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Increased Peptide YY blood concentrations, not decreased acyl-ghrelin, are associated with reduced hunger and food intake in healthy older women: preliminary evidence.

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Abstract (245 words)

With ageing there is frequently a loss of appetite, termed anorexia of ageing, which can result in under-nutrition. We do not know how appetite control alters with ageing. The objective of this study was to investigate whether differences in the release of, and response to, gastrointestinal appetite hormones is altered in young compared to old healthy volunteers. We hypothesised that an increase in PYY and GLP-1 or a decrease ghrelin may result in a decreased appetite. A comparative experimental design, using a cross-sectional sample of ages from a healthy population, matched for sex and BMI was used. The study compared total ghrelin, acyl-ghrelin, PYY, GLP-1 and subjective appetite responses to ingestion of a standardised 2781kj (660kcal) test meal. 31 female volunteers aged between 21-92yrs took part. Multiple linear regression showed that both age and sex had an independent effect on energy intake. Subjective appetite scores showed that hunger, pleasantness to eat, and prospective food intake were significantly lower in the older age groups. PYY incremental area under the curve (IAUC) was greater in the oldest old compared to younger ages $f(3,27)=2.9$, $p=0.05$. No differences in GLP-1, ghrelin or acyl-ghrelin were observed in the older compared to younger age groups. Our data suggest that there may be increases in postprandial PYY(3-36) levels in female octogenarians, potentially resulting in reduced appetite. There does not appear to be any change in ghrelin or acyl-ghrelin concentrations with ageing.

Key words

Ageing, appetite, ghrelin, PYY, anorexia.

Introduction

The ageing population is increasing and with ageing there is frequently a loss of appetite, occurring even in the absence of disease, termed 'anorexia of ageing', which can result in under-nutrition (Morley & Silver 1988). Under-nutrition results in a decline in functional status, impaired muscle function, poor wound healing, higher hospital admission rates, and increased mortality, and contributes to increasing healthcare costs (Norman et al. 2008). Current consensus suggests that under-nutrition is an under diagnosed problem and this is reflected in national and international guidance about nutritional screening (Kondrup et al. 2003). In the UK, prevalence is estimated to be three million, based on prevalence data from the community, nursing homes, residential homes, and hospitals (BAPEN Malnutrition Advisory Group 2009). Centres for Disease Control and Prevention in USA estimate that up to 3000 people die each year from malnutrition related causes (Lee & Berthelot 2010). In 2006, the rate of malnutrition mortality for all adults was 0.8 per 100,000 people, but for 75-84 years it was 5.2, and for over 85 years it was 20.9 (Lee & Berthelot 2010).

Under-nutrition is normally treated using nutritional supplements, which are effective but not universally (Milne et al. 2009). Studies have also looked at additional food and eating assistance, but these also have mixed outcomes (Wade & Flett 2012). Poor outcome of nutritional interventions appear to be mediated by appetite; if appetite is suppressed it will limit the consumption of enough food and/or supplements.

Evidence exists to suggest a dysregulation of appetite control with ageing; older adults have been shown to have an inability up-regulate appetite after periods of under-nutrition compared to younger. This was related to differences in the sensations of hunger and fullness and resulted in a failure to regain lost weight (Moriguti et al. 2000).

Appetite is controlled by hormonal and neural factors communicating between the gut and brain (Hameed S, 2009). Briefly, there are several hypothesised satiety hormones including cholecystokinin, peptide tyrosine tyrosine (PYY_{3-36}) and glucagon-like-peptide-1 (GLP-1), and there is only one gastrointestinal hormone which increases food intake, ghrelin. Our team has demonstrated that the appetite loss in elderly people with fractured neck of femur is associated with dysregulation of PYY and ghrelin (Nematy et al. 2006b). However, changes seen during illness may be different to those related only to ageing. Therefore, there is a need to investigate whether there are changes in appetite control that occur in healthy older adults, before overt under-nutrition or illness is evident, that may precede these conditions and place the older person at greater risk of developing these conditions. In this study we carefully recruited individuals with no diagnosed health conditions.

Our study hypothesis was that appetite would be suppressed leading to lower energy intakes in healthy older people, compared with young, and this would be related to either a decrease in ghrelin or an increase in PYY and GLP-1.

Methods

The Riverside Research Ethics Committee granted ethical approval for the study (REC No 08/H0706/128). All volunteers gave written informed consent. The study was carried out between 2009 and 2011, at John McMichael Clinical Research Facility, Imperial College Healthcare NHS Trust.

Our aim was to investigate the effects of ageing alone on appetite regulation therefore we recruited healthy subjects from the local community (via posters, adverts in local organisational newsletters, and through the local General Practitioner research network), ensuring equal numbers in each age range: 20-39; 40-59; 60-79, 80+ years, and matched

groups for sex and BMI (within ±3kg/m²). We recognise that healthy subjects over 60 years are not necessarily representative of their population due to the lack of ill health, but excluding illness was necessary to test whether ageing per se is associated with changes in appetite control. The inclusion criteria were no diagnosed acute disease and no chronic disease or medication known to interfere with gut peptide secretion or appetite (this excludes virtually all chronic diseases; stable hypertension controlled by medication was the most common condition allowed). The main exclusion criteria were: history of alcoholism or substance abuse; raised blood pressure (>90/140mmHg); pregnant or breastfeeding women; recent treatment with an investigational drug; recent blood donation; current smokers; scoring 15 or more in restraint section of the Three Factors Eating Questionnaire.

The primary outcome was total ghrelin concentration. Secondary outcomes included acyl-ghrelin, PYY and GLP-1 concentration, insulin and glucose levels, appetite visual analogue scores, food intake at the *ad libitum* meal, and gastric emptying.

