Melanin-concentrating hormone in peripheral circulation in the human

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Abstract

Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide with a well-characterised role in energy homeostasis and emergent roles in diverse physiologic functions such as arousal, mood and reproduction. Work to date has predominantly focused on its hypothalamic functions using animal models; however, little attention has been paid to its role in circulation in humans. The aims of this study were to (a) develop a radioimmunoassay for the detection of MCH in human plasma; (b) establish reference ranges for circulating MCH and (c) characterise the pattern of expression of circulating MCH in humans. A sensitive and specific RIA was developed and cross-validated by RP-HPLC and MS. The effective range was 19.5–1248 pg MCH/mL. Blood samples from 231 subjects were taken to establish a reference range of 19.5–55.4 pg/mL for fasting MCH concentrations. There were no significant differences between male and female fasting MCH concentrations; however, there were correlations between MCH concentrations and BMI in males and females with excess fat ($P<0.001$ and $P=0.020$) and between MCH concentrations and fat mass in females with excess fat ($P=0.038$). Plasma MCH concentrations rose significantly after feeding in a group of older individuals ($n=50$, males $P=0.006$, females $P=0.023$). There were no robust significant correlations between fasting or post-prandial MCH and resting metabolic rate, plasma glucose, insulin or leptin concentrations although there were correlations between circulating MCH and leptin concentrations in older individuals ($P=0.029$). These results indicate that the role of circulating MCH may not be reflective of its regulatory hypothalamic role.

Introduction

Melanin-concentrating hormone (MCH) is an orexigenic neuropeptide; rodent studies indicate it has multiple and diverse physiologic functions including a key role in the central control of energy metabolism. Intracerebroventricular (ICV) administration of MCH results in hyperphagia and increased adiposity (Qu \textit{et al.} 1996, Gomori \textit{et al.} 2002, Santollo & Eckel 2008), whilst decreased availability of hypothalamic MCH results in...
hyperphagia or hypophagia accompanied by reduced body weight and fat mass depending on whether a pharmacological or genetic model is used (Marsh et al. 2002, Segal-Lieberman et al. 2003, Mashiko et al. 2005). Ablation of functional MCH results in increased energy expenditure via increased metabolic rate, increased locomotor activity or both (Shimada et al. 1998, Segal-Lieberman et al. 2003). MCH is expressed in the central nervous system (CNS), primarily in the rostral zona incerta/ incerto-hypothalamic and the lateral hypothalamic areas (Bittencourt et al. 1992, Sita et al. 2007, Bittencourt 2011). Mch/MCH and MCH have also been reported in rodent and human peripheral tissue (Hervieu & Nahon 1995, Verlaet et al. 2002, Sandig et al. 2007). Circulating MCH has been detected in both rodents (Bradley et al. 2000, Stricker-Krongrad et al. 2001, Sun et al. 2004) and humans (Gavrilta et al. 2005, Schmidt et al. 2015); however, there has been published debate concerning the validity of the detection methods used in the earlier human study (Mantzoros 2005, Waters & Krause 2005). Both central and peripherally derived MCH are implicated in glucose homeostasis (Ludwig et al. 2001, Pereira-da-Silva et al. 2005, Bjursell et al. 2006), and there is evidence of local production of MCH in the endocrine pancreas in rodents and humans (Pissios et al. 2007). However, the physiological role of circulating MCH remains largely unexplored at present.

The overall aims of these studies were to determine whether circulating concentrations of MCH are related to body weight regulation and metabolism by developing and validating a competitive RIA for the detection of MCH in human plasma, and to establish reference ranges for circulating MCH in both the fasted and fed states were examined in association with circulating glucose, insulin and leptin concentrations. Additionally, associations between circulating MCH and resting metabolic rate (RMR) were investigated.

Materials and methods

**MCH RIA development and validation**

**Reverse phase-high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS)** RP-HPLC was conducted using a modified version of a previously described method (Maulon-Ferraille et al. 2002). The optimum dilution for detection of MCH in plasma was 1:9 plasma:0.1 M HCl (v:v). The mixture was centrifuged at 4°C and 10,000g for 10 min, and the supernatant was analysed by HPLC in a RP column (C18 Phenomenex, UK) with a gradient of 20–60% (0.1% trifluoroacetic acid in HPLC water: acetonitrile) for 60 min 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 the determination of analyte mass. MS was performed by single quadrupole mass spectrometric detector (Dionex MSQ Plus, Dionex Corp., Massachusetts, USA), and MS data were analysed using Chromeleon LC/ MS software (Dionex Corp.).

