, H., Iwaasa, H., Pan, J., Sailer, A.W., Hreniuk, D.L., Feighner, S.D., Palyha, O.C., Pong, S-S., Figueroa, D.J., Austin, C.P., Jiang, M.M., Yu, H., Ito, J., Ito, M., Ito, M., Guan, X.M., MacNeil, D.J., Kantani, A., Van der Ploeg, L.H.T., Howard, A.D., (2002). Melanin-concentrating hormone receptor subtypes 1 and 2: species-specific gene expression. *Genomics,* 79: 785 - 792

Tarasuk, V., Beaton, G.H., (1991). Menstrual-cycle patterns in energy and macronutrient intake. *American Journal of Clinical Nutrition*, 53: 442 - 447

Teff, K.L., Elliott, S.S., Tschop, M., Kieffer, T.J., Rader, D., Heimann, M., Townsend, Keim, N.L., D'Alessio, D., Havel, J., (2004). Dietary fructose reduces circulating insulin and leptin, attenuates postprandial suppression of ghrelin and increases trigylcerides in women. *Journal of Clinical Endocrinology and Metabolism,* 89: 2963 - 2972

Thompson, R.C., Watson, S.J., (1990). Nucleotide sequence and tissue-specific expression of the rat melanin concentrating hormone gene. *DNA & Cell Biology,* 9: 637 – 645

Tritos, N.A., Mastaitis, J.W., Kokkotou, D., Maratos-Flier, E., (2001). Characterisation of melanin concentrating hormone and preproorexin expression in the murine hypothalamus. *Brain Research,* 895: 160 - 166

Tritos, N.A.,Vincent, D., Gillette, J., Ludwig, D.S., Flier, E.S., Maratos-Flier, E., (2004). Estradiol-induced anorexia is independent of leptin and melanin-concentrating hormone. *Obesity Research,* 12: 716 - 724

Tsukamura, H., Thompson, R, C., Tsukahara, S., Ohkura, S., Maekawa, F., Moriyama, R., Niwa, Y., Foster, D.L., Maeda, K-I., (2000). Intracerebroventricular administration of melanin-

concentrating hormone suppresses pulsatile luteinizing hormone release in the female rat. *Journal of Neuroendocrinology,* 12: 529 – 534

Urban, J.H., Bauer-Dantoin, A.C., Levine, J.E., (1993). Neuropeptide Y gene expression in the arcuate nucleus: sexual dimorphism and modulation by testosterone. *Endocrinology*; 132 - 139

Vaughan, J.M., Fisher, W.H., Hoeger, C., River, J., Vale, W., (1989). Characterisation of melanin-concentrating hormone from rat hypothalamus. *Endocrinology,* 125: 1660 – 1665

Verlaet, M., Adamantidis, A., Coumans, B., Chanas, G., Zorzi, W., Heinen, E., Grisar, T., Lakaye, B., (2002). Human immune cells expresss ppMCH mRNA and functional MCHR-1 receptor. *FEBS Lett* ,527: 205 – 210

Viale, A., Kerdelhue, B., Nahon, J.L., (1999). 17-β Estradiol regulation of melanin-concentrating hormone and neuropeptide-E-I contents in cynomolgus monkeys: a preliminary study. *Peptides,* 20: 553 - 559

Vitale, M.L., Chicchio, S.R., Tramezzani, J.H., (1993). Serotonin, a neurotransmitter involved in the regulation of luteinising hormone release. *Endocrine Reviews,*14: 480 b-493

Wade, G.N., (1986). Sex steroids and energy balance: sites and mechanisms of action. *Annals of New York Academy of Science,* 474: 389 – 399

Wade, G.N., Jones, J.E., (2004). Neuroendocrinology of nutritional infertility. *American Journal of Physiology, Regulatory and Comparative Physiology*, 287 : R1277 – R1296

Wahrenburg, H., Hertel, K., Leijonhufvud, B-M., Persson, Lars-Goran, Toft, E., Arner, P., (2005). Use of waist circumference to predict insulin resistance: retrospective study. *British Medical Journal, 330*: 1363 - 1364

Wang, Z., Heshka, S., Gallagher, D., Boozer, C.N., Kotler, D.P., Heymsfield, S.B., (2000). Resting energy expenditure-fat-free mass relationship: new insights provided by body composition modelling. *American Journal of Physiology, Endocrinology and Metabolism,* 279: E539 – E545

Ward, D.R., Dear, F.M., Ward, I.A., Anderson, S.I., Spergel, D.J., Smith. P.A., Ebling, F.J.P., (2009). Innervations of Gonadotropin-Releasing Hormone neurons by peptidergic neurons conveying circadian or energy balance information in the mouse. *PLos One*, 4: 1 – 9

Waters, S.M., Krause, J.E., (2005). Letter re: Melanin-concentrating hormone and energy balance. *Journal of Clinical Endocrinology and Metabolism,* 90: 6337

Webb, P., (1986). 24-h energy expenditure and the menstrual cycle. *American Journal of Clinical Nutrition*, 44: 614 - 619

Weinsier, R.L., Schutz, Y., Bracco, D., (1992). Re-examination of the relationship of resting metabolic rate to fat-free mass and to the metabolically active components of fat-free mass in humans. *American Journal of Clinical Nutrition*, 55: 790 - 794

Weir J.B., (1949). New methods for calculating metabolic rate with special reference to protein. Journal of Physiology,109:1-9.

Williamson-Hughes, P.S., Grove, K.L., Smith, M.S., (2005). Melanin-concentrating hormone (MCH): A novel neural pathway for regulation of GnRH neurons. *Brain Research,* 1041: 117 – 124

Wolf, B.A., Daft, M.C., Koenig, J.W., Fly, M.W., Turk, J.W., Scott, M.G., (1989). Measurement of clyclosporine concentrations in whole blood: HPLC and radioimmunoassay with a specific monoclonal antibody and ^{3}H or ^{125}I-labelled ligand compared. *Clinical Chemistry,* 35: 120 – 124

Woods, S.C., (2005). Signals that influence food intake and body weight. *Physiology and Behaviour,* 86: 709 -716

Woods, S.C., Gotoh, K., Clegg, D.J., (2003). Gender differences in the control of energy homoeostasis. *Experimental Biology and Medicine*, 228: 1175 – 1180

World Health Organisation (2003) Obesity and overweight factsheet, *WHO Global Strategy on Diet, Physical Activity and Health,* p 1 - 2

Wu, M., Dumalska, I., Morozoova, E., van den Pol, A., Alreja, M., (2009). Melanin-concentrating hormone directly inhibits GnRH neurons and blocks kisspeptin activation, linking energy balance to reproduction. *Proceedings of the National Academy of Sciences, USA* 106 (40): 17217 - 17222

Yang, Y., Zhou, L-B., Liu, S-Q., Tang, J-F., Li, F-Y., Li, R-Y., Song, H-D., Chen, M-D., (2005). Expression of feeding-related peptide receptors mRNA in GT1-7 cell line and roles of leptin and orexins in control of GnRH secretion. *Acta Pharmacologica Sinica,* 26 (8) : 976 – 981

Young, G.S., Kirkland, J.B., (2007). Rat models of caloric intake and activity: relationships to animal physiology and human health. *Applied Physiology, Nutrition and Metabolism,* 32: 161 – 176