Volunteers were invited to a screening visit where their body mass index (BMI: kg/m^2), and body fat percentage (multi-frequency segmental bioelectrical impedance analysis; Tanita Body Composition Analyser, Amsterdam, Netherlands) were calculated. They were screened for blood abnormalities (full blood count, urea and electrolytes and liver function tests) and asked about any current medications and illnesses. Participants then completed the restraint section of the three factors eating questionnaire (Stunkard & Messick 1985) to exclude people with high eating restraint scores that may affect food intake at the test meal. Finally, subjects chose which *ad libitum* meal they would prefer. These meals were manufactured composite meals chosen for their similar macronutrient profiles (see

Table 1) and homogenous consistency.

Table 1: Nutritional Composition of ad libitum meals

Values/100g	Bolognese bake	Chicken Tikka Masala	Macaroni Cheese
Energy KJ (kcal)	665 (160)	665 (160)	631 (151)
Protein (g)	8.5	7.3	7.0
Carbohydrate (g)	15.6	16.6	14.0
of which sugars (g)	1.5	3.6	1.0
Fat (g)	6.8	6.9	7.4
of which saturates (g)	3.5	2.4	4.8

Mono-unsaturates (g)	2.7	3.0	2.3
Poly-unsaturates (g)	0.3	1.1	0.3

Subjects then attended for two ½ day visits, arriving at 08.30 fasted (no food and only water from 9pm the night before) and having avoided alcohol and excessive exercise prior to the visit. The first visit was a sham visit, mimicking the process for the true test meal except that minimal blood was taken at each sampling point. Gut hormones are influenced by stress and therefore a sham visit was necessary to accustom volunteers to the environment and protocol. (The process for the study is shown in Figure 1S, supplementary data). The second visit followed this process and the required blood sample volumes were taken.

The standard meal was consumed immediately after the first fasting blood sample and consisted of 600ml nutritionally complete supplement drink (Ensure plus, Abbott, USA). This is routinely used in our laboratory as it provides a fixed known amount of macro-nutrients and energy that can be consumed quickly, ensuring a standardised stimulus for gut hormone release. Subjects consumed all of the drink over a 15 minutes period (2781kj/660kcal, 27.5g protein, 89g CHO, 21.6g fat). Directly after the test meal the subjects took 1.5g of soluble paracetamol to measure gastric emptying. Blood samples and visual analogue scales were then completed at regular intervals for 3 hours. After 180 minutes the participants were offered a pre-weighed excess of the chosen meal and asked to eat until comfortably full. The remaining food was weighed to calculate the energy and nutrient intake at the meal. Between one and three volunteers were studied at any given time and no contact was allowed between participants.

All plasma peptide hormones and insulin were measured in duplicate in a single assay. Total ghrelin (Patterson et al. 2005), total PYY (Adrian et al. 1985b, 1987), GLP-1(1-36) (Kreymann et al. 1987), and insulin by radioimmunoassay; glucose using hexokinase and G-6-PDH with

Abbott ci8200 analysers (Abbott Diagnostics, Maidenhead, UK); acyl-ghrelin using sandwich ELISA kit (Millipore, USA) specific for human acyl-ghrelin; and paracetamol using Enzymatic/Colorimetric method with Abbott ci800 analysers (Abbott Diagnostics, Maidenhead, UK). The time to peak concentration for paracetamol was used as a measurement of gastric emptying (Heading et al. 1973). An index of fasting insulin resistance was measured (HOMA) (Matthews et al. 1985).

Appetite visual analogue scales (VAS) include five scales to assess hunger, nausea, pleasantness to eat, prospective desire to eat, and fullness (Stubbs et al. 2000). Each scale is a 100mm line which is marked by the participant and measured to the mark, providing a score out of 100 assuming 1mm equals a score of one.

Gut hormone concentration and VAS scores are measured over time and thus the area under the curve (AUC) and the incremental area under the curve (IAUC) were used to quantify these measures over the whole test meal period. The IAUC includes the area between the plasma level curve and the fasting concentration (T=0), with any area beneath fasting being ignored. It is calculated using the sum of the areas of the triangles and rectangles making up the area, calculated geometrically by applying the trapezoid rule (Wolever & Jenkins 1986). The IAUC is the preferred method to determine changes over time since it takes into account the baseline value, however, it could mask the overall effect of ageing. For example, hunger levels may not decrease significantly more in ageing in response to a test meal, but overall hunger levels may be consistently lower, demonstrating an overall effect of ageing rather than just a response to a test meal, therefore AUC was also used.

For a power of 90% and $\alpha=0.05$, with a sd=256.6 (Neary et al. 2004) and to detect a difference of 100pmol/l (difference in total ghrelin levels seen in our previous work and

previously shown to result in altered food intake) 72 patients were needed in total (18 in each age group).

The sample was grouped by age as follows: 20-39, 40-59, 60-79 and 80 + years. Age groups were compared either using a one-way ANOVA followed by a Hochberg post-hoc test (for data judged to be normally distributed using Kolmogorov-Smirnov test) or a Kruskall Wallis test followed by a Mann Whitney U test (for data judged to be non-normal).

Multiple regression analysis was also used to explore the association of age with hormone concentration, and the factors potentially affecting total energy intake. Exploratory analyses were performed with composite variables constructed from the main endpoints, and the relationship of these composite variables with age was tested with a linear regression analysis. To construct the composite variables, they were first standardised to a common scale by dividing each endpoint by its standard deviation, and subtracting the mean value. Residuals from the regression analysis testing the composite variables were tested for normality using the Shapiro-Wilk statistic and no statistically significant deviations from normality were detected.