Blood from the same individual was collected in different vacutainers (lithium heparin, silica + gel, fluoride oxalate, EDTA and sodium citrate) to establish if there was any effect on the detection of MCH by RP-HPLC. To determine the lability of MCH in plasma, a separate blood sample was subjected to the following conditions: room temperature 1 h; 4°C 1 h; room temperature overnight; 4°C overnight; −20°C overnight; and −20°C before being thawed, refrozen and thawed again. The samples were processed as described previously and compared with a freshly prepared sample.

**RIA for MCH** A double antibody RIA for MCH was developed using commercially available reagents, that is, MCH antibody (M8440: Sigma-Aldrich), radiolabelled MCH (125I-MCH; NEX373010UC: PerkinElmer) and anti-rabbit SacCel (AA-SAC1: IDS Ltd., UK). Phosphate buffered saline with 1% bovine serum albumin (A3294: Sigma-Aldrich) was used throughout. Day 1: MCH antibody (1:30,000 in 100 µL) with normal rabbit serum (1:300) was added to diluted standards and unknowns and left at 4°C. Day 2: 125I-MCH (10,000 cpm/100 µL) diluted in buffer supplemented with EDTA (0.025 M) was added to each tube and left at 4°C. Day 3: SacCel (solid-phase anti-rabbit IgG-coated cellulose suspension: IDS Ltd.) was added following the manufacturer’s instructions, that is, 0.1 mL SacCel were added to each tube (except total counts), left for 30 min at room temperature and then 1 mL deionised water was added before all tubes were centrifuged at 180g and 4°C for 10 min. The supernatant was aspirated and the resultant pellet was counted for 1 min on a gamma counter. Data were analysed using AssayZap (Biosoft, Cambridge, UK).

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 treated as unknowns in the MCH assay. Biomolecules tested were human atrial natriuretic peptide (ANP; A1663: Sigma-Aldrich) (Hervieu et al. 1996); human α-MSH (H1075: Bachem, Switzerland) (Barber et al. 1987, Ludwig et al. 1998); human ACTH (H1160: Bachem) (Baker et al. 1985) and neuropeptide–E-I-MCH (NEI-MCH; H4714: Bachem) (Maulon-Ferraille et al. 2002).

Comparison between RP-HPLC and RIA  Plasma samples collected in EDTA tubes were diluted with either 0.1 N HCl or buffer with EDTA (1:9 dilution) with/without purified MCH and subjected to RP-HPLC. Fractions were collected at 1-min intervals, and the aliquots were analysed for MCH by RIA.

Circulating MCH  

Subjects  The experiments involving human subjects were approved by the University of Westminster’s Ethics Sub-Committee. Each subject gave full informed consent. Fasting blood samples were taken from all subjects between 08:00 and 11:30 h.

Cross-sectional study  Fasting venous blood samples were collected from 135 females and 96 males. Weight to the nearest 0.1 kg, height to the nearest 0.1 cm, waist and hip circumference were measured. Total fat and lean body mass were measured by air displacement plethysmography (BodPod: Body Composition Tracking System, version 4.1; Life Measurement Instruments, Concord, CA, USA). All venous blood samples were collected in EDTA vacutainers and plasma was recovered after centrifugation. Plasma was stored at −20°C until MCH concentrations were determined by RIA (intra- and inter-assay CVs were 2.4% and 3.7%, respectively).

Intervention studies  Two cohorts were recruited. Cohort A, 18–30 years: 21 females and 11 males. The inclusion criteria for females were pre-menopausal (however, the stage of the menstrual cycle was not recorded); non-hormonal contraceptive using and a body mass index (BMI) of ≤24.9. The inclusion criterion for males was BMI of ≤24.9. Cohort B, Over 40 years: (a) lean individuals (11 females and 11 males) and (b) those with excess body fat (13 females and 15 males). Lean (L) was defined as <31% body fat in females and <21% body fat in males. Excess body fat (E) was defined as ≥31% body fat in females and ≥21% body fat in males (ACSM 1996). The inclusion criterion for both males and females was that they should be over 40 years of age. In both cohorts, those on medication(s) for chronic illness or known to cause hypoglycaemia or hyperglycaemia or affect metabolic rate and females who were pregnant, lactating or recently lactating were excluded.

