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1 TITLE: Melanin concentrating hormone in peripheral circulation in the human

2

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33 SHORT TITLE: MCH in peripheral circulation in the human

34 KEY WORDS: circulating melanin concentrating hormone, radioimmunoassay,

35 human, metabolism, leptin

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37

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) characterize 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.

Word Count: 248

63

64 **INTRODUCTION**

65 Melanin concentrating hormone (MCH), is an orexigenic neuropeptide; rodent
66 studies indicate it has multiple and diverse physiologic functions including a key role
67 in the central control of energy metabolism. Intracerebroventricular (ICV)
68 administration of MCH results in hyperphagia and increased adiposity (Qu *et al.,*
69 1996; Gomori *et al.,* 2002; Santollo and Eckel, 2008), whilst decreased availability of
70 hypothalamic MCH results in hyper- or hypophagia accompanied by reduced body
71 weight and fat mass depending on whether a pharmacological or genetic model is
72 used (Marsh *et al.,* 2002; Segal-Lieberman *et al.,* 2003; Mashiko *et al.,* 2005).
73 Ablation of functional MCH results in increased energy expenditure via increased
74 metabolic rate, increased locomotor activity, or both (Shimada *et al.,* 1998; Segal-
75 Lieberman *et al.,* 2003). MCH is expressed in the central nervous system (CNS),
76 primarily in the rostral zona incerta/incerto-hypothalamic and the lateral hypothalamic
77 areas (Bittencourt *et al.,* 1992; Sita *et al.,* 2007; Bittencourt, 2011). Prepro-MCH
78 mRNA and MCH have also been reported in rodent and human peripheral tissue
79 (Hervieu and Nahon, 1995; Verlaet *et al.,* 2002; Sandig *et al.,* 2007). Circulating
80 MCH has been detected in both rodents (Bradley *et al.,* 2000; Stricker-Krongrad *et*
81 *al.,* 2001; Sun *et al.,* 2004) and humans (Gavrila *et al.,* 2005; Schmidt *et al*., 2015)
82 however there has been published debate concerning the validity of the detection
83 methods used in the earlier human study (Mantzoros, 2005; Waters and Krause
84 2005). Both central and peripherally-derived MCH are implicated in glucose
85 homeostasis (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005; Bjursell *et al.,* 2006)
86 and there is evidence of local production of MCH in the endocrine pancreas in

87 rodents and humans (Pissios *et al.,* 2007). However the physiological role of
88 circulating MCH remains largely unexplored at present.
89
90 The overall aim of these studies was to determine whether circulating concentrations
91 of MCH are related to body weight regulation and metabolism by developing and
92 validating a competitive RIA for the detection of MCH in human plasma, and
93 conducting a cross-sectional study in order to establish reference ranges for
94 circulating MCH. Two intervention studies were also conducted to investigate
95 whether circulating MCH concentrations were acutely responsive to food stimuli,
96 furthermore plasma MCH concentrations in both the fasted and fed states were
97 examined in association with circulating glucose, insulin and leptin concentrations.
98 Additionally, associations between circulating MCH and resting metabolic rate (RMR)
99 were investigated.
100

101 MATERIALS AND METHODS

102 MCH RIA development and validation

103 *Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Mass*
104 *Spectrometry (MS)*

105 RP-HPLC was conducted using a modified version of a previously described method
106 (Maulon-Ferraille *et al.,* 2002). The optimum dilution for detection of MCH in plasma
107 was 1:9 plasma: 0.1 N HCl (v:v). The mixture was centrifuged at 4°C and 1000 rpm
108 for 10 minutes and the supernatant analyzed by HPLC in a RP column (C18
109 Phenomenex, UK) with a gradient of 20–60% (0.1% trifluoroacetic acid in HPLC
110 water: acetonitrile) for 60 minutes at a flow rate of 0.5 ml/min. Purified MCH was
111 serially diluted and treated similarly for comparison. MCH was detected using UV