Results

58 volunteers were recruited (details are shown in figure 2S, supplementary data). All age group recruitment targets were met except those over 80 years. This proved an extremely challenging group to recruit, and despite accessing several General Practitioner patient databases, inviting hundreds of potential patients and screening many, we were only able to recruit 6 females eligible for the study. Most individuals were not suitable due to comorbidities and/or current medications. Therefore we have presented here the data for females only due to the absence of males in the oldest age group (data for the whole group is in supplementary information).

191 Demographic details are shown in Table 2. As expected body composition of the older
192 participants was different to the younger; fat percentage increased and lean percentage
193 decreased with increasing age. Matching for weight and BMI was effective with no
194 significant differences between the age groups.

195 **Table 2: Female Participants' Characteristics**

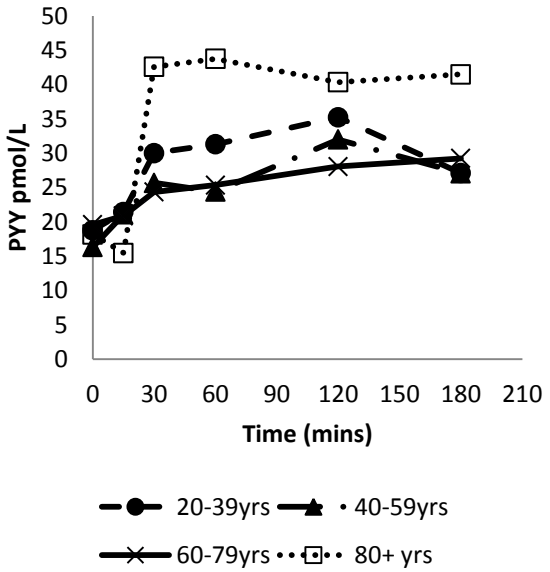
	Mean (SD)				
	Total N=31	***20-39 years N=9***	***40-59 years N=9***	***60-79 years N=7***	***80+ years N=6***
Age (years)	54.0 (21.8)	27.2 (5.0)	51.2 (7.3)	66.4 (4.8)	85.0 (4.8)
BMI (kg/m^2)	22.9 (1.8)	22.2 (1.4)	23.2 (1.7)	23.4 (1.6)	23.5 (2.7)
Body Fat (%)	31.9 (7.9)[a]	28.4 (3.2)	33.5 (13.0)	32.5 (5.4)[a]	34.0 (4.2)
Lean Mass (%)	18.5 (1.3)[a]	19.0 (1.1)	19.0 (1.3)	17.7 (1.1)[a]	17.7 (1.1)
Weight (kg)	63.2 (8.1)	61.6 (6.4)	65.1 (6.7)	64.0 (12.2)	61.8 (8.1)

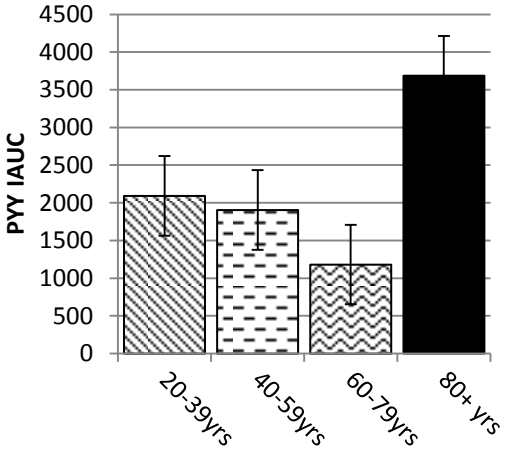
196 [a]=1 missing value; BMI=Body Mass Index.

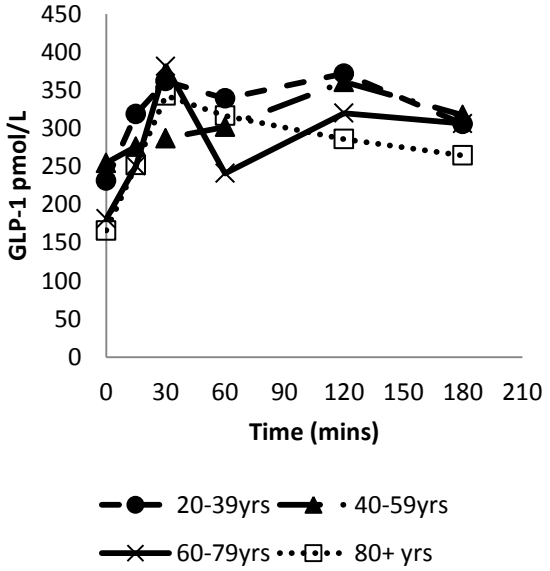
197

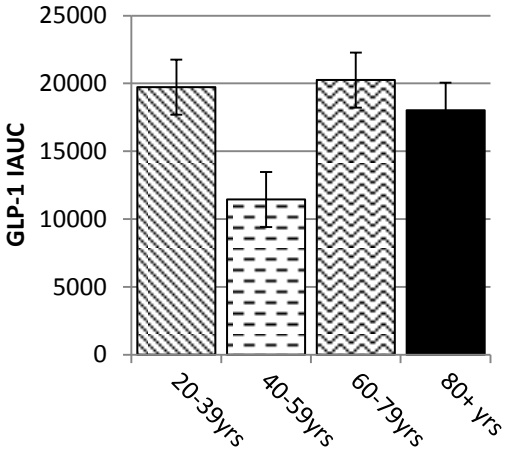
198 Plasma hormone analysis can be seen in Figure 1 and shows statistically significant
199 differences between the female age groups for PYY IAUC ($f(3,27)=2.9$, $p=0.05$). Post hoc
200 analysis using Hochberg test showed that the 80+ age group had a greater IAUC compared
201 to the 60-79 at the 0.05 level. All other comparisons were not significant. No significant
202 differences in GLP-1, acyl or total ghrelin were observed. The regression analysis found no
203 associations with age for either PYY, GLP-1 or ghrelin. Further post hoc tests exploring
204 different age cut offs for the oldest group (either 75+ or 70+) did not change the pattern of
205 results and only the *a priori* planned analyses are presented.