Protocol  Subjects arrived after an overnight fast, and anthropometric, body composition and resting metabolic rate (RMR) (Deltatrac II Metabolic Monitor, Datex Instrumentarium Corp., Helsinki, Finland) measurements were taken. Fasting venous and fingerprick blood samples were obtained before subjects were fed a controlled meal of mixed macronutrient content (388 k/cal females; 510 k/cal males). Eight fingerprick samples were obtained at 15-min intervals and 3 venous blood samples at 30, 60 and 120 min after meal. Plasma was recovered from the venous blood samples and stored at −20°C until assayed for MCH, leptin (HL-81HK, Millipore; intra-assay CV: 8.3% at 4.9 ng/mL; 3.4% at 25.6 ng/mL) and insulin (DSL-1600, Diagnostic Systems Inc.; intra-assay CV: 8.3% at 4.8 µIU/mL; 6.4% at 54.6 µIU/mL). All samples for each cohort were assayed for each hormone in a single assay. The fingerprick blood samples were immediately analysed for blood glucose concentrations using the HemoCue Glucose 201+ Analyser (HemoCue AB, Sweden; intra-assay CV <1.8%).

Data and statistical analyses  

RMR  The group was subdivided based on percentage of ‘standard BMR’ (Fleisch 1951). A BMR of ±10% standard is considered normal (McArdle et al. 2001). The groups were low (L) = ≤89.9% standard BMR; normal (N) = within 10% of standard BMR and high (H) = ≥110% standard BMR. RMR has been used synonymously with BMR as the only condition specific to BMR that was not met was that subjects did not sleep at the facility overnight.

Body composition  Subjects were subdivided into four groups based on the American College of Sports Medicine’s body fat percentage cut-off points: 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). The subdivision of subjects by body composition was done after data collection for the cross-sectional study and both intervention studies.

Inter-gender differences between anthropometric characteristics and circulating hormone concentrations...
were established by independent samples t-tests. A one-way between-group ANOVA with Tukey’s multiple comparison test was conducted to determine whether there was an effect of body composition on plasma MCH concentrations. Associations between fasting plasma MCH concentrations and body composition parameters were determined by Pearson product-moment correlational analysis. Differences in pre- and post-prandial circulating hormone concentrations were assessed by paired samples t-tests. Leptin concentrations were not normally distributed and so the data were transformed using the square root before the analyses. Comparisons between circulating hormone concentrations at the four sampling times were assessed by repeated-measures design ANOVA. When analysing the AUC data, only individuals with data from all four blood samples were included in the analyses and hence the lower ‘n’ values. Data were analysed using the Statistical Package for the Social Sciences (SPSS, version 16.0 for Windows) or Prism (Prism 5 for Mac OS X; GraphPad Software). Statistical significance was set at \( P < 0.05 \).

Results

RIA development and validation

The gold standard method for the detection of MCH is RP-HPLC and MS; hence, this method was used to demonstrate that MCH is present in plasma. Using RP-HPLC, the retention time for purified MCH was found to be between 21 and 28 min (Fig. 1A). It was predicted that product ions of \( m/z \) 796 and 2 of \( m/z \) 1194 would be generated specifically for MCH, and these were detected at the corresponding elution times when either purified MCH or human plasma samples were analysed by MS (Fig. 1B). MCH was only detected by RP-HPLC and MS in samples collected in the lithium heparin, silica + gel and EDTA vacutainers. No effect of storage under the conditions described could be detected when compared to freshly prepared samples measured by RP-HPLC and MS (data not shown). Purified MCH, plasma and buffer alone were each separately fractionated by RP-HPLC, and eluates were collected at one-minute intervals. Immunoreactive MCH, as determined by RIA, was detected in eluates collected between 19 and 28 min for purified MCH and eluates collected between 18 and 24 min for plasma (Fig. 1C).