Yu, W.H., Kimura, M., Walczewska, A., Karanth, S., McCann, S.M., (1997). Role of leptin in hypothalamic-pituitary function. *Proceedings of the National Academy of Sciences, USA,* 94: 1023 – 1028

Zheng, H., Patterson, L.M., Morrison, C., Banfield, B.W., Randall, J.A., Browning, K.N., Travagli, R.A., Berthould, H-R., (2005). Melanin-concentrating hormone innervation of caudal brainstem areas involved in gastrointestinal functions and energy balance. *Neuroscience,* 135: 611 – 625

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., (1994). Positional cloning of the mouse *obese* gene and its human homologue, *Nature*, 372: 425 – 431

Appendix

Appendix 1 Ethics Application

OFFICE USE: ___ / ___/ _____

University of Westminster

University Ethics Committee

Application for Approval of a Proposed Investigation, Demonstration, Research or Experiment

Section 1 – to be completed by all applicants

1.1 Project Title Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH)

1.2 Applicant Details	
Name: Jane Naufahu	**Email:** janenaufahu@hotmail.com
Address: 84 Finborough Rd London SW10 9ED	**Telephone Number:** 07765 717761
Please tick relevant box: ☐ **Undergraduate** ☐ **Postgraduate** ✓ **PhD Student** ☐ **Staff**	

Section 2 – to be completed when applicable

Please note that all applicants with a supervisor(s) must ensure that the supervisor signs the declaration. All staff must obtain the signature of their Dean of School, or School Research Director, as appropriate.

2.1 Supervisor Details

Name: Dr Joanne Murray	**Email:** J.F.Murray@wmin.ac.uk
School/Department: Human and Health Sciences	**Telephone Number**: 0207 911 5000 ex 5366

Declaration:

In accordance with the University's Code of Practice Governing the Ethical Conduct of Investigation, Demonstrations, Research and Experiments, I agree that the applicant named in 1.2 above should submit their proposal to the Ethics Committee for consideration.

Signed:

Date:

Section 3

3.1 Does your work relate to any of the following areas? Please tick box

☐ Human Participants in Health and Community settings

☐ Work with prescription drugs

☐ Work involving foetal tissue

☐ Drug Studies on human participants

☐ Pre-marketing drug trials

3.2 Are you proposing work using participants from any of the following categories? Please tick box:

☐ Prisoners sectioned under the Mental Health Act

☐ Prisoners or arrestees

☐ Persons with severe mental illness

☐ Persons with learning difficulties or brain damage

☐ Persons with a reduced level of consciousness

3.3 Any work where a qualified clinical person is required to:

☐ Be responsible for all work carried out

☐ Be in attendance when certain procedures are carried out

☐ Ensure that facilities for emergency medical care are at hand

If you have ticked one or more boxes in Section 3, please contact your supervisor and discuss sending your proposal for external approval. More information can be found at:

www.wmin.ac.uk/page-3380

In addition, please complete this form up to Section 3, sign the declaration in Section 11 and send it to:

Carl Hornsey

Assistant Registrar (Student Information)

Academic Registrar's Department

University of Westminster

115 New Cavendish Street

London W1W 6UW

If you have not ticked any boxes in Section 3, please continue to Section 4

Section 4

4.1 Is your work related to any of the following areas? Please tick relevant box:

☐ Any work involving patients

☐ Non-clinical work involving bodily fluids

☐ Administering of a non-food substance

☐ Work with children

☐ Deception of participants – Possibly Study 3 option (c)

☐ Data not already in the public domain that bears on the issues of criminality

☐ Work which requires participants to reveal medical history

If you have ticked one or more boxes in Section 4, your proposal will need approval from the University Ethics Committee – please continue to fill out the rest of this form giving as much detail as possible.

If you have not ticked any boxes in Section 3 or Section 4, your work does not require approval by the University Ethics Committee. Please refer to the University of Westminster 'Code of Practice Governing the Ethical Conduct of Investigations, Demonstrations, Research and Experiments' and consult with your supervisor.

Section 5

5.1 Please provide a brief description of your proposed work below:

MCH is known to have multiple physiological effects including modulation of energy balance, reproductive function and more complex behaviours such as stress response, anxiety and depression. Despite this very little is known about circulating MCH in human populations. The initial objective of this study will be to determine the pattern of release of circulating human MCH. Depending on the results of the initial characterisation, further studies will examine the pattern of circulating MCH in response to interventions such as specific food intake, exercise and stress.

Specifically the objectives of the research will be as follows:

Study 1 : To investigate the pattern of circulating MCH throughout the normal menstrual cycle

Study 2 : To determine whether there is a diurnal rhythm of circulating MCH, to determine whether there is any correlation between circulating MCH concentrations and plasma cortisol, to determine post-prandial effects on circulating MCH concentrations

Study 3 : To investigate the pattern of circulating MCH concentrations in response to food intake/exercise/stress

5.2 What are the specific aims of the work you plan to carry out?

The relationship between reproductive function and nutritional status is well established,

though the mechanisms involved are complex and incompletely understood. In particular this investigation will examine the dual roles of MCH in the modulation of energy balance and reproductive function. Specifically whether or not circulating concentrations of MCH mediate either or both of its food intake/metabolic and reproductive functions and to determine whether MCH provides a link between nutritional status and fertility. Also to explore associations between biochemical markers of stress and circulating MCH since stress affects both reproductive function and eating behaviours.

MCH is an orexigenic neuropeptide (stimulates eating). Manipulation of the availability of MCH has been shown to decrease food intake, body weight and fat mass and to increase energy expenditure. The potentially therapeutic value of MCH antagonists in the treatment of human obesity is currently being taken seriously by both the scientific community and the pharmaceutical industry. Clearly there are clinical implications for the treatment of under/overnutrition syndromes and related fertility. The incidence of obesity in many Western countries is currently endemic and its many associated afflictions such as Type II diabetes, metabolic syndrome, hypertension, heart attack, stroke, some cancers, as well as fertility problems impose a huge burden on the community as well as considerable personal distress to the individual.

This research aims to further the understanding of the mechanisms which link energy balance to reproductive function and how they are integrated and regulated. An understanding of the natural fluctuations of circulating MCH and pattern of expression in various peripheral tissues will enhance understanding of how its actions relate to particular reproductive and metabolic behaviours and the nutritional management of anovulation.

5.3 Please outline the design and methodology of your work

A series of 3 consecutive studies using different cohorts of volunteers (n.b. some volunteers may take part in more than one study), will be performed in which blood samples will be collected and assayed for MCH, leptin, glucose, insulin, appropriate reproductive hormones, appropriate biochemical markers and possibly other metabolically active molecules. Prior to commencement of the main studies a pilot study will be performed in order to determine inter and intra-subject variations of plasma MCH concentrations since (to our knowledge) no such data exists. The reliability and reproducibility of the assay will also be assessed using data from the pilot study.

NOTE 1 : Written informed consent will be obtained from all participants prior to participation (pilot and main studies, copies attached).

NOTE 2 : All of the studies will be undertaken in the human performance laboratory at the University of Westminster. Since some subjects will be expected to spend several hours in the laboratory (maximum 24 hours), every effort will be made to ensure they are relaxed,

comfortable and reassured for the duration of their stay.