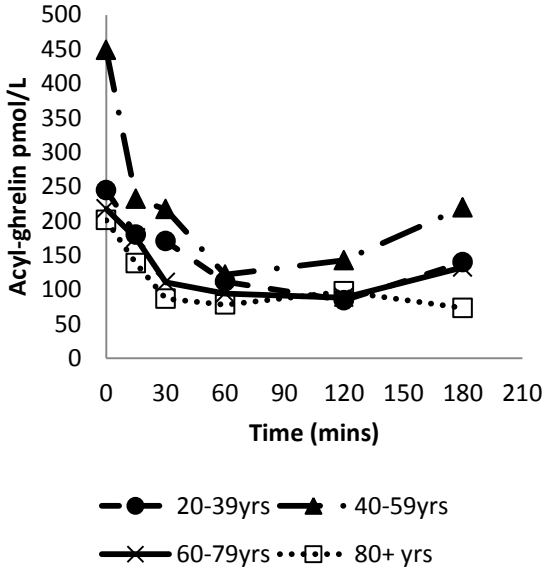
Figure 1: Difference in PYY, GLP-1 and Acyl-ghrelin blood concentrations between age groups

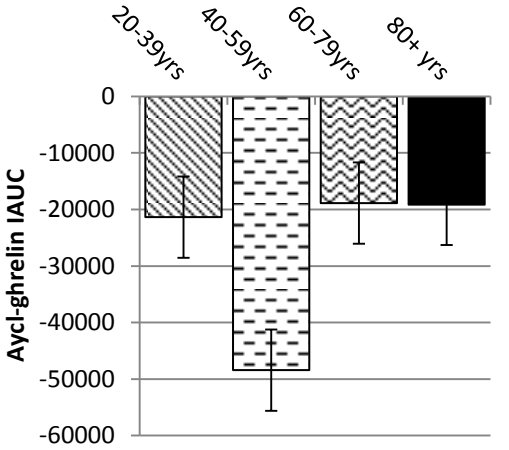












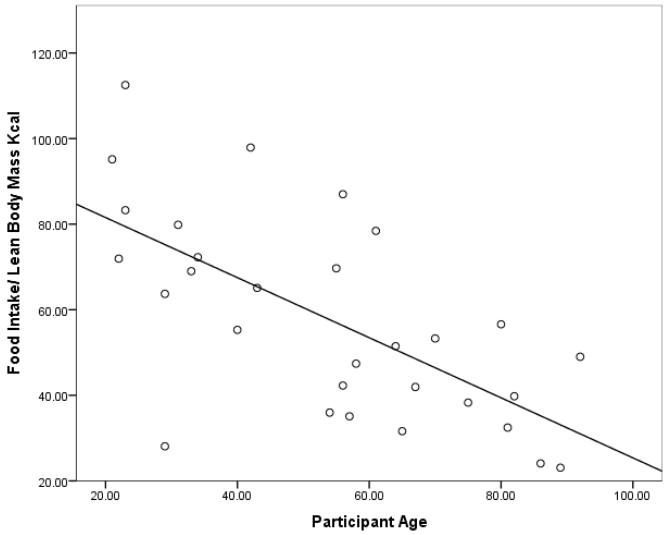
There was a decline in energy intake at the *ad libitum* meal from the young to the old group (ANOVA: f(3,27)=2.9, p=0.05) as shown in Table 3.

Table 3: Energy intake from ad libitum meal (women only)

Age group	Mean energy (kcal) intake at *ad libitum* meal (±sem)
20-39 yrs	885.5 (±107.0)
40-59 yrs	744.0 (±105.3)
60-79 yrs	684.0 (±130.4)
80+ yrs	415.6(±72.4)

Post hoc testing (Hochberg) revealed significant differences between youngest compared to oldest age group at the 0.05 level. All other comparisons were not significant. These differences were maintained when lean body mass was controlled for, showing a negative correlation between energy intake (kcal)/lean body mass (kg) ($p<0.001$, $r=-0.66$) (see Figure 2).

Figure 2: Association between age and energy intake (kcal)/kg lean mass from the *ad libitum* meal.



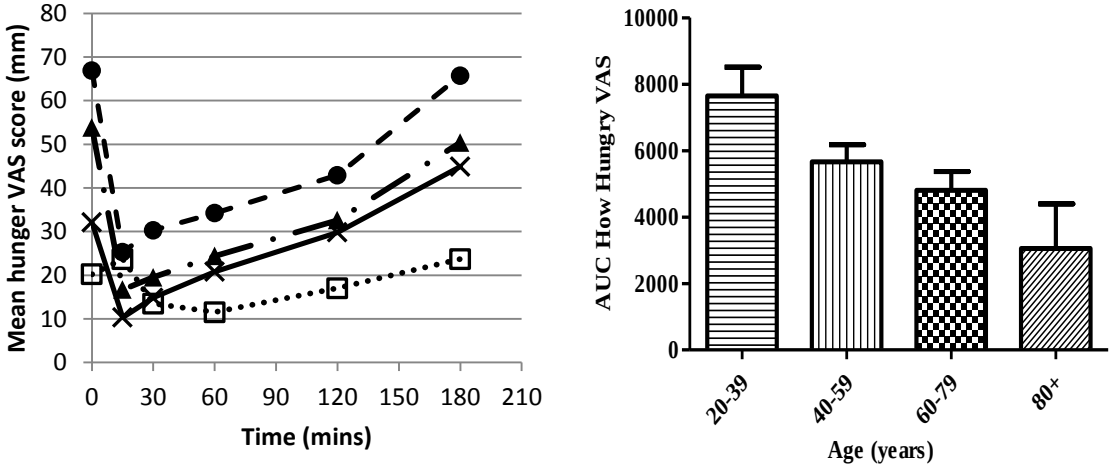
A multiple linear regression analysis (including men and women) showed that only age ($p<0.001$) and sex ($p=0.03$) had an independent effect on energy intake at this meal, and