Figure 1

(A) Chromatogram of a human plasma sample. The retention time for the elution of purified MCH or MCH in plasma ranged from 21 to 28 min depending on the conditions. (B) Product ion mass spectrum obtained from a human plasma sample. The arrows indicate peaks at \( m/z \) 796 and 1194. (C) MCH immunoreactivity of samples fractionated by RP-HPLC. Solid bars = purified MCH; open bars = human plasma; grey bars = buffer. The dotted line represents the level of detection of the RIA.
An effective range of 19.5–1248 pg MCH/mL was established, and 19.5 pg/mL was taken as the level of detection. Serial dilutions of ANP, α-MSH and ACTH failed to displace the MCH antibody demonstrating the specificity of the radioimmunoassay. Only supraphysiological concentrations of NEI-MCH showed any potential for cross-reactivity with the MCH antibody (at concentrations 100× greater than MCH: data not shown). Standard curves diluted in buffer or plasma (with unknown initial concentrations of MCH: data not shown) were parallel.

### Table 1 Demographic, anthropometric and hormonal variables of cross-sectional study participants.

<table>
<thead>
<tr>
<th></th>
<th><strong>Female</strong> (n=135)</th>
<th><strong>Male</strong> (n=96)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean ± s.d.</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>36.9 ± 13.6</td>
<td>18.0–76.0</td>
</tr>
<tr>
<td><strong>Height (m)</strong></td>
<td>1.66 ± 0.07</td>
<td>1.42–1.86</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>61.3 ± 9.8</td>
<td>42.1–98.5</td>
</tr>
<tr>
<td><strong>Fat mass (%)</strong></td>
<td>27.7 ± 8.9***</td>
<td>10.5–54.8</td>
</tr>
<tr>
<td><strong>Lean mass (%)</strong></td>
<td>72.2 ± 8.9</td>
<td>45.2–89.5</td>
</tr>
<tr>
<td><strong>Fat mass (kg)</strong></td>
<td>17.6 ± 8.3</td>
<td>6.1–51.2</td>
</tr>
<tr>
<td><strong>Lean mass (kg)</strong></td>
<td>43.7 ± 5.1</td>
<td>28.4–55.6</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>22.3 ± 3.9</td>
<td>16.5–39.7</td>
</tr>
<tr>
<td><strong>Fasting MCH (pg/mL)</strong></td>
<td>37.3 ± 9.2</td>
<td>19.5–70.4</td>
</tr>
</tbody>
</table>

***Significantly greater than the other gender (P<0.001).
BMI, body mass index.

Circulating MCH in humans

**Cross-sectional study** Demographic and anthropometric measurements of 231 subjects are presented in Table 1. Fasting plasma MCH concentrations were detected in the range 19.5–70.4 pg/mL with the exception of one subject who had fasting MCH concentrations in excess of 150 pg/mL (this outlier was not included in Table 1 or in the statistical analyses). Within this assay, 95% of the sample population would be expected to have fasting MCH concentrations between 19.5 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 BMI, there were no significant differences in plasma MCH concentrations between the groups (Fig. 2A) except between males with a BMI <20 compared with those with a BMI >30 (p < 0.0473, ANOVA with Tukey’s multiple comparison test). Fasting plasma MCH concentrations were not significantly correlated with percent fat mass, percent lean mass, height, weight or age. There were, however, significant correlations between fasting plasma MCH concentrations and: (a) both body fat mass weight (kg) and BMI in females with excess fat (≥21% body fat) (n = 41, r = −0.326, P = 0.038; n = 39, r = −0.372, P = 0.020, respectively); (b) male BMI (n = 96, r = 0.230, P = 0.030) and (c) BMI in males with excess fat (≥21% body fat) (n = 44, r = 0.513, P < 0.001: Fig. 2B). Note that in females, the correlations were inverse, whereas in males, correlations were positive.

**Intervention studies** Demographic, anthropometric and fasting MCH measurements are presented in Tables 2 and 3 for Cohorts A and B, respectively.