NOTE 3 : Prior to participation in any study volunteers will be required to give a finger prick blood sample to screen for anaemia (haematocrit count). Any subject with a haematocrit count outside of the normal range will not be allowed to participate

Pilot study

Ten male and ten female volunteers will be recruited by various methods including advertisements in local press, radio, family planning clinics, polyclinic and posters. Female subjects will be pre-menopausal, non-oral contraceptive using women aged 18 – 30yrs, body mass index (BMI) 19 – 25. Males will be aged 18 – 30yrs, BMI 19 – 25. Those on medication for chronic illnesses, lactating females, those who have been lactating within the last 12 months and pregnant women will be excluded. Female subjects should have regular menstrual cycles of normal length (26 – 30 days). The study will be undertaken in the human performance laboratory at the University of Westminster. Subjects will be expected to attend in the morning after an over-night fast. On arrival resting energy expenditure will be measured via Delta-trac calorimeter. Subjects will be required to wear a ventilated hood attached to a metabolic cart (Delta-trac). Some subjects may find wearing the hood slightly unsettling at first and a subject-specific period of acclimatisation will be allowed. Measurements will then be taken continuously whilst the subject reclines prone for a period of one hour. It is known that MCH affects metabolic energy expenditure based on rodent models. On completion of the energy expenditure measurements an indwelling intravenous catheter will be inserted into a forearm vein by a trained phlebotomist. After a 30 minute recovery period, the 1st blood sample will be withdrawn. A standard controlled meal will then be consumed by subjects (mixed macronutrient composition) 15 minutes after 1st blood sampling. Blood plasma will then be sampled at 15 minute intervals over the next 2 hours to determine changes in plasma MCH concentrations in response to feeding and to determine if there is a relationship between the post-prandial glucose and insulin response and circulating MCH concentrations. Subjects will then be served a standard controlled mid-day meal (mixed macronutrient composition). Two further post-prandial samples will be withdrawn at 2 hours and 4 hours post-feeding. The first of these samples will be dispensed into 3 vacutainers containing different anti-coagulants to assess the effect of these anti-coagulants on plasma MCH concentrations. Over the study period the total volume of blood drawn is not expected to exceed 250ml. Note : This is a relatively small amount of blood compared to 470ml, the amount normally donated to the National Blood Service. Subjects will be expected to attend for a total of no more than 9 hours. During this period fat mass will be measured by Air Displacement Plethysmography (ADP). During this procedure subjects sit comfortably in a sealed chamber (Bodpod) for 1 minute, whilst body volume is estimated by measuring the amount of air displaced by the subjects body. Subjects will also complete a questionnaire to assess their stress/anxiety levels since there are conflicting reports on the effects of MCH administration/MCH receptor antagonists on anxiety/stress in rodents.

Summary : Aims of Pilot Study as follows :

1. To validate the MCH assay developed by researchers at University of Westminster
2. To determine the range of plasma MCH concentrations which will inform how many subjects will be required in the main studies
3. To detect any obvious relationships between MCH and BMI, fat mass, resting energy expenditure, plasma cortisol, glucose, insulin concentrations and gender

Study 1

Objective : To investigate the pattern of circulating MCH throughout the normal menstrual cycle

Design : Observational Study

Female volunteers will be recruited as above. Sample size will be calculated using the results of the pilot study in conjunction with a standard sampling equation. Exclusion/inclusion criteria will be as Pilot Study above. Subjects will be asked to keep a menstrual diary for 3 full cycles prior to attendance at the laboratory, this will include recording daily morning resting shell temperature (taken orally). Thermometers to be supplied by the University of Westminster. Subjects will attend a minimum of 3, maximum of 6 occasions during one full menstrual cycle. The 1st occasion should be during menses (days 1 – 3 menstrual cycle). At each visit a blood sample will be withdrawn (on arrival between 08.00 – 10.00am) from a forearm vein by a trained phlebotomist. No more than 15ml will be withdrawn during each visit. Each subsequent visit will be at agreed times between subject and researchers according to the individual subject's self-described menstrual cycle. Ideally each subject will attend (in addition to during menses): at least once in late follicular phase, peri-ovulatory stage; once in early to mid-luteal phase; and once in late luteal phase. Blood samples will be assayed for the appropriate hormones and metabolically active molecules as above (see below for assay details). Additionally during 2 of the visits resting energy expenditure will be measured by Delta-trac metabolic cart as above, on these occasions subjects will attend for no longer than a 2 hour period, on visits when blood plasma only is sampled subjects will attend for no longer than a 30 minute period. Core temperature will be measured tympanically on each visit before blood sampling.

Assays :

1. In-house MCH assay validated by researchers at University of Westminster
2. In-house steroid assay (radioimmunoassay)
3. Commercial kits will be obtained to assay for insulin, glucose, cortisol (radioimmunoassy or ELISA)

Study 2

Objectives :

- **To determine whether there is a diurnal rhythm of circulating MCH**
- **To determine whether there is any correlation between circulating MCH levels and plasma cortisol**
- **To determine post-prandial effects on circulating MCH levels**

Design : Observational Study

Male and female volunteers will be recruited as above. Sample size will be calculated as Study 1. Inclusion/exclusion criteria will be as Pilot study and Study 1 above. Male and female subjects will be age and BMI matched. Female subjects will be required to attend on Day 2 of their menstrual cycle for a period of 24 hours. Matched male subjects will be required to attend on a mutually agreed date with researchers for a period of 24 hours. Subjects will be expected to arrive between 08.00am – 10.00am. On arrival an indwelling intravenous catheter will be inserted into a forearm vein by a trained phlebotomist. After a 30 min recovery period blood plasma sampling will be initiated hourly except for in the 2 hour period after meals when sampling will be every 15 minutes. During the 24 hour period no more than 300ml blood plasma will be withdrawn by a trained phlebomist. Again less than the amount normally donated to the National Blood Service (470ml). Subjects will be free to move around and will be encouraged to take exercise using on-site exercise equipment in the morning or late afternoon within defined time slots. Exercise intensity should not exceed that which would elicit a heart rate of more than 60% age-related maximum. Sleeping will be encouraged between 11.00pm and 07.00am and not permitted at other times. During the night the intravenous tubing will be extended beyond a screen so that blood samples can be withdrawn with minimum disturbance to the subject. On awakening resting energy expenditure will be measured by Delta-trac metabolic cart as described in Pilot Study. Over the 24 hour period subjects will be served standardised meals at set times. Eating between meals will not be permitted but water may be consumed ad libitum, non-caffeinated drinks will also be available at set times. During the day fat mass will be assessed by Air

Displacement Plethysmography (ADP) as Pilot Study. Subjects will be encouraged to keep an activity diary during their stay at the laboratory. They will also complete a stress/anxiety questionnaire(s). Blood samples will be assayed for hormones and appropriate metabolically active molecules as Study 1 above.