229 energy expenditure, lean mass and water consumption were all excluded from the final
230 model.

231 We also explored the VAS results in the whole cohort, men and women; the Kruskall-
232 Wallis test showed that hunger ($H(3)=10.8$, $p=0.013$), pleasantness to eat ($H(3)=16.6$,
233 $p=0.001$), and prospective desire to eat ($H(3)=18.4$, $p=0.001$) scores were significantly
234 affected by age. Pairwise comparisons with adjusted p values showed that hunger was
235 significantly lower in the oldest compared to the youngest ($p=0.016$); pleasantness to eat
236 was reduced for 80+ ($p=0.001$) and 60-79yrs ($p=0.023$) compared to the youngest; and
237 similarly prospective desire to eat was reduced for 80+ ($p=0.001$) and 60-79yrs ($p=0.011$)
238 compared to the youngest. (See Figure 3). Fullness and nausea scores were not different
239 (data not shown).

240

241 **Figure 3: Mean VAS score over time and AUC for hunger, pleasantness to eat, and prospective desire to eat.**



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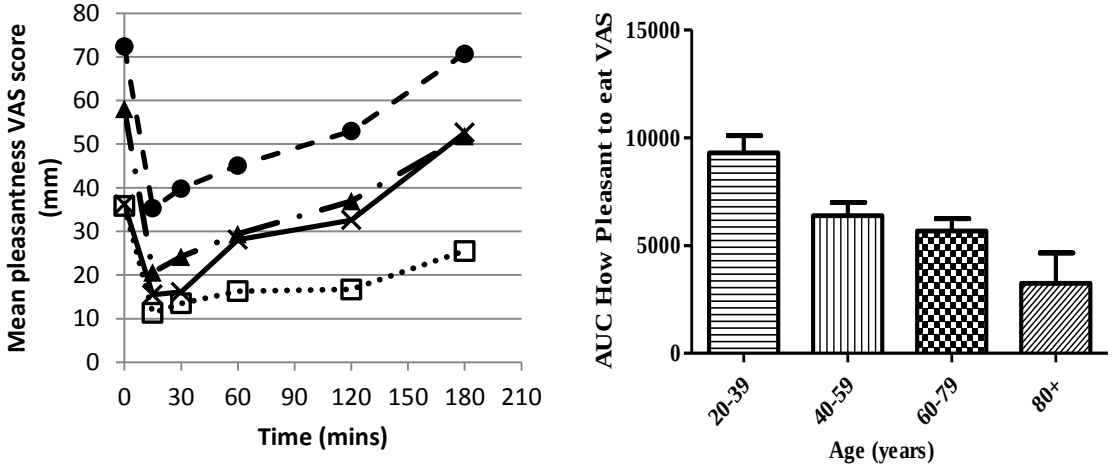

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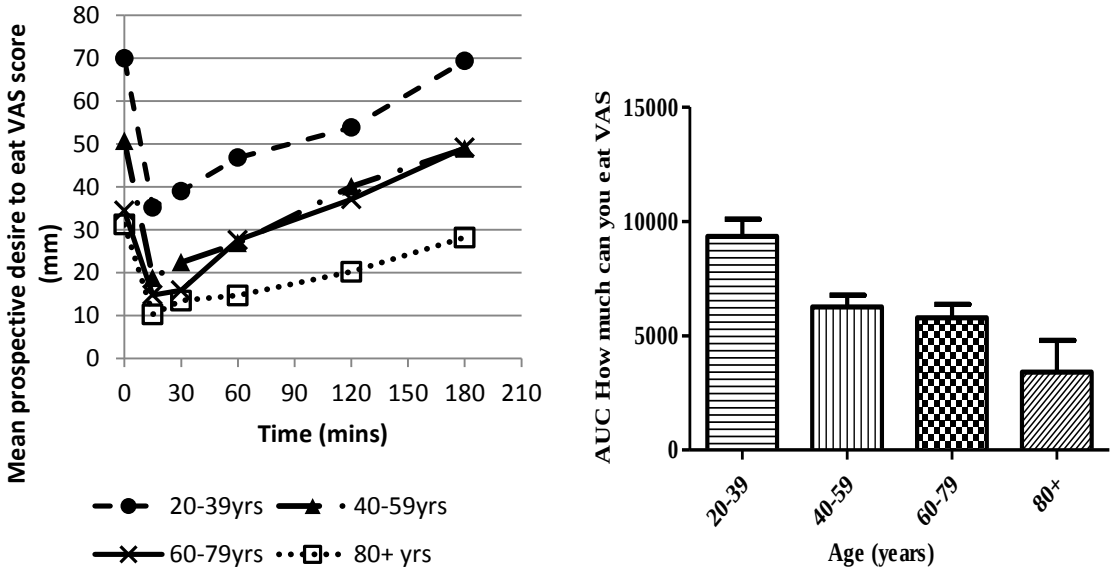

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Insulin, glucose and insulin resistance were all raised in the older age groups compared to the younger group, as expected from previous research data (Fraze et al. 1987). Peak paracetamol concentrations did not change with age, indicating no difference in gastric emptying between the four age groups. Further details of this data can be found in the supplementary data.