**Table 2** Demographic, anthropometric, hormonal and RMR characteristics of study participants in Cohort A (aged ≤30 years).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Male (n = 9)</th>
<th>Female (n = 17)</th>
<th>Male (n = 2)</th>
<th>Female (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± s.d.</td>
<td>25.5 ± 3.4a</td>
<td>27.2 ± 2.9a</td>
<td>26.7 ± 4.6a</td>
<td>28.8 ± 1.9a</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.79 ± 0.09a</td>
<td>1.67 ± 0.05b</td>
<td>1.72 ± 0.04b</td>
<td>1.65 ± 0.09b</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.7 ± 10.1a</td>
<td>60.3 ± 6.3b</td>
<td>75.4 ± 3.5b</td>
<td>65.9 ± 4.8b</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>13.1 ± 3.8a</td>
<td>25.0 ± 4.5b</td>
<td>26.6 ± 4.2b</td>
<td>33.7 ± 2.8c</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>86.9 ± 3.9a</td>
<td>74.9 ± 4.5b</td>
<td>73.3 ± 4.2b</td>
<td>66.3 ± 2.8c</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 1.7a</td>
<td>21.7 ± 2.3a</td>
<td>25.5 ± 0.7a</td>
<td>23.9 ± 1.1a</td>
</tr>
<tr>
<td>Fasting plasma MCH (pg/mL)</td>
<td>44.7 ± 11.0a</td>
<td>44.7 ± 10.8a</td>
<td>58.2 ± 5.5a</td>
<td>42.1 ± 12.7a</td>
</tr>
<tr>
<td>RMR (kcal/day)</td>
<td>1678 ± 219a</td>
<td>1338 ± 148b</td>
<td>1602 ± 112a</td>
<td>1251 ± 83b</td>
</tr>
</tbody>
</table>

Mean ± s.d., within a category values with different superscripts are significantly different from each other P < 0.05. Participants were categorized as lean or with excess fat based on their % body fat (male lean <21%; male excess fat ≥21%; female lean <31%; female excess fat ≥31%). BMI, body mass index; RMR, resting metabolic rate.

There were differences in the post-prandial plasma MCH concentrations between Cohort A (n = 32) and Cohort B (n = 50). In Cohort A, there were no differences in plasma MCH concentrations at any of the four time points (P = 0.772; Fig. 3). In Cohort B, plasma MCH concentrations increased after eating (females P = 0.023, males P = 0.006; Fig. 4).

There were no differences in circulating concentrations of glucose or insulin between males and females and no effect of % body fat in either Cohort A or Cohort B. In Cohort A, although there were no correlations with mean plasma MCH concentrations or the MCH area under the curve (AUC) and the glucose AUC, both the mean plasma MCH concentrations and the MCH AUC were correlated with the insulin AUC in all individuals (that is, both females and males) with excess fat only (that is, ≥31% body fat in females and ≥21% in males) (n = 6, r = 0.907, P = 0.013 and n = 6, r = 0.932, P = 0.007, respectively).

In Cohort B, there were no significant associations between mean plasma MCH concentrations or the MCH AUC and the glucose and insulin AUCs.

Mean circulating leptin concentrations were greater in individuals with excess fat compared to those in lean

**Table 3** Demographic, anthropometric, hormonal and RMR characteristics of study participants in Cohort B (aged ≥40 years).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Male (n = 11)</th>
<th>Female (n = 11)</th>
<th>Male (n = 15)</th>
<th>Female (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± s.d.</td>
<td>50.6 ± 6.1a</td>
<td>48.9 ± 8.0a</td>
<td>51.9 ± 7.6a</td>
<td>52.4 ± 5.9a</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.81 ± 0.04a</td>
<td>1.64 ± 0.06b</td>
<td>1.78 ± 0.05a</td>
<td>1.66 ± 0.09b</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.1 ± 6.0a</td>
<td>59.5 ± 8.2b</td>
<td>89.6 ± 11.6c</td>
<td>76.7 ± 11.4a</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>13.4 ± 4.1a</td>
<td>25.8 ± 3.6b</td>
<td>26.9 ± 5.2b</td>
<td>38.9 ± 5.4c</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>86.6 ± 4.1a</td>
<td>74.2 ± 3.6b</td>
<td>73.0 ± 5.2b</td>
<td>61.1 ± 5.4c</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 1.9a</td>
<td>22.1 ± 2.4a</td>
<td>28.0 ± 3.6b</td>
<td>28.0 ± 4.6b</td>
</tr>
<tr>
<td>Fasting plasma MCH (pg/mL)</td>
<td>36.5 ± 6.4a</td>
<td>33.7 ± 6.3a</td>
<td>34.3 ± 6.8a</td>
<td>33.3 ± 6.0b</td>
</tr>
<tr>
<td>RMR (kcal/day)</td>
<td>1691 ± 162a</td>
<td>1216 ± 140b</td>
<td>1680 ± 157a</td>
<td>1332 ± 152b</td>
</tr>
</tbody>
</table>