Study 3

Objective : To investigate the circulating MCH levels in response to food intake/exercise/stress

Study Design : Intervention study

Male and/or female volunteers (depending on results of Studies 1 and 2) will be recruited as above. Sample size will be calculated as Studies 1 and 2. Inclusion/exclusion criteria to be defined. Male and female subjects will be age and BMI matched. The study will be performed in the human performance laboratory at the University of Westminster and subjects may be required to attend the laboratory a number of times. The study design will be somewhat dependent upon results of the Pilot Study and Studies 1 and 2 and in conjunction with the *in vitro* rodent studies which will be running concurrently. The latter studies aim to identify factors regulating the pattern of secretion of MCH. The human studies aim to assess the applicability of these regulatory factors to human populations. Study 3 will be designed to determine the role of yet to be determined variables in the secretion of MCH. These variables could include any one of or a combination of the following 1) food intake/macronutrient content of food, 2) energy expenditure, 3) stress. Depending on the final study design the following procedures in relation to the variables above may be implemented :

(a) Food intake/macronutrient content of food : Subjects will attend the laboratory a maximum of 3 times, minimum once for (a) 12 hour period(s) and consume 3 equicaloric single meals consisting of a) carbohydrate, b) protein and c) lipid at 4 hourly intervals in randomised order. Subjects will be cannulated on arrival as Pilot Study and Study 2, blood samples will be withdrawn at 15 minutes intervals for the 1st 2 hours post-feeding, then hourly for 2 hours post-feeding. No more than 150ml of blood plasma will be withdrawn over the study period. Samples will be assayed for glucose, insulin and other appropriate biochemical markers to determine post-prandial plasma MCH response to single meals.

(b) Physical exercise : Subjects will attend the laboratory on no more than 4 occasions for a period of no more than 4 hours each visit. On Visit 1 aerobic fitness will be assessed by the Astrand sub-maximal cycle test. Subjects will be required to review and sign "Informed Consent" forms and complete "pre-test Medical Questionnaire" forms (copies attached) prior to testing. The Astrand test is a sub-maximal aerobic test of no more than 9 minutes duration. It is performed on a cycle ergometer and subjects' heart rates are monitored (by Polar heart rate monitor) and recorded at 60 second intervals. The load on the cycle ergometer will be manipulated so that the subject reaches a steady state heart rate (no more than 10 beats per minute difference between minutes 5 and 6) at not more than 75% age-predicted maximum heart rate. An estimation of maximal oxygen consumption (VO_2max) may then be derived by nomogram. The subject may feel slightly breathless but healthy subjects should feel no discomfort since maximal exertion is not required. On subsequent visits subjects will be cannulated as above and a blood sample (5ml) will be withdrawn from a forearm vein by a trained phlebotomist on arrival. Subjects will then perform physical activity on a stationary cycle or treadmill for a period of no longer than 20 minutes. The intensity at which the subject will exercise will be randomised and prescribed by different percentages of Heart Rate Reserve (HRR). This is a method of prescribing exercise intensity which corresponds approximately to maximal oxygen consumption (VO_2max). The American College of Sports Medicine recommends exercising at intensities ranging from 40 – 50%HRR to 85%HRR several times per week for the attainment/maintenance of cardiorespiratory fitness. An intensity of 40 – 50% HRR is at the lower end of the range and is considered mild to moderate. Subjects will be randomised to perform 1 bout of exercise (either mild, moderate or higher intensity) at each visit within the HRR range above. Less

aerobically fit individuals or those unused to regular exercise will not be expected to exercise at higher intensities. After the exercise period a further blood sample will be withdrawn (5ml). Subjects will then perform a 5 minute cool down. Subjects will remain in the laboratory for a further 2 hours and 2 further hourly blood samples will be taken. No more than 40ml blood will be withdrawn in total.

(c) Stress : The final study design (sample size, gender, inclusion/exclusion criteria) will be partly contingent on results from Studies 1 and 2. However it is envisaged that subjects will attend the laboratory on 2 occasions (S1 Protocol and S2 Protocol)) in the early afternoon at least 2 weeks between visits. Subjects will be instructed not to have consumed food or drink (except water) or to have done strenuous exercise for at least one hour prior to their visit to the laboratory. S1 Protocol will proceed as follows : On arrival subjects will be fitted with an indwelling intravenous catheter as Study 2. A blood sample will be withdrawn immediately after which samples will be withdrawn at intervals of 30 minutes until commencement of the stress challenge. After a 30 minute recovery period subjects will complete (a) stress/anxiety questionnaire(s) then be encouraged to relax for one hour. Subjects will then participate in a controlled psychosocial stress challenge (modified version of Trier Social Stress Test). This test is a widely used standardized laboratory stress protocol which reliably invokes a strong cortisol stress response in humans. During the challenge subjects will be asked to present a short oral presentation to 2 researchers on a pre-determined subject. Subjects will be informed that their performance (both audio and visual) will be recorded by video camera. Subjects will be given a short time to prepare before presentation. They will also be asked to complete a mental arithmetic task. After the task subjects will be fully debriefed that they in fact were not recorded. The duration of the challenge is 65 minutes which includes 10 minutes relaxation time, 10 minutes preparation time, 10 minutes task performance and 35 minutes recovery period (debriefed). During the challenge blood will be sampled approximately every 10 minutes. After completion of the challenge subjects will remain in the laboratory for a further 2 hours and 2 further hourly blood samples will be taken. Subjects are expected to attend the laboratory for a period of not more than 5 hours. During S2 Protocol subjects will cannulated as above and blood sampling initiated immediately, then at 30 minute intervals for the next 5 hours. Subjects will complete the stress/anxiety questionnaire(s) as above but will not take part in the stress challenge. The order in which subjects undertake either S1 Protocol or S2 Protocol will be randomized thereby subjects will act as their own controls (pattern of cortisol release). This will also ensure collection of maximum data if subjects fail to return for their second session. In total no more than 150ml of blood will be withdrawn. Blood samples will be assayed for MCH, cortisol and possibly other metabolically active molecules.

5.4

Start Date: January 2007

Estimated duration of work: 3 years

5.5 If your work is a multi-centred study, please provide details of any other organisations involved N/A

Contact Name	**Contact Name**
Address	**Address**
Telephone Number	**Telephone Number**

Please provide a copy of any agreement between the organisations

Section 6

6.1 Describe any potential physical/emotional discomforts to participants in the investigation:

Where multiple blood samples are to be obtained an indwelling intravenous catheter will be placed in a forearm vein by a trained phlebotomist. The level of discomfort will be no more than that normally associated with giving a blood sample in a clinical setting i.e. a slight sting upon entry of the needle. It is possible some subjects may experience minor bruising around the point of entry. This method is less likely to cause tissue damage than the withdrawal of multiple samples. Where single blood samples are required the sample (no more than 15ml on each occasion) will be withdrawn from a forearm vein by a trained phlebotomist. The level of discomfort is expected to be transient as per cannulation above.

Participants may be required to undertake physical activity which may be somewhat physically challenging but not expected to cause discomfort to healthy individuals.

During the psychosocial stress challenge (Trier Social Stress Test) subjects may experience subjective feelings of stress and anxiety in varying degrees during the first part of the challenge (approximately 20 minutes).

6.2 Aside from 6.1 above, describe potential hazards which may be suffered by the participants? Please give details of any measures taken e.g. COSH, Risk Assessment etc. No additional potential hazards envisaged to participants. Risk assessment will take the form of a medical screening questionnaire (copy attached), any contra-indications would become evident at this point and the participant would not be allowed to participate.

6.3 Outline the degree to which these risks are balanced against potential benefits

N/A – no risks envisaged

6.4 What criteria will be employed for deciding the end point at which:

a) the investigation will stop because of unjustifiable further risk?
b) one method is declared the preferred option and the investigation terminates?

a) No risks envisaged to participants
b) There will be no comparison of methodologies therefore not relevant

6.5 What monitoring mechanisms will be in place to decide when participants should be withdrawn from the research?

Subjects will be advised that they are free to terminate participation in the research at any time without personal disadvantage. If any subject complains of feeling unwell during participation in the research they will be withdrawn from the study and medical advice sought, it is not anticipated that any ill effect would be the consequence of participation in the study.