Discussion

This data confirms that healthy older adults have lower energy intake at the *ad libitum* meal and lower daily energy expenditure, with age as an independent factor, when controlled for body composition. The subjective appetite data (VAS) shows a corresponding picture of significantly less hunger and desire to eat in older subjects explaining lower intakes.

We hypothesised that a difference in the pattern of concentration of gut hormones would account for appetite suppression in older healthy people. The results are inconsistent. These data suggest that the age related difference in energy intake in those aged over 80 years may result from higher blood concentrations of PYY but not from an increase in GLP-1 or a reduction in ghrelin concentration. The regression analysis using age as a continuous variable did not support this finding and found no relationship between age and any hormone blood concentration.

We also found higher insulin, glucose and insulin resistance in the oldest age group as would be expected, and this may be explained to some extent by the differences in body fat mass (Paolisso et al. 1999). We found no difference in gastric emptying between young and old, thus eliminating this factor as contributing to the reduced food intake.

To date most research to explore appetite control and ageing has focussed on ghrelin concentration (Moss et al. 2012), testing the hypothesis that a reduction in this appetite stimulating hormone may explain the decrease in appetite seen with ageing (Moss et al. 2012). The results from these studies are mixed. Part of the reason for this is because older studies did not measure the two forms of ghrelin; desacyl (inactive) and acyl (biologically active), but only total ghrelin. Only four of the nine published studies investigating ageing and ghrelin measured acyl ghrelin (Bauer et al. 2010; Di Francesco et al. 2008; Nass et al. 2013; Schneider et al. 2008) but even these have used different methods for acyl-ghrelin analysis (RIA and Sandwich ELISA) which could in part explain the inconsistent results found in these studies (Prudom et al. 2010). One study compared ghrelin concentration over 24hr in young and old subjects (Nass et al. 2013) and found lower levels of ghrelin concentration during the night but not post-prandially. This supports our results in part, but it is problematic to compare this study to ours as the purpose and methodologies are different. Only one other study shows a lower fasting and post-prandial ghrelin concentrations in older adults (Di Francesco et al. 2008), supporting the theory that a reduction in ghrelin concentration causes a reduced appetite with ageing. This study uses the most robust methodology in terms of the calorie content of the study meals provided, time of day, and the matching of groups on age range, body mass index and sex. In contrast our study, also carefully designed to limit many of these methodological problems, does not support the theory that ghrelin concentration is reduced with ageing. It should be noted that our study was powered to detect a 100pmol/l difference between groups and the sample size was too low in the oldest age group.

Many studies have demonstrated that $PYY_{3–36}$ exerts an anorectic effect in humans (Batterham et al. 2003; Degen et al. 2005; Sloth et al. 2006). PYY concentrations increase

293 significantly after a meal in proportion to the amount of calories consumed reaching a
294 plateau after approximately 1 h and remains at the plateau for up to 6h (Adrian et al.
295 1985a). We and others have demonstrated that in a number of chronic conditions and in
296 critical care, appetite dysregulation is related to increased PYY and/or decreased ghrelin
297 (le Roux et al. 2005; Nematy et al. 2006a; Nematy et al. 2006b; Oner-Iyidogan et al. 2011).
298 Increased circulating PYY concentrations, early PYY production post-prandially or
299 increased sensitivity to PYY in ageing could all result in elevated feelings of fullness,
300 leading to the cessation of food intake, and may explain the changes in appetite seen
301 during ageing. Studies exploring the sensitivity to PYY in young healthy adults have shown
302 ambiguous results and that even near physiological doses can elicit nausea and vomiting
303 (Batterham et al. 2003; Degen et al. 2005; Sloth et al. 2006), thus such studies in older
304 adults are challenging to carry out.

305 There are currently only two studies that have explored the effect of ageing on PYY
306 production (Di Francesco et al. 2005; MacIntosh et al. 1999). MacIntosh et al (MacIntosh
307 et al. 1999) found no effect of age when comparing PYY concentrations in young (20-
308 34yrs, n=7) and older (65-80yrs, n=8) male adults (healthy and matched for BMI) during an
309 infusion of lipid or glucose directly into the duodenum. These researchers aimed to study
310 small intestine nutrient-mediated feedback, making the method of nutrient delivery
311 different to normal eating, where the effects of food consumption, nutrients in the
312 stomach and gastric emptying play a role. It is therefore difficult to compare this evidence
313 with the current study, but in fact show data in line with those reported here, since the
314 age of the older group was less than 80 years. The other study aimed, like ours, to
315 replicate normal eating, albeit in a controlled manner and environment. Di Francesco et al
316 (Di Francesco et al. 2005) used a study protocol involving an overnight fast followed by a
317 3347kJ (800kcal; 15% protein, 45% fat, 40% carbohydrates) solid meal with water,

318 comparing young (25-53yrs, n=9) and older (72-82yrs, n=10) men and women (M:F=10:9),
319 all healthy and matched for BMI. Their study demonstrated that older subjects had no
320 difference in fasting PYY or PYY concentrations up to 120 minutes post-prandially, but a
321 significantly higher concentration at 180 and 240 minutes. An absence of an altered GLP-1,
322 total ghrelin, acyl ghrelin and gastric emptying with ageing was also observed in this
323 investigation. These data are in agreement with our findings and are consistent with the
324 post-prandial satiety effects of PYY which appears to be released in greater quantity in
325 response to food intake in older individuals. In addition, our data suggest that these
326 effects are only clearly apparent in the very old healthy population. This information
327 supports the hypothesis that ageing is associated with increased PYY production, resulting
328 in satiety, and offering some explanation for the anorexia of ageing. Our data also showed
329 that in the older group there appears to be a suppression of PYY at 15 minutes following
330 the meal. The physiological relevance of this is not known. However there is no
331 suggestion from the VAS that this cause a decrease in satiety at this time point. It is also
332 worth noting that both these studies measured cholecystokinin (CCK) (not done in this
333 study) and found higher concentrations in older subjects both fasting and post prandially.
334 Another group has also reported elevated CCK (Sturm, 2003) but Serra-Prat et al was
335 unable to reproduce this observation (Serra-Prat et al 2009).