Mean ± s.d., within a category values with different superscripts are significantly different from each other P < 0.05. Participants were categorized as lean or with excess fat based on their % body fat (male lean <21%; male excess fat ≥21%; female lean <31%; female excess fat ≥31%). BMI, body mass index; RMR, resting metabolic rate.
individuals (Cohort A, \( P = 0.025 \); Cohort B, \( P > 0.001 \)) and women have higher concentrations than males (Cohort A, \( P > 0.001 \); Cohort B, \( P > 0.001 \); Figs 3 and 4). In both cohorts in females, circulating leptin concentrations had decreased 1 h post-prandial (Cohort A: \( P = 0.012 \); Cohort B: \( P = 0.037 \)). In males, circulating leptin concentrations did not decrease significantly until 2 h post-prandial in Cohort B only (\( P = 0.028 \)). In Cohort A, there were no significant correlations between plasma MCH and leptin at any time point or the MCH and leptin AUCs. In Cohort B, there were three significant correlations between plasma MCH and leptin concentrations. There were negative correlations between plasma MCH concentrations and leptin concentrations in fasted males with excess fat (that is, \( \geq 21\% \) body fat) (n = 9, \( r = -0.672, P = 0.047 \)) and at 30 min post-prandial in females with excess fat (that is, \( \geq 31\% \) body fat) (n = 8, \( r = -0.757, P = 0.030 \)). By contrast, there was a positive correlation between mean plasma MCH concentrations and mean plasma leptin concentrations in lean males (that is, \( < 21\% \) body fat) (n = 11, \( r = 0.654, P = 0.029 \)). The MCH AUC was not significantly correlated with the leptin AUC.

In both cohorts, there were no significant correlations between fasted or mean post-prandial plasma MCH

**Figure 3**
Mean (\( \pm \text{s.e.m. or s.d.} \)) circulating concentrations of MCH (closed circle), leptin (closed square), glucose (open circle) and insulin (open square) in lean and with excess fat females (n = 12–17 and n = 4, respectively) and males (n = 7–9 and n = 2, respectively). All participants were 30 years or younger. The first sample was taken while subjects were in fasted state (f). After feeding of a controlled meal of mixed macronutrient content (388kcal females; 510kcal males), blood samples were removed at regular intervals for 120 min. ACSM (1996) body fat % cut-off points were used to classify subjects as lean or excess fat (refer to Body composition section of the ‘Material and methods’ section for further details). \( a \) mean leptin concentrations were greater in excess fat compared to lean subjects, \( P = 0.025 \); \( b \) mean leptin concentrations are greater in females than those in males, \( P > 0.001 \); \( c \) post-prandial decrease in leptin concentrations at 60 min in females, \( P = 0.012 \).

**Figure 4**
Mean (\( \pm \text{s.e.m.} \)) circulating concentrations of MCH (closed circle), leptin (closed square), glucose (open circle) and insulin (open square) in lean and with excess fat females (n = 4–11 and n = 4–13, respectively) and males (n = 5–11 and n = 5–15, respectively). All participants were aged 40 years or older. The first sample was taken while subjects were fasted (f). After feeding of a controlled meal of mixed macronutrient content (388kcal females; 510kcal males), blood samples were removed at regular intervals for 120 min. ACSM (1996) body fat % cut-off points were used to classify subjects as lean or excess fat (refer to Body composition section of the ‘Material and methods’ for further details). \( a \) post-prandial increase in MCH concentrations in females, \( P = 0.023 \); \( b \) post-prandial increase in MCH concentrations in males, \( P = 0.006 \); \( c \) mean leptin concentrations were greater in excess fat compared to those in lean subjects, \( P > 0.001 \); \( d \) mean leptin concentrations are greater in females than those in males, \( P > 0.001 \); \( e \) post-prandial decrease in leptin concentrations at 60 min in females, \( P = 0.037 \); \( f \) post-prandial decrease in leptin concentrations at 120 min in males, \( P = 0.028 \).
concentrations and RMR (for values, Tables 2 and 3) in either males or females or when categorised by adiposity.