6.6 What procedures and subsequent observations are to be made on participants for the purpose of detecting any complication arising from the investigation?

No complications are expected to arise from the taking of blood samples by a trained phlebotomist or by healthy subjects performing physical exercise of individually-prescribed intensity.

6.7 Do participants have any previous or existing professional relationship with the investigator?

Possibly, students, staff and researchers from the University of Westminster may volunteer to participate in the studies

If yes, please explain the circumstances:

Section 7 – Consent of Applicants

7.1 What type of consent will you seek?

☐ Written (including email)

☐ Verbal only

☐ Not applicable (please give justification below as to why consent is not applicable)

7.2 How and where will you make contact with the participant(s) in order to obtain consent?

Copies of “Informed Consent Form and Information Sheet” (pilot and main studies, copies attached) will be forwarded to participants at least one week prior to date of initial participation. Completed forms will be returned to researchers on the initial day of participation

7.3 Is there a subject information sheet?

☐ Yes (Please enclose a copy with this application) in addition to the above a separate “Subject Information Sheet” is attached for each study

☐ No

7.4 Is parental consent required?

☐ Yes

☐ No

Section 8 – Confidentiality of Information

8.1 Will the sharing of information be communicated to others working on the project?

☐ Yes (Please attach a copy of the Participant Confidentiality Code of Practice which will be used) Copy attached

☐ No

8.2 Will the work include:

☐ Named participants

☐ Participants whose names have been separately coded

☐ Unnamed participants

8.3 Where will locked files of investigation material be stored?

All data files will be coded and stored in a locked filing cabinet in a lockable room at the University of Westminster

8.4 If the investigation involves storage of computerised data which might enable the participant it be identified, please name the investigator in charge of Computer System Security for the investigation?

N/A – a handwritten list of coded names will be kept in the locked filing cabinet as above. Each subject will be allocated a code and all samples and data will be identified only by code. A photocopy of the handwritten list will be kept in a locked cabinet in the office of Dr Joanne Murray at the University of Westminster, therefore there will be no computerised record which may identify participants.

8.5 Does the investigation and any planned publication include the use of photographs or videos either of individuals or tissues?

Tissues Yes ☐ No ☐

Individuals Yes ☐ No ☐

If yes to either of these, please provide a copy of the consent form which participants will be asked to sign for this purpose.

Section 9 - Finance

9.1 Will expenses be paid to participants?

☐ Yes (If yes, how much?) Travel expenses within Greater London (public transport only)

☐ No

9.2 Will a reward over and above expenses be made to participants?

☐ Yes (If yes, please give more details)

☐ No

9.3 Is this study initiated/sponsored by a pharmaceutical or other industrial company?

☐ Yes (If yes, what is the name of the company?)

☐ No

9.4 Detail any financial or other direct interest to you or to your department arising from this study.

N/A

9.5 Will this project increase work/cost to any other Department or School?

☐ Yes (If yes, obtain and include the name and signature of the relevant Dean(s) of School(s) concerned:

Name	**Signature**

☐ No - The study costs will be covered by the applicants (Jane Naufahu) School of Bioscience Scholarship award, University of Westminster

Section 10 – Insurance

10.1 Are manufacturers providing insurance cover?

☐ Yes (If yes, please enclose a letter confirming insurance cover, including the names of all

covered)

☐ No

10.2 Are all of the investigators employees or students of the University of Westminster?

☐ Yes

☐ No

If no, please provide evidence of insurance cover, including:

- list of all people involved in the investigation
- details of the form this cover will take

10.3 Does the investigation involve the use of equipment or medicines?

☐ Yes

☐ No

If yes, please give details of *manufacturer's indemnity*:

Statement of Cover attached

10.4 Does the investigation involve the use of equipment or medicines which are manufactured on site but are not covered by insurance?

☐ Yes

☐ No

If yes, appropriate insurance cover must be arranged and written confirmation of such cover must be attached

Section 11 – Declaration – this Section must be completed by all applicants

Please Read and Sign

The information I have given on this form is true and to the best of my knowledge correct:

Signed:

Date:

Send the completed form to:

Carl Hornsey

Assistant Registrar (Student Information)

Academic Registrar's Department

University of Westminster

115 New Cavendish Street

London W1W 6UW

Appendix 2 Informed Consent Form Study 1

INFORMED CONSENT FORM AND INFORMATION SHEET

PILOT STUDY FOR The project entitled "Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH) "

Thank you for showing an interest in this pilot study. Please read all the information carefully. Think about whether or not you want to take part. I will contact you again to ask about your decision.

If you decide to take part you will be asked to sign this form.

You do not have to take part. If you decide that you do not want to participate, there will be no disadvantage to you.

Melanin-concentrating hormone (MCH) is a recently discovered molecule known to be active in the control of eating behaviours, energy expenditure and in reproductive function. Recently pharmaceutical companies have become interested in its potential as an anti-obesity treatment since manipulation of the availability of MCH has been shown to decrease food intake and increase energy expenditure. MCH also affects many aspects of female reproduction and hence fertility status. It is known that MCH circulates in the human blood system but little is known about what regulates its activities.

The main aim of the pilot study is to derive data which will inform the normal range of blood concentrations of the hormone, melanin-concentrating hormone (MCH) in healthy men and women in order to design further studies.

If you agree to take part you will be required to attend the human performance laboratory at the University of Westminster. You will arrive at an agreed time in the morning and will stay for a period of approximately 4 hours. Your stay will involve the following procedures:

- On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

- Four blood samples (2 from each arm) will be taken by a trained phlebotomist at intervals during the morning. No more than 50ml of blood will be withdrawn in total. (Note: this is a relatively small amount of blood compared to 470ml normally withdrawn during blood donation). You may also be required to give some finger-prick blood samples (one or two drops of blood per sample).

- You will be served one meal of mixed macronutrient composition after energy expenditure has been taken in the morning.

- Your total body fat and lean mass will be measured via "Bodpod". During this procedure you sit comfortably in a chamber for one minute.

- You will complete a stress/anxiety questionnaire to assess your stress levels.

Risks and discomfort

The risks to yourself are minimal. You will be required to give blood samples a number of times over the morning as indicated above. A trained phlebotomist will take all samples. You may experience a slight stinging sensation exactly like giving a regular blood sample in a clinical setting, after that you should feel no further discomfort. Some volunteers may also experience minor bruising around the point of entry. A momentary pricking sensation may be experienced when the finger-prick samples are taken.

Safety

You will complete a medical questionnaire prior to taking part to identify any health related reasons why you may not participate. You will be informed if you are deemed unsuitable to participate. Blood samples will be taken only by a trained phlebotomist.

Injury

In the unlikely event you are injured during the testing and have questions about your treatment you should contact Jane Naufahu, who will be present and who will liaise with the appropriate medical service.

Benefits

Your resting energy expenditure will be measured and your basal metabolic rate (BMR) will be calculated. BMR indicates how many calories your body requires per day for basic functioning of your vital organs. This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised.

Your stress hormone levels will be measured and you may be asked to complete a stress/anxiety questionnaire, results of these tests are useful for anyone interested in identifying personal stress levels. Note: results of these tests are not intended to identify/evaluate specific life stressors such as jobs or relationships.