112 absorbance at 230 nm. Protein fragments obtained by RP-HPLC were subject to MS
113 for determination of analyte mass. MS was performed by single quadropole mass
114 spectrometric detector (Dionex MSQ Plus, Dionex Corp., Massachusetts, USA) and
115 MS data analysed using Chromeleon LC/MS software (Dionex Corp.,
116 Massachusetts, USA).
117
118 Blood from the same individual was collected in different vacutainers (lithium
119 heparin, silica+gel, fluoride oxalate, EDTA and sodium citrate) to establish if there
120 was any effect on the detection of MCH by RP-HPLC. To determine the lability of
121 MCH in plasma, a separate blood sample was subjected to the following conditions*:*
122 room temperature 1 hour; 4°C 1 hour; room temperature overnight; 4°C overnight; -
123 20°C overnight; and -20°C before being thawed, refrozen and thawed again. The
124 samples were processed as described above and compared with a freshly prepared
125 sample.
126
127 *RIA for MCH*
128 A double antibody RIA for MCH was developed using commercially available
129 reagents; that is, MCH antibody (M8440: Sigma-Aldrich, UK), radiolabelled MCH
130 (^{125}I-MCH; NEX373010UC: PerkinElmer Inc., USA) and anti-rabbit SacCel (AA-
131 SAC1: IDS Ltd., UK). Phosphate buffered saline with 1% bovine serum albumin
132 (A3294: Sigma-Aldrich, UK) was used throughout. Day 1: MCH antibody (1:30,000
133 in 100 μl) with normal rabbit serum (1:300) was added to diluted standards and
134 unknowns and left at 4°C. Day 2: ^{125}I-MCH (10,000 cpm/100 μl) diluted in buffer
135 supplemented with EDTA (0.025 M) was added to each tube and left at 4°C. Day 3:
136 SacCel (solid phase anti-rabbit IgG coated cellulose suspension: IDS Ltd., Boldon,

137 UK) was added following the manufacturer's instructions; that is, 0.1 ml SacCel were
138 added to each tube (except total counts), left for 30 minutes at room temperature
139 and then 1 ml deionized water was added before all tubes were centrifuged at 1000
140 rpm and 4°C for 10 min. The supernatant was aspirated and the resultant pellet was
141 counted for 1 minute on a gamma counter. Data were analysed using AssayZap
142 (Biosoft, Cambridge, UK).
143
144 To determine possible cross-reactivity, a series of dilution curves (range 0.1 pg–0.1
145 mg) of biomolecules reported to have a competitive or agonistic relationship with
146 MCH were treated as unknowns in the MCH assay. Biomolecules tested were:
147 human atrial natriuretic peptide (ANP; A1663: Sigma-Aldrich, UK) (Hervieu *et al.,*
148 1996); human α-MSH (H1075: Bachem, Switzerland) (Barber *et al.,* 1987; Ludwig *et*
149 *al.,* 1998); human ACTH (H1160: Bachem, Switzerland) (Baker *et al.,* 1985); and
150 neuropeptide–E-I-MCH (NEI-MCH; H4714: Bachem, Switzerland) (Maulon-Ferraille
151 *et al.,* 2002).
152
153 *Comparison between RP-HPLC and RIA*
154 Plasma samples collected in EDTA tubes were diluted with either 0.1 N HCl or buffer
155 with EDTA (1:9 dilution) with/without purified MCH and subjected to RP-HPLC.
156 Fractions were collected at 1 minute intervals and the aliquots analysed for MCH by
157 RIA.
158
159 <u>Circulating MCH</u>
160 *Subjects*
161 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 8 and 11.30 am.

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 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 hypo- or hyperglycaemia or affect metabolic rate and females who were pregnant, lactating or recently lactating were excluded.

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

201

202 *Data and Statistical Analyses*

203 RMR: The group was sub-divided based on percentage of "Standard BMR" (Fleisch,
204 1951). A BMR of ±10% Standard is considered normal (McArdle *et al.,* 2001). The
205 groups were Low (L)= ≤89.9% Standard BMR; Normal (N)=within 10% of Standard
206 BMR; High (H)= ≥110% Standard BMR. RMR has been used synonymously with
207 BMR since the only condition specific to BMR that was not met was that subjects did
208 not sleep at the facility overnight.

209

210 Body composition: subjects were sub-divided into four groups based on the
211 American College of Sports Medicine's body fat percentage cut-off points: male lean

212 (ML)=body fat % <21%; male excess fat (ME)=body fat % ≥21%; female lean
213 (FL)=body fat % <31%; female excess fat (FE)=body fat % ≥31% (ACSM, 1996).
214 Subjects were sub-divided by body composition after data collection for the cross-
215 sectional and both intervention studies.