Discussion

A sensitive and specific RIA for the quantifiable measurement of MCH in human plasma has been successfully developed. To confirm that MCH is detectable and measurable in human plasma, cross-validation was performed by RP-HPLC and MS. A peak was detected between 21 and 28 min when plasma was run through the HPLC column, which corresponds 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. Immunoreactive MCH was detected by RIA only in the eluates collected between 18 and 28 min of either purified MCH or human plasma. In the RIA, the only molecule assessed showing evidence of cross-reactivity was NEI-MCH, though only at supraphysiological concentrations. 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 confirmed that other plasma components have no adverse effects on the curve. Plasma MCH retained stability under various conditions including freeze-thaw cycles and being left at room temperature overnight. MCH was only detected in plasma collected in lithium heparin or EDTA vacutainers or in serum tubes containing a clotting agent. Other anti-coagulants interfered with detection. These results indicate that collection methods for plasma MCH should be standardised, though variability in storage conditions is not detrimental.

Using the RIA described herein, repeatable measures of the relative concentrations of MCH in circulation have been obtained. The range of values obtained do vary significantly, however, from the two other studies published to date (Gavrila et al. 2005, Schmidt et al. 2015). The two other research groups used different assays from the same commercial supplier, and it is not known if the assays utilise the same antibody. Neither group appeared to validate the assay they have used within their own laboratories, and there is little information supplied by the company to suggest that the assays have been validated by the company itself. Although it is not uncommon, as Schmidt and coworkers noted in their discussion on the differences in measurements for MCH in their studies and Gavrila and coworkers’, for there to be a wide range of baseline values reported depending on the method of assaying (for example, B-type natriuretic peptide (as reviewed by Fischer et al. 2001) and oxytocin (as reviewed by Leng & Ludwig 2016)), the paucity of validation data available for the two commercial assays does preclude direct comparisons being made between their findings and those described herein.

Fasting blood samples from 135 females and 96 males were obtained to establish a reference range. Subjects were recruited from a range of ethnicities, ages and phenotypes. The mean fasting plasma MCH concentration was $36.7 \pm 9.3$ pg/mL, and 95% of the population would be expected to have plasma MCH concentrations between 19.4 and 55.4 pg/mL. In rodents, increased availability of hypothalamic MCH is associated with adiposity (Ludwig et al. 2001, Gomori et al. 2002), whereas decreased availability is associated with leanness (Marsh et al. 2002, Kowalski et al. 2004); therefore, it was hypothesised that circulating MCH concentrations would also be aligned to fat mass in humans. Although there were no associations between circulating MCH concentrations and percent fat mass, percent lean mass, age, height or weight, there were significant correlations between circulating MCH concentrations and BMI in males and females with excess fat (that is, $\geq 31$% body fat in females and $\geq 21$% in males). There was also a correlation between circulating MCH concentrations and body fat weight (kg) in females with excess fat. It is worth noting that the correlations between BMI and MCH were inverse for women and positive for men. Hence, it appears there may be some gender- and age-related regulation, which differs in the presence of adiposity. In young males, MCH may be indexed to leanness rather than adiposity as there was a positive correlation between BMI and lean body mass (kg) in the younger cohort. 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. 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: whether or not fat interferes with MCH signalling either directly or indirectly via leptin resistance or some other perturbation of the system is not currently known.

There were no differences between male and female fasting circulating MCH concentrations. In this respect, our results agree with those of Gavrila and coworkers (Gavrila et al. 2005). Age-related changes in body composition did not appear to influence circulating MCH.
concentrations as there were no differences in circulating MCH concentrations between groups when all subjects in the cross-sectional study were categorised by gender, age (≤30 years, 31–39 years and ≥40 years) and % body fat (male lean <21%; male excess fat ≥21%; female lean <31%; female excess fat ≥31%). Nor was there a significant correlation between age and absolute circulating MCH concentrations. However, in the intervention studies, an effect of age was observed, both in fasting MCH concentrations, which were greater in the younger group compared to the older group and in the post-prandial response (compare Figs 3 and 4).