You can change your mind and decide not to take part at any time. If you decide to stop, you do not have to give any reasons for your decision, and you will be placed at no disadvantage whatsoever.

What information will be collected, how it will be used

Your age, height, weight, fat mass and resting energy expenditure will be recorded. Blood concentrations of MCH, glucose, insulin, cortisol, reproductive hormones and possibly other biochemical markers and metabolically active molecules will established. All or any combination of these can be detected in a single blood sample, it is not necessary to take separate blood samples. The reason for taking multiple samples will be to look for changes in blood levels of these metabolically active molecules over time or in response to an intervention such as eating a meal.

The results will be stored in a locked container and accessed only by researchers involved in the study. All samples and data files will be coded and participants will not be identifiable.

The results of the final project may be published, but the information will not be linked to any specific person. A copy of your results (excluding blood parameters) will be given to you if you ask for them. Please note your blood will only be screened for hormones, biochemical markers and metabolically active molecules as above. It will not be screened for clinical disease or medical conditions.

You can ask questions about the pilot study at any time. Please contact Jane Naufahu at the testing session or telephone 0207 911 5000 ext 2830, e:mail jane@cavresearch.org

Statement by subject:

- I have volunteered to take part in this project
- I know I can stop taking part an any time without being disadvantaged
- I am satisfied that the results will be stored securely
- I know that the results of the main project may be published
- I am aware of any possible risks and discomfort
- I agree to inform the researcher immediately if I am in pain, or if I feel uncomfortable
- I have had the chance to ask questions
- I know that I will not receive any money for taking part

I have read this form and I understand it. I agree to take part in the pilot study for the project titled **"Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH)"**

Signed (Subject) : **Date :**

Signed (Witness) : **Date :**

Appendix 3 Subject Information Sheet Study 1

SUBJECT INFORMATION SHEET

Study 1

How do I prepare for the visit to the laboratory?

Location

All research will be conducted in the Human Performance Laboratory (4th floor), University of Westminster, 115 New Cavendish Street, London W1W 6UW

What to bring

- Signed Informed Consent Form
- Food diary
- Swimwear
- Something to occupy you during short rest periods e.g. reading material, ipod, laptop

General Preparation

You will be required to keep a food diary for three days prior to the visit. This will be emailed to you and you simply fill in what you eat and how much of it. You will also be required to complete an initial medical screening questionnaire.

In the laboratory

You will be expected to arrive in the laboratory in the morning having eaten or drunk nothing since the night before and will spend approximately 4 hours in the laboratory. On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

Afterwards a blood sample will be taken from a forearm vein by a trained phlebotomist, this is the same procedure as giving a regular blood sample in a clinical setting. You may feel a slight sting as the sample is taken. Some volunteers may experience minor bruising around the point of entry.

You will be served a breakfast of mixed macronutrient content in the morning after your resting energy expenditure has been measured. Although the meal will be of controlled content your personal preferences will be taken into account.

After breakfast 3 more blood samples will be taken over the next 2 hours. Therefore a total of 4 blood samples (2 from each arm) will be taken over the course of the morning. No more than 50ml of blood will be withdrawn in total, this is a comparatively small amount compared 470ml of blood which is normal given when donating blood and is easily tolerated by healthy people. In addition we will take some small finger-prick blood samples, this involves one or two drops of blood being withdrawn from a fingertip, again you may feel a slight sting as the sample is taken.

Your total body fat and lean mass will be also be measured by a procedure where you sit in a chamber for 2 – 3 minutes. During this procedure you should wear swimwear or underwear. This procedure measures the amount of air displaced by your body in the chamber and is quite comfortable.

You will also complete a stress/anxiety questionnaire to assess your stress levels.

Thank you for your co-operation, by agreeing to take part you will help to inform the normal range of blood concentrations of melanin-concentrating hormone (MCH) in healthy men and women. This is important since (to our knowledge) no such data exists and it is essential that researchers are equipped with such knowledge in order to design further studies. Currently very little is known about circulating MCH in humans though MCH is known to affect food intake, energy expenditure and reproductive function. The results of the pilot study will help researchers design experiments which will examine whether or not circulating concentrations of MCH mediate its multiple functions and whether MCH provides a link between nutritional status and fertility.

Also you will get an accurate assessment of your basal metabolic rate (minimum number of calories you need per day for basic functioning of your vital organs), This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised. You will also receive the results of your stress

questionnaire which is useful for anyone interested in identifying personal stress levels.

Thank you once again for your interest, if you would like to be involved please email jane@cavresearch.org to arrange a mutually convenient date for the testing.

Appendix 4 Medical Screening Questionnaire

MEDICAL SCREENING QUESTIONNAIRE

The information obtained in this form will be kept as **CONFIDENTIA**L. Only the researchers related to the above named project may have access to the information.

All sections are to be completed by the participant.

Participant's Surname (Miss/ Ms/ Mrs): _____

First name:_____

Date of Birth: _____

Address: _____

_____

_____Postcode:_____

Contact Tel. No:_____ (Home)

_____(Office)

Mobile No: _____

Medical History

Have you suffered any of the following conditions at any time?

(Please tick the appropriate column. If 'Yes' please give some details.)

	No	Yes	Details
Rheumatic or scarlet fever			
Heart trouble or murmur			
Heart Palpitations			
High Blood Pressure			
Heart Attack			
Chest pain/Angina			
Stroke			
Disease of arteries or veins			
Haemophilia			
Fainting or blackout			
Epilepsy			
Lung or bronchial disease			
Asthma			
Hay fever			
Anaemia			
Diabetes			
Thyroid Disease			
Any other medical conditions not mentioned above			

Having normal or regular periods			
Currently pregnant or breast feeding			

Current Medication

State the name and dosage of any drugs or medicines including ORAL CONTRACEPTIVES that you are taking regularly.

Drug/ Oral contraceptives	Dose	Time of last dose

Signed : _____

Name : _____

Date: _____

Appendix 5 Food Diary

3-DAY FOOD DIARY

Dear Participant,

The purpose of the food diary is to find your average daily calorie intake and the percentage of calories obtained from protein carbohydrate and fat.

Directions for Using the Food Diary

1. Do not specifically alter your diet during the period you keep this diary.
2. Keep your food diary current. List foods immediately after they are eaten.

   Please print all entries.
3. Record only one food item per line on the record sheet
4. Record amounts in household measures, e.g. **ounces, tablespoons, cups**, **slices** or **units**, as in one cup non-fat milk, two slices of wheat toast, or one raw apple
5. Include the method that was used to prepare the food item, e.g. **fresh, frozen, stewed, fried, baked, canned, broiled, raw,** or **braised,** also any oils or fats used in preparation.
6. For canned foods, include the liquid in which it was canned, e.g. **sliced peaches in heavy syrup, fruit cocktail in light syrup,** or **tuna in water.**
7. Remember to record the amounts of visible fats (oils, butter, salad dressings, margarine, etc) you eat or use in cooking.
8. Remember to record drinks and indicate whether diet or non-diet version.
9. It would be useful if you could also keep the labels or cut out the ingredients listed on any packaging to give in with the food diary.