336 The main limitation of this study is the low number of healthy participants over 80yrs
337 (n=6) and that they were all female, thus it is possible that these results are a type 1
338 statistical error or due to the sex of the subjects. We have presented the female only
339 analysis but the analysis for all data is shown in the supplementary information,
340 suggesting few differences between the sexes.. There is little published data to suggest an
341 effect of sex on gut hormone concentration and appetite control and this evidence relates
342 to phases of the menstrual cycle (Brennan et al. 2009). Larger studies, ideally with both

men and women, using similar standardised protocols are required to confirm our results. Another limitation is that total PYY was measured not PYY_{3-36} only.

It is difficult to establish from the published literature exactly what age anorexia may start; it appears to be a gradual process but is most common along-side chronic disease (Visvanathan, 2015). Our data shows that food intake decreases even with no ill health continuing up to 92 years.

We also did not identify an incremental change in PYY concentration in the 60-79yr age group, as would be expected assuming a gradual alteration in gut hormone concentration with ageing. However, this is not necessarily a sound assumption as anorexia of ageing effects the very old to a greater extent (Malafarina et al. 2013). It may be that these changes in PYY concentration are limited to the oldest age groups. However, the VAS data do show a linear reduction of appetite with age, suggesting other factors may be at play. Appetite remains under the influence of many external factors, such as lifestyle (retirement), psychological (bereavement, depression) or hormonal (menopause) changes. These changes may precede later physiological changes suggested from our results. Although subjects were matched for BMI, percentage body fat was found to be higher in the oldest age group compared to the youngest and so could be a possible confounder. However, there is scant evidence that PYY is affected by BMI or fat mass. One study (Batterham et al. 2003) found lower PYY levels in obese compared to lean young adults, and a negative relationship between BMI and PYY, suggesting that PYY would be lower with higher fat mass. Therefore, the difference in fat mass is unlikely to be a significant factor in the finding that PYY was increased in the oldest age group.

Gastric emptying was also not different between age groups eliminating this factor as one which contributed to the reduction in food intake seen in our aged study sample. In

theory; increased time to empty the stomach could reduce appetite and speed up satiety. The use of a small volume meal may have ensured that gastric emptying was not affected allowing us to conclude that the reduction in food intake is due to the changes in PYY concentration.

Our data suggest that there may be an increase in the concentration of PYY after meals with healthy ageing in females, potentially resulting in a reduced appetite. There does not appear to be any change in ghrelin or acyl-ghrelin concentration with ageing.

Acknowledgements

Our thanks go to the GPs who were part of the North West London Research Network for assistance with recruiting our older volunteers. MH, GF, WD, CM designed research; statistical advice regarding the power calculation was received from Joseph Eliahoo, Statistical Advice Service, Imperial College, London; advice on the regression analysis was received from Les Huson; CM, JB conducted research; MG provided essential reagents or provided essential materials; MH, CM, WD & GF analyzed data; MH wrote paper, all authors reviewed the final manuscript and MH has primary responsibility for final content.

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Conflict of Interest

All authors declare no conflicts of interest in relation to this paper.

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Supplementary data

Title: Increased Peptide YY blood concentrations, not decreased acyl-ghrelin, are associated with reduced hunger and food intake in healthy older women: preliminary evidence

Included here is additional information to supplement the methods and results sections of the main manuscript, including an analysis of the whole sample (men and women), the results for glucose, insulin, insulin resistance and gastric emptying times between the four age groups and details of the female only analysis.

Methods

Figure 4S: Overview of the test meal process, including all blood sampling points