216

217 Inter-gender differences between anthropometric characteristics and circulating
218 hormone concentrations were established by independent samples t-tests. A one-
219 way between groups ANOVA with Tukey's Multiple Comparison test was conducted
220 to determine whether there was an effect of body composition on plasma MCH
221 concentrations. Associations between fasting plasma MCH concentrations and
222 body composition parameters were determined by Pearson product-moment
223 correlational analysis. Differences in pre- and post-prandial circulating hormone
224 concentrations were assessed by paired samples t-tests. Leptin concentrations were
225 not normally distributed and so the data were transformed using the square root
226 before the analyses. Comparisons between circulating hormone concentrations at
227 the four sampling time-points were assessed by repeated measures design ANOVA.
228 When analyzing the AUC data, only individuals with data from all four blood samples
229 were included in the analyses and hence the lower 'n' values. Data were analysed
230 using the Statistical Package for the Social Sciences (SPSS version 16.0 for
231 Windows; Chicago, IL, US) or Prism (Prism 5 for Mac OS X; GraphPad Software,
232 Inc.). Statistical significance was set at $p<0.05$.

233

234 **RESULTS**

235 <u>RIA development and validation</u>

236 The gold standard method for the detection of MCH is RP-HPLC and MS hence this
237 method was used to demonstrate that MCH is present in plasma. Using RP-HPLC,
238 the retention time for purified MCH was between 21 and 28 minutes (Figure 1a). It
239 was predicted that product ions of m/z 796 and 2 of m/z 1194 would be generated
240 specifically for MCH and these were detected at the corresponding elution times
241 when either purified MCH or human plasma samples were analysed by MS (Figure
242 1b). MCH was only detected by RP-HPLC and MS in samples collected in the
243 lithium heparin, silica+gel and EDTA vacutainers. No effect of storage under the
244 conditions described could be detected when compared to freshly prepared samples
245 measured by RP-HPLC and MS (data not shown). Purified MCH, plasma and buffer
246 alone were each separately fractionated by RP-HPLC and eluates collected at one
247 minute intervals. Immunoreactive MCH, as determined by RIA, was detected in
248 eluates collected between 19 and 28 minutes for purified MCH and eluates collected
249 between 18 and 24 minutes for plasma (Figure 1c).

250

251 An effective range of 19.5–1248 pg MCH/ml was established and 19.5 pg/ml was
252 taken as the level of detection. Serial dilutions of ANP, α-MSH and ACTH failed to
253 displace the MCH antibody demonstrating the specificity of the radioimmunoassay.
254 Only supraphysiological concentrations of NEI-MCH showed any potential for cross-
255 reactivity with the MCH antibody (at concentrations 100x greater than MCH: data not
256 shown). Standard curves diluted in buffer or plasma (with unknown initial
257 concentrations of MCH: data not shown) were parallel.

258

259 <u>Circulating MCH in humans</u>

260 Cross-sectional study: Demographic and anthropometric measurements of 231

261 subjects are presented in Table 1. Fasting plasma MCH concentrations were
262 detected in the range 19.5–70.4 pg/ml with the exception of one subject who had
263 fasting MCH concentrations in excess of 150 pg/ml (this outlier was not included in
264 Table 1 or in the statistical analyses). Within this assay 95% of the sample
265 population would be expected to have fasting MCH concentrations between 19.5 and
266 55.4 pg/ml. There were no significant differences in mean fasting plasma MCH
267 concentrations between males and females. When the sample population was
268 grouped by gender and BMI, there were no significant differences in plasma MCH
269 concentrations between the groups (Figure 2a) except between males with a BMI
270 <20 compared with those with a BMI >30 ($p<0.0473$, ANOVA with Tukey's Multiple
271 Comparison test). Fasting plasma MCH concentrations were not significantly
272 correlated with percent fat mass, percent lean mass, height, weight or age. There
273 were however significant correlations between fasting plasma MCH concentrations
274 and: a) both body fat mass weight (kg) and BMI in females with excess fat (≥31%
275 body fat) ($n=41$, $r=-0.326$, $p=0.038$; $n=39$, $r=-0.372$, $p=0.020$, respectively); b) male
276 BMI ($n=96$, $r=0.230$, $p=0.030$); and c) BMI in males with excess fat **(**≥21% body fat)
277 ($n=44$, $r=0.513$, $p<0.001$: Figure 2b). Note that in females the correlations were
278 inverse whilst in males correlations were positive.

279

280 Intervention studies: Demographic, anthropometric and fasting MCH measurements
281 are presented in Tables 2 and 3 for Cohorts A and B, respectively. There were
282 differences in the post-prandial plasma MCH concentrations between Cohort A
283 ($n=32$) and Cohort B ($n=50$). In Cohort A, there were no differences in plasma MCH
284 concentrations at any of the four time-points ($p=0.772$; Figure 3). In Cohort B,
285 plasma MCH concentrations increased after eating (females $p=0.023$, males

286 p=0.006; Figure 4).