Name:

Day 1: Date:

Time	Food Item and Method of Preparation	Amount Eaten
Breakfast		
Mid Morning		
Lunch		

Tea		
Evening Meal		
Evening Snack		

Name:

Day 2: Date:

Time	Food Item and Method of Preparation	Amount Eaten
Breakfast		
Mid Morning		
Lunch		

Tea		
Evening Meal		
Evening Snack		

Name:

Day 3: Date:

Time	Food Item and Method of Preparation	Amount Eaten
Breakfast		
Mid Morning		
Lunch		

Tea		
Evening Meal		
Evening Snack		

Appendix 6 Subject Information Sheet Study 2

SUBJECT INFORMATION SHEET

Study 2

How do I prepare for the visit to the laboratory?

Location

All research will be conducted in the Human Performance Laboratory (4th floor), University of Westminster, 115 New Cavendish Street, London W1W 6UW

What to bring

- Signed Informed Consent Form
- Food diary
- Something to occupy you during short rest periods e.g. reading material, ipod, laptop

General Preparation

You will be required to keep a food diary for three days prior to the visit. This will be emailed to you and you simply fill in what you eat and how much of it. You will also be required to complete an initial medical screening questionnaire. Anyone whose medical screening questionnaire results indicate that they should not be involved will not be allowed to participate in the main study.

In the laboratory

You will be expected to arrive in the laboratory in the morning having eaten or drunk nothing except water before you arrive that morning and will spend approximately 4 hours in the laboratory. On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

Afterwards a blood sample will be taken from a forearm vein by a trained phlebotomist, this is the same procedure as giving a regular blood sample in

a clinical setting. You may feel a slight sting as the sample is taken. Some volunteers may experience minor bruising around the point of entry.

You will be served a breakfast of mixed macronutrient content in the morning after your resting energy expenditure has been measured. Although the meal will be of controlled content your personal preferences will be taken into account.

After breakfast we will ask you for 3 more blood samples spaced out over 2 hours. Therefore a total of 4 blood samples (2 from each arm) will be taken over the course of the morning. No more than 50ml of blood will be withdrawn in total, this is a comparatively small amount compared to 470ml of blood which is normal given when donating blood and is easily tolerated by healthy people. In addition we will ask you for some small finger-prick blood samples, this involves one or two drops of blood being withdrawn from a fingertip, again you may feel a slight sting as the sample is taken.

Your total body fat and lean mass will be also be measured by a procedure where you sit in a chamber for 2 – 3 minutes. During this procedure you should wear swimwear or underwear. This procedure measures the amount of air displaced by your body in the chamber and is quite comfortable.

You will also complete some stress/anxiety questionnaires to assess your stress levels.

Thank you for your co-operation, by agreeing to take part you will help to inform the normal range of blood concentrations of melanin-concentrating hormone (MCH) in men and women. Currently very little is known about circulating MCH in humans though MCH is known to affect food intake, energy expenditure and reproductive function. The results of this study will help researchers determine whether or not circulating concentrations of MCH mediate its multiple functions and particularly the role of MCH in energy balance, nutritional status and blood glucose levels

Also you will get an accurate assessment of your basal metabolic rate (minimum number of calories you need per day for basic functioning of your

vital organs), This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised. You will also receive the results of your stress questionnaire which is useful for anyone interested in identifying personal stress levels.

Thank you once again for your interest, if you would like to be involved please email jane@cavresearch.org to arrange a mutually convenient date for the testing.

Appendix 7 Informed Conent Form Study 2

INFORMED CONSENT FORM AND INFORMATION SHEET

FOR The project entitled "Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH) "

Thank you for showing an interest in this study. Please read all the information carefully. Think about whether or not you want to take part. I will contact you again to ask about your decision.

If you decide to take part you will be asked to sign this form.

You do not have to take part. If you decide that you do not want to participate, there will be no disadvantage to you.

Melanin-concentrating hormone (MCH) is a recently discovered molecule known to be active in the control of eating behaviours, energy expenditure and in reproductive function. Recently pharmaceutical companies have become interested in its potential as an anti-obesity treatment since manipulation of the availability of MCH has been shown to decrease food intake and increase energy expenditure. MCH also affects many aspects of female reproduction and hence fertility status. It is known that MCH circulates in the human blood system but little is known about what regulates its activities.

The main aim of this study is to derive data which will inform the normal range of blood concentrations of the hormone, melanin-concentrating hormone (MCH) in men and women over the age of 40 particularly with regard to its correlation with blood glucose levels.

If you agree to take part you will be required to attend the human performance laboratory at the University of Westminster. You will arrive at an agreed time in the morning and will stay for a period of approximately 4 hours. Your stay will involve the following procedures:

- On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

- Four blood samples (2 from each arm) will be taken by a trained phlebotomist at intervals during the morning. No more than 50ml of blood will be withdrawn in total. (Note: this is a relatively small amount of blood compared to 470ml normally withdrawn during blood donation). You will also be required to give some finger-prick blood samples (one or two drops of blood per sample).

- You will be served one meal of mixed macronutrient composition after energy expenditure has been taken in the morning.

- Your total body fat and lean mass will be measured via "Bodpod". During this procedure you sit comfortably in a chamber for about two minutes.

- You will complete a stress/anxiety questionnaire to assess your stress levels.

Risks and discomfort

The risks to yourself are minimal. You will be required to give blood samples a number of times over the morning as indicated above. A trained phlebotomist will take all samples. You may experience a slight stinging sensation exactly like giving a regular blood sample in a clinical setting, after that you should feel no further discomfort. Some volunteers may also experience minor bruising around the point of entry. A momentary pricking sensation may be experienced when the finger-prick samples are taken.

Safety

You will complete a medical questionnaire prior to taking part to identify any health related reasons why you may not participate. You will be informed if you are deemed unsuitable to participate. Blood samples will be taken only by a trained phlebotomist.

Injury

In the unlikely event you are injured during the testing and have questions about your treatment you should contact Jane Naufahu, who will be present and who will liaise with the appropriate medical service.

Benefits

Your resting energy expenditure will be measured and your basal metabolic rate (BMR) will be calculated. BMR indicates how many calories your body requires per day for basic functioning of your vital organs. This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised.

Your stress hormone levels will be measured and you may be asked to complete a stress/anxiety questionnaire, results of these tests are useful for anyone interested in identifying personal stress levels. Note: results of these tests are not intended to identify/evaluate specific life stressors such as jobs or relationships.

You can change your mind and decide not to take part at any time. If you decide to stop, you do not have to give any reasons for your decision, and you will be placed at no disadvantage whatsoever.

What information will be collected, how it will be used

Your age, height, weight, fat mass and resting energy expenditure will be recorded. Blood concentrations of MCH, glucose, insulin, cortisol, reproductive hormones and possibly other biochemical markers and metabolically active molecules will be established. All or any combination of these can be detected in a single blood sample, it is not necessary to take separate blood samples. The reason for taking multiple samples will be to look for changes in blood levels of these metabolically active molecules over time or in response to an intervention such as eating a meal.

The results will be stored in a locked container and accessed only by researchers involved in the study. All samples and data files will be coded and participants will not be identifiable.

The results of the final project may be published, but the information will not be linked to any specific person. A copy of your results (excluding blood parameters) will be given to you. Please note your blood will only be screened for hormones, biochemical markers and metabolically active molecules as above. It will not be screened for clinical disease or medical conditions.