In the older group, circulating MCH concentrations rose significantly during the 2 h post-prandial sampling period in males and females, and in both lean individuals and those with excess fat. In the younger group, post-prandial circulating MCH concentrations did not change significantly. In both groups, post-ingestive circulating leptin concentrations declined significantly. Whether or not this was related to the meal, MCH concentrations or the morning nadir of leptin requires qualification (Sinha et al. 1996). In the lipostatic model of energy homeostasis (for review see Woods 2005), leptin inhibits the anabolic pathway through which MCH operates. Leptin and MCH may also interact in the periphery; for example, the MCH receptor has been detected on rodent adipocytes (Bradley et al. 2000). Although there is ample evidence that MCH and leptin can both inhibit and stimulate each other (Huang et al. 1999, Bradley et al. 2000, Kokkotou et al. 2001), few studies have attempted to evaluate the association between circulating MCH and leptin. Except for in 3 small sub-groups (within Cohort B), there were no consistent significant associations between circulating MCH and leptin concentrations; in this respect, our results broadly concur with Gavrila and coworkers (Gavrila et al. 2005) who found no associations with serum leptin concentrations in a younger population (17 ± 1.7 years). Leptin action is altered with ageing and is characterised by increased adiposity and the development of leptin resistance: it is not known which precedes which (Carrascosa et al. 2009). In the older individuals, it may be that the differential direction of the plasma MCH/plasma leptin relationship between those with excess fat and lean phenotypes (negative vs 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. Overall, there was a trend for circulating MCH and leptin concentrations to be inversely correlated which, although non-significant, was consistent. This would be expected if the inhibitory effect of hypothalamic leptin on hypothalamic MCH is reflected in the periphery.

MCH has been shown to stimulate insulin release from beta cells in vitro, and it has been suggested that MCH may be necessary for normal β-cell function (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. Although there was a gender difference in the magnitude of the AUC insulin, which did not appear to be related to adiposity, our results indicate that there were no robust associations between the MCH AUC or mean circulating MCH concentrations and the glucose or insulin AUCs. However, in the younger cohort, there was a significant positive relationship between the respective AUCs for insulin and MCH and with the insulin AUC and mean circulating MCH concentrations but only in individuals with excess fat. Although in this group it would seem that the MCH and insulin response to food stimuli moves in the same direction, the small sample size of these subgroups precludes broader application, and a larger scale enquiry should be undertaken.

At the outset of the intervention study with Cohort B, it was the intention to compare an older cohort with excess fat 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 >30 kg/m² and waist-to-hip ratio of >1.0 for men and >0.8 for women, had fasting and 2 h post-prandial blood glucose concentrations within the normal range (<6.1 mmol/L fasting, <7.8 mmol/L 2 h 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 (data not shown). There appears to be no reference values for HOMA scores, which represent insulin resistance; however, scores in excess of 2.00 and 3.99 have been taken as definitive in other studies (Bakari & Onyemelukwe 2005, Wahrenburg et al. 2005). Plasma insulin concentrations were not significantly different between those with excess fat and lean individuals at any time point, and 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. The effect of insulin resistance on circulating MCH concentrations therefore requires further investigation.

Contrary to rodent studies, in humans there is little evidence to support a role for circulating MCH in energy homeostasis; therefore, it was deemed important to describe associations between RMR and circulating MCH concentrations in young healthy and older individuals. We found no evidence of a relationship between fasted or fed plasma MCH concentrations and RMR in either group. 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, as well as RMR per se do not contribute significantly to the variance in fasted or post-prandial MCH concentrations. These results suggest that circulating MCH cannot be considered a biomarker of resting energy expenditure in humans.

In conclusion, we have demonstrated that circulating MCH can be reliably and quantifiably measured in humans by RIA. Overall, circulating MCH concentrations are not overtly reactive, and no robust physiological effects of circulating MCH were observed. There does, however, appear to be some differential regulation in the presence of a combination of gender and adiposity, which is variable depending on the population under examination. Hence, in the subjects studied here, circulating MCH is not a marker of energy homeostasis, contrary to the suggestion of Gavrila and coworkers (Gavrila et al. 2005). Rather our results suggest that circulating MCH may not have a signalling role in this context although a detailed 24h profile of circulating MCH should be established, which would lend contextual relevance to the limited body of knowledge regarding circulating MCH in humans to date.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
J N was in receipt of a University of Westminster, School of Life Sciences PhD scholarship.

Author contribution statement
J N, F A, M F B, B D, A C and J F M planned the research; J N, F A, M F B and B D recruited subjects and collected samples; J N, F A, M F B, B D and T V did the laboratory analyses; J N did the statistical analyses; and J N, A C and J F M wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements
The authors would like to thank Carole Seaward, Laboratory Technician for her help with data collection, particularly her venepuncture skills.

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