287

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

298

299 Mean circulating leptin concentrations were greater in individuals with excess fat
300 compared to lean individuals (Cohort A, p=0.025; Cohort B, p>0.001) and women
301 have higher concentrations than males (Cohort A, p>0.001; Cohort B, p>0.001):
302 Figures 3 and 4). In both cohorts in females, circulating leptin concentrations had
303 decreased 1 hour post-prandial (Cohort A: p=0.012; Cohort B: p=0.037). In males,
304 circulating leptin concentrations did not decrease significantly until 2 hours post-
305 prandial in Cohort B only (p=0.028). In Cohort A, there were no significant
306 correlations between plasma MCH and leptin at any time-point or the MCH and leptin
307 AUCs. In Cohort B there were three significant correlations between plasma MCH
308 and leptin concentrations. There were negative correlations between plasma MCH
309 concentrations and leptin concentrations in fasted males with excess fat (that is,
310 ≥21% body fat) (n=9, r=-0.672, p=0.047) and at 30 minutes post-prandial in females

311 with excess fat (that is, ≥31% body fat) (n=8, r=-0.757, p=0.030). By contrast, there
312 was a positive correlation between mean plasma MCH concentrations and mean
313 plasma leptin concentrations in lean males (that is, <21% body fat) (n=11, r=0.654,
314 p=0.029). The MCH AUC was not significantly correlated with the leptin AUC.

315

316 In both cohorts, there were no significant correlations between fasted or mean post-
317 prandial plasma MCH concentrations and RMR (for values, Tables 2 and 3) in either
318 males or females or when categorized by adiposity.

319

320 **DISCUSSION**

321 A sensitive and specific RIA for the quantifiable measurement of MCH in human
322 plasma has been successfully developed. To confirm that MCH is detectable and
323 measurable in human plasma cross-validation was performed by RP-HPLC and MS.
324 A peak was detected between 21 and 28 minutes when plasma was run through the
325 HPLC column which corresponds to the elution time of purified MCH. Additionally,
326 when human plasma was subject to MS, product ions of identical mass to those
327 generated by purified MCH were observed. Immunoreactive MCH was detected by
328 RIA only in the eluates collected between 18 and 28 minutes of either purified MCH
329 or human plasma. In the RIA, the only molecule assessed showing evidence of
330 cross-reactivity was NEI-MCH, though only at supra-physiological concentrations.
331 Currently there is little evidence to suggest that NEI-MCH circulates therefore at
332 physiological concentrations this assay is specific for MCH. Furthermore, parallelism
333 of the dilution curves of plasma to the standard curve confirmed that other plasma
334 components have no adverse effects on the curve. Plasma MCH retained stability
335 under various conditions including freeze-thaw cycles and being left at room

336 temperature overnight. MCH was only detected in plasma collected in lithium
337 heparin or EDTA vacutainers or in serum tubes containing a clotting agent. Other
338 anti-coagulants interfered with detection. These results indicate that collection
339 methods for plasma MCH should be standardized, though variability in storage
340 conditions is not detrimental.
341

342 Using the RIA described herein, repeatable measures of the relative concentrations
343 of MCH in circulation have been obtained. The range of values obtained do vary
344 significantly however from the two other studies published to date (Gavrila *et al*.,
345 2005; Schmidt *et al*., 2015). The two other research groups used two different
346 assays from the same commercial supplier and it is not known if the assays utilize
347 the same antibody. Neither group appeared to validate the assay they have used
348 within their own laboratories and there is little information supplied by the company
349 to suggest that the assays have been validated by the company itself. Whilst it is
350 not uncommon, as Schmidt and colleagues noted in their discussion on the
351 differences in measurements for MCH in their studies and Gavrila and colleagues',
352 for there to be a wide range of baseline values reported depending on the method of
353 assaying (for example, B-type natriuretic peptide [as reviewed by Fischer *et al*.,
354 2001] and oxytocin [as reviewed by Leng and Ludwig, 2016], the paucity of validation
355 data available for the two commercial assays does preclude direct comparisons
356 being made between their findings and those described herein.
357

358 Fasting blood samples from 135 females and 96 males were obtained to establish a
359 reference range. Subjects were recruited from a range of ethnicities, ages and
360 phenotypes. The mean fasting plasma MCH concentration was 36.7±9.3 pg/ml and