You can ask questions about the pilot study at any time. Please contact Jane Naufahu at the testing session or e:mail jane@cavresearch.org

Statement by subject:

- I have volunteered to take part in this project
- I know I can stop taking part an any time without being disadvantaged
- I am satisfied that the results will be stored securely

- I know that the results of the main project may be published
- I am aware of any possible risks and discomfort
- I agree to inform the researcher immediately if I am in pain, or if I feel uncomfortable
- I have had the chance to ask questions
- I know that I will not receive any money for taking part

I have read this form and I understand it. I agree to take part in this study for the project titled **"Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH)"**

Signed (Subject) : **Date :**

Signed (Witness) : **Date :**

Appendix 8 Subject Information Sheet Study 3

SUBJECT INFORMATION SHEET

Study 3

How do I prepare for the visits to the laboratory?

Location

All research will be conducted in the Human Performance Laboratory (4th floor), University of Westminster, 115 New Cavendish Street, London W1W 6UW

What to bring

- Signed Informed Consent Form
- Food diaries
- Swim suit or close fitting underwear

General Preparation

You will be required to take your temperature at the same time every morning and record it on a record sheet provided for a 1 – 3 weeks depending on where you are in your cycle.

You will also be required to keep two 3 day food diaries (each diary 2 weekdays and one weekend day). These will be provided and you simply fill in what you eat and how much of it. You will also be required to complete an initial medical screening questionnaire. Anyone whose medical screening questionnaire results indicate that they should not be involved will not be allowed to participate in the main study.

Four dates will be booked for you to come in to the laboratory at around 09.00 am each visit. The first visit will be during the early follicular phase, the second will be 2/3 days before the expected day of ovulation, the third will be 2/3 days after ovulation and the fourth and final visit will be during the late luteal phase of your monthly cycle. You should also carry on keeping your temperature diary during this month.

In the laboratory

You should arrive in the laboratory in the morning having eaten or drunk nothing except water before you arrive that morning and will spend approximately 1 hour in the laboratory. On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

Your total body fat and lean mass will be also be measured by a very accurate procedure where you sit in a chamber for 2 – 3 minutes. During this procedure you should wear swimwear or underwear. This procedure measures the amount of air displaced by your body in the chamber and is quite comfortable.

Afterwards a small blood sample will be taken from a forearm vein by a trained phlebotomist, this is the same procedure as giving a regular blood sample in a clinical setting. You may feel a slight sting as the sample is taken. Some volunteers may experience minor bruising around the point of entry. No more than 10ml of blood will be taken (about 2 tsps).

We will also provide a light breakfast after your measurements have been taken.

Also you will get an accurate assessment of your basal metabolic rate (minimum number of calories you need per day for basic functioning of your vital organs), This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised. Your body mass index and waist/hip ratio will also be calculated.

Thank you for your co-operation, by agreeing to take part you will help to inform the normal range of blood concentrations of melanin-concentrating hormone (MCH) in women at different stages of their menstrual cycle. Currently very little is known about circulating MCH in humans though MCH is known to affect food intake, energy expenditure and reproductive function.

The results of this study will help researchers determine whether or not there is any association between circulating MCH concentrations throughout the menstrual cycle, body fat mass and metabolic rate and hence whether MCH plays a role in the integration of energy balance with the reproductive axis.

All data obtained will be confidential.

Thank you once again for your interest, if you would like to be involved please email jane@cavresearch.org to arrange a mutually convenient date you to collect your thermometer, food diaries and other relevant information.

Appendix 9 Informed Consent Form Study 3

INFORMED CONSENT FORM AND INFORMATION SHEET

STUDY FOR The project entitled "Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH) "

Thank you for showing an interest in this study. Please read all the information carefully. Think about whether or not you want to take part. I will contact you again to ask about your decision.

If you decide to take part you will be asked to sign this form.

You do not have to take part. If you decide that you do not want to participate, there will be no disadvantage to you.

Melanin-concentrating hormone (MCH) is a recently discovered molecule known to be active in the control of eating behaviours, energy expenditure and in reproductive function. Recently pharmaceutical companies have become interested in its potential as an anti-obesity treatment since manipulation of the availability of MCH has been shown to decrease food intake and increase energy expenditure. MCH also affects many aspects of female reproduction and hence fertility status. It is known that MCH circulates in the human blood system but little is known about what regulates its activities.

The main aim of the study is to derive data which will inform the normal range of blood concentrations of the hormone, melanin-concentrating hormone (MCH) in healthy women throughout their menstrual cycle.

If you agree to take part you will be required to attend the human performance laboratory at the University of Westminster. You will arrive at an agreed time in the morning and will stay for a period of approximately 1 hour 4 times during your menstrual cycle. Your stay will involve the following procedures:

- On arrival your resting energy expenditure will be measured. This involves relaxing on a bed for about 40 minutes wearing a ventilated hood whilst measurements are taken, you will not feel any physical discomfort from this procedure.

- A blood sample will be taken by a trained phlebotomist. No more than 10ml (about 2tsp) of blood will be withdrawn each visit.

- Your total body fat and lean mass will be measured via "Bodpod". During this procedure you sit comfortably in a chamber for one minute.

Risks and discomfort

The risks to yourself are minimal. You will be required to give a blood sample on each visit as indicated above. A trained phlebotomist will take all samples. You may experience a slight stinging sensation exactly like giving a regular blood sample in a clinical setting, after that you should feel no further discomfort. Some volunteers may also experience minor bruising around the point of entry.

Safety

You will complete a medical questionnaire prior to taking part to identify any health related reasons why you may not participate. You will be informed if you are deemed unsuitable to participate. Blood samples will be taken only by a trained phlebotomist.

Injury

In the unlikely event you are injured during the testing and have questions about your treatment you should contact Jane Naufahu, who will be present and who will liaise with the appropriate medical service.

Benefits

Your resting energy expenditure will be measured and your basal metabolic rate (BMR) will be calculated. BMR indicates how many calories your body requires per day for basic functioning of your vital organs. This is useful if you are interested in maintaining a healthy body weight. Additionally your total body fat and lean mass will be accurately measured and categorised.

You can change your mind and decide not to take part at any time. If you decide to stop, you do not have to give any reasons for your decision, and you will be placed at no disadvantage whatsoever.

What information will be collected, how it will be used

Your age, height, weight, fat mass and resting energy expenditure will be recorded. Blood concentrations of MCH, insulin, cortisol, reproductive hormones and possibly other biochemical markers and metabolically active molecules will established. All or any combination of these can be detected in a single blood sample, it is not necessary to take separate blood samples.

The results will be stored in a locked container and accessed only by researchers involved in the study. All samples and data files will be coded and participants will not be identifiable.

The results of the final project may be published, but the information will not be linked to any specific person. A copy of your results (excluding blood parameters) will be given to you if you ask for them. Please note your blood will only be screened for hormones, biochemical markers and metabolically active molecules as above. It will not be screened for clinical disease or medical conditions.

You can ask questions about the study at any time. Please contact Jane Naufahu at the testing session or e:mail jane@cavresearch.org

Statement by subject:

- I have volunteered to take part in this project
- I know I can stop taking part an any time without being disadvantaged
- I am satisfied that the results will be stored securely
- I know that the results of the main project may be published
- I am aware of any possible risks and discomfort
- I agree to inform the researcher immediately if I am in pain, or if I feel uncomfortable
- I have had the chance to ask questions
- I know that I will not receive any money for taking part

I have read this form and I understand it. I agree to take part in the pilot study for the project titled **"Investigating the anti-obesogenic effects and potential links to fertility of the neuropeptide, melanin-concentrating hormone (MCH)"**

Signed (Subject) : **Date :**

Signed (Witness) : **Date :**