361 95% of the population would be expected to have plasma MCH concentrations
362 between 19.4-55.4 pg/ml. In rodents, increased availability of hypothalamic MCH is
363 associated with adiposity (Ludwig *et al.,* 2001; Gomori *et al.,* 2002) whilst decreased
364 availability is associated with leanness (Marsh *et al.,* 2002; Kowalski *et al.,* 2004),
365 therefore it was hypothesized that circulating MCH concentrations would also be
366 aligned to fat mass in humans. Whilst there were no associations between percent
367 fat mass, percent lean mass, age, height or weight: there were significant
368 correlations between circulating MCH concentrations and BMI in males and females
369 with excess fat (that is, ≥31% body fat in females and ≥21% in males). There was
370 also a correlation between circulating MCH concentrations and body fat weight (kg)
371 in females with excess fat. These results are curious since the correlations between
372 BMI and MCH were inverse for women and positive for men. Hence it appears there
373 may be some gender and age related regulation which differs in the presence of
374 adiposity. In young males MCH may be indexed to leanness rather than adiposity
375 since there was a positive correlation between BMI and lean body mass (kg) in the
376 younger cohort. The two major peripheral adiposity signals; leptin and insulin are
377 processed differently in males and females, female brains being more sensitive to
378 leptin and male brains being more sensitive to insulin. Leptin correlates better with
379 total body fat in females and insulin correlates better with total body fat in males
380 (Clegg *et al.,* 2003; Woods *et al.,* 2003). It could be that MCH also displays a
381 sexually dimorphic sensitivity: whether or not fat interferes with MCH signalling either
382 directly or indirectly via leptin resistance or some other perturbation of the system is
383 not currently known.
384

385 There were no differences between male and female fasting circulating MCH
386 concentrations. In this respect, our results agree with those of Gavrila and
387 colleagues (Gavrila *et al.,* 2005). Age-related changes in body composition did not
388 appear to impact on circulating MCH concentrations since there were no differences
389 in circulating MCH concentrations between groups when all subjects in the cross-
390 sectional study were categorized by gender, age (≤30 years, 31-39 years and ≥40
391 years) and % body fat (male lean=<21 %; male excess fat=≥ 21%; female lean=<31
392 %; female excess fat=≥31%). Nor was there a significant correlation between age
393 and absolute circulating MCH concentrations. However in the intervention studies
394 an effect of age was observed, both in fasting MCH concentrations which were
395 greater in the younger group compared to the older group and in the post-prandial
396 response (compare Figures 3 and 4).
397
398 In the older group circulating MCH concentrations rose significantly during the 2 hour
399 post-prandial sampling period in males and females, and in both lean individuals and
400 those with excess fat. In the younger group, post-prandial circulating MCH
401 concentrations did not change significantly. In both groups, post-ingestive circulating
402 leptin concentrations declined significantly. Whether or not this was related to the
403 meal, MCH concentrations or the morning nadir of leptin requires qualification (Sinha
404 *et al.,* 1996). In the lipostatic model of energy homeostasis (for review see Woods,
405 2005), leptin inhibits the anabolic pathway through which MCH operates. Leptin and
406 MCH may also interact in the periphery; for example, the MCH receptor has been
407 detected on rodent adipocytes (Bradley *et al.,* 2000). Whilst there is ample evidence
408 that MCH and leptin can both inhibit and stimulate each other (Huang *et al.,* 1999;
409 Bradley *et al.,* 2000; Kokkotou *et al.,* 2001)), few studies have attempted to evaluate

410 the association between circulating MCH and leptin. Except for in 3 small sub-groups
411 (within Cohort B) there were no consistent significant associations between
412 circulating MCH and leptin concentrations: in this respect our results broadly concur
413 with Gavrila and colleagues (2005) who found no associations with serum leptin
414 concentrations in a younger population (17±1.7 yrs). Leptin action is altered with
415 aging and is characterized by increased adiposity and the development of leptin
416 resistance: it is not known which precedes which (Carrascosa *et al.,* 2009). In the
417 older individuals, it may be that the differential direction of the plasma MCH/plasma
418 leptin relationship between those with excess fat and lean phenotypes (negative
419 *versus* positive) is symptomatic of disruption between MCH and leptin signaling, in
420 this context it is possible that MCH may be responding to some leptin resistant state.
421 Overall there was a trend for circulating MCH and leptin concentrations to be
422 inversely correlated which, whilst non-significant, was consistent. This would be
423 expected if the inhibitory effect of hypothalamic leptin on hypothalamic MCH is
424 reflected in the periphery.
425
426 MCH has been shown to stimulate insulin release from beta cells *in vitro* and it has
427 been suggested that MCH may be necessary for normal β-cell function (Pissios *et*
428 *al.,* 2007). Whether or not MCH acts in a paracrine or autocrine manner within the
429 pancreas or is released into the circulation is not known. If MCH is active at the level
430 of the endocrine pancreas it was hypothesised that the post-prandial insulin
431 response might be related to the post-prandial MCH response and could be altered
432 in the presence of insulin resistance. Whilst there was a gender difference in the
433 magnitude of the AUC insulin, which did not appear to be related to adiposity, our
434 results indicate that there were no robust associations between the MCH AUC or

435 mean circulating MCH concentrations and the glucose or insulin AUCs. However in
436 the younger cohort, there was a significant positive relationship between the
437 respective AUCs for insulin and MCH, and with the insulin AUC and mean circulating
438 MCH concentrations but only in individuals with excess fat. Whilst in this group it
439 would seem that the MCH and insulin response to food stimuli moves in the same
440 direction, the small sample size of these sub-groups precludes broader application
441 and a larger scale enquiry should be undertaken.

442

443 At the outset of the intervention study with Cohort B, it was the intention to compare
444 an older cohort with excess fat with an older leaner cohort reasoning that the more
445 corpulent group would be more likely to have some degree of insulin resistance.
446 However even those displaying morphological characteristics which would incline
447 them towards insulin resistance; that is a BMI of > 30 kg/m^2 and waist-hip ratio of >
448 1.0 for men and > 0.8 for women, had fasting and 2 hour post-prandial blood glucose
449 concentrations within the normal range (< 6.1 mmol/l fasting, < 7.8 mmol/l 2 hrs post-
450 prandial). Furthermore the individual Homeostatic Model Assessment (HOMA)
451 scores (Matthews *et al.,* 1985), which is a mathematical model method for detecting
452 insulin resistance, only exceeded 2.0 in 2 individuals (data not shown). There
453 appears to be no reference values for HOMA scores which represent insulin
454 resistance, however scores in excess of 2.00 and 3.99 have been taken as definitive
455 in other studies (Bakari and Onyemelukwe, 2005; Wahreneburg *et al.,* 2005).
456 Plasma insulin concentrations were not significantly different between those with
457 excess fat and lean individuals at any time point and the AUCs for insulin and
458 glucose were not different between the excess fat and lean groups of either gender.
459 Therefore it would seem that glucose homeostasis was still normal in both the lean

460 group and the excess fat group, hence the hypothesis that an altered MCH response
461 may have been observed in the presence of insulin resistance could not be further
462 explored in the current study. The effect of insulin resistance on circulating MCH
463 concentrations therefore requires further investigation.
464
465 Contrary to rodent studies, in humans there is little evidence to support a role for
466 circulating MCH in energy homeostasis, therefore it was deemed important to
467 describe associations between RMR and circulating MCH concentrations in young
468 healthy and older individuals. We found no evidence of a relationship between
469 fasted or fed plasma MCH concentrations and RMR in either group. To further
470 explore the relationship between metabolic rate and circulating MCH regression
471 analyses were performed. Results indicate that factors associated with variance in
472 RMR, that is percent fat-free mass, percent fat-mass, fat-free mass (kg) and gender,
473 as well as RMR *per se* do not contribute significantly to the variance in fasted or
474 post-prandial MCH concentrations. These results suggest that circulating MCH
475 cannot be considered a biomarker of resting energy expenditure in humans.
476
477 In conclusion, we have demonstrated that circulating MCH can be reliably and
478 quantifiably measured in humans by RIA. Overall circulating MCH concentrations
479 are not overtly reactive and no robust physiological effects of circulating MCH were
480 observed. There does however appear to be some differential regulation in the
481 presence of a combination of gender and adiposity which is variable depending on
482 the population under examination. Hence, in the subjects studied here circulating
483 MCH is not a marker of energy homeostasis, contrary to the suggestion of Gavrila
484 and colleagues (2005). Rather our results suggest that circulating MCH may not

485 have a signalling role in this context although a detailed 24 hour profile of circulating
486 MCH should be established which would lend contextual relevance to the limited
487 body of knowledge regarding circulating MCH in humans to date.
488
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