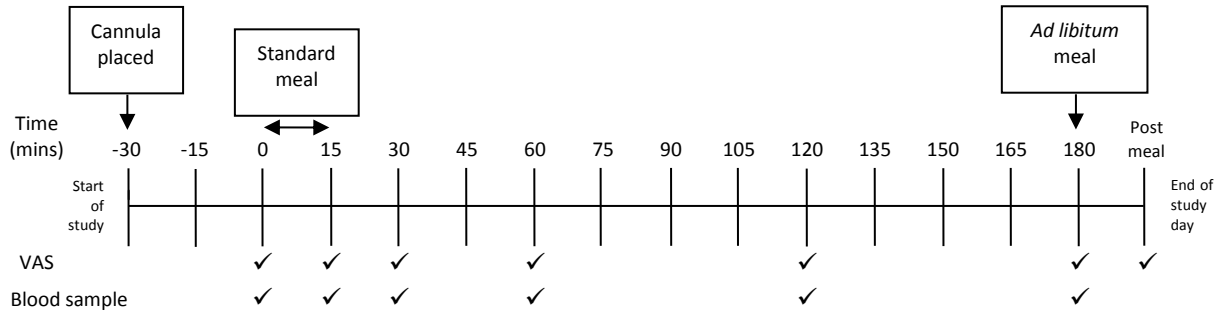
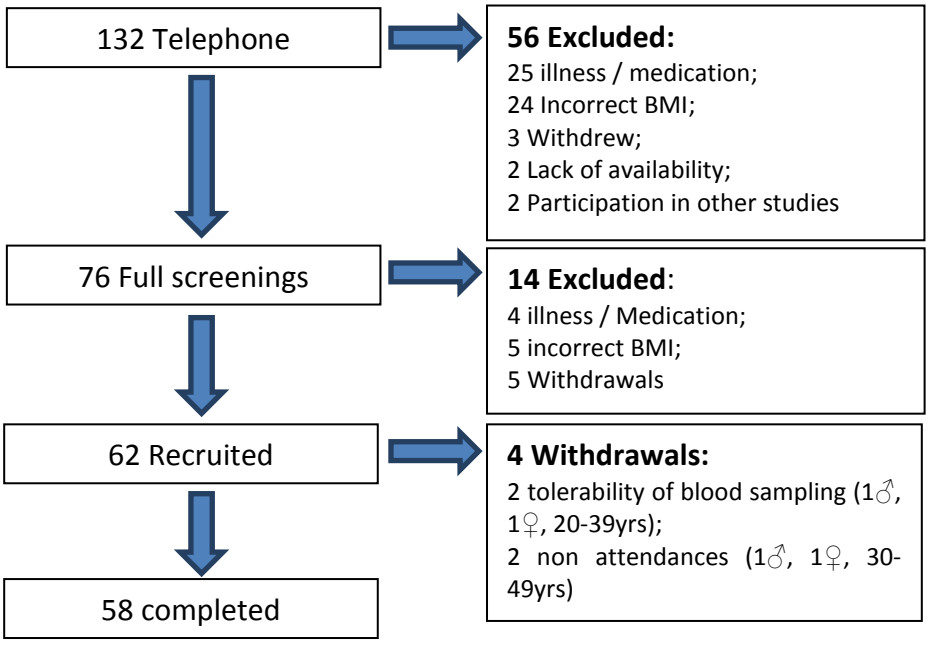


Figure 5S: Flow chart of screening process.



Results

The characteristics of all subjects (both men and women) are shown in Table 4S.

532 Table 5S shows the AUC and IAUC for each hormone, and

Figure 6S shows the differences between age groups.

Table 4S: Participants' Characteristics (men and women)

	Total N=58	*20-39 years N=18*	*40-59 years N=18*	*60-79 years N=16*	*80+ years N=6*
Sex (male:female)	27:31 (47%:53%)	9:9	9:9	9:7	0:6
			Mean (SD)		
BMI (kg/m^2)	23.5 (1.9)	22.6 (1.6)	23.7 (1.7)	24.1 (2.0)	23.5 (2.7)
Lean Mass (%)*	20.1 (2.4)[b]	21.4 (2.8)[a]	20.1 (1.7)	19.7 (2.0)[a]	17.7 (1.1)
			Median (Inter-quartile range)		
Weight (kg)	70.3 (61.8-77.6)	66.6 (59.4-72.7)	73.1 (64.2-81.3)	75.3 (61.4-80.2)	61.8 (57.2-65.9)
Body Fat (%)$^{\$}$	25.3 (17.1-31.3)[a]	20.2 (9.15-28.27)	23.3 (18.4-30.4)	25.3 (20.2-30.8)[a]	33.9 (31.4-37.0)
Age (years)	54 (29.8-65.5)	25 (22.8-29.3)	53 (43.8-56.0)	66 (61.5-71.5)	84 (80.8-89.8)

[a]=1 missing value; [b]=2 missing values; BMI=Body Mass Index.

* There was significant effect of age on lean mass percentage $F(3,52)=4.84$, $p=0.005$. Post hoc analysis using Hochberg test showed that the oldest group lower % lean mass compared to the youngest group at the 0.05 level.

$^{\$}$Body fat percentage was significantly different between age groups, $H(3)=12.7$, $p=0.005$. Jonckheere's test revealed a significant trend as expected; with increasing age the median fat % increased $J=816.5$, $z=3.34$, $p=0.001$.

Table 5S: Mean area under the curve (AUC) and incremental AUC (IAUC) values for the plasma analysis (whole sample – men and women)

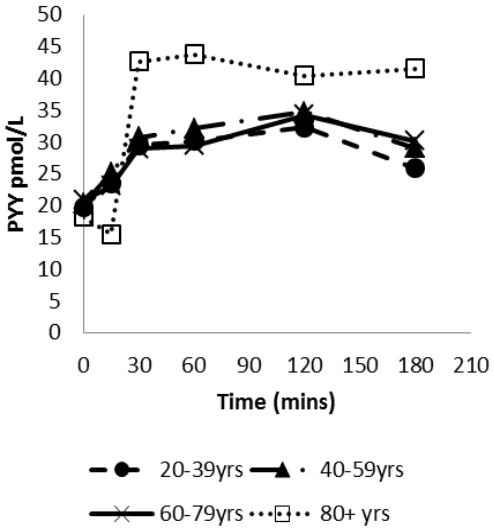
Mean (sd)	*Total N=58*	*20-39 years N=18*	*40-59 years N=18*	*60-79 years N=16*	*80+ years N=6*	*P value*
Total Ghrelin AUC	82007.0 (44799)	68315.5 (32696)	93323.3 (46464)	75895.4 (50931)	105430.5 (46995)	0.12
IAUC	-39409.0 (46134)	-29995.4 (25917)	-48104.9 (44918)	-35836.2 (64145)	-51089.8 (44490)	0.35
Acyl Ghrelin AUC	21011.6 (15336)	19875.4 (13150)	23294.5 (19472)	21194.6 (14485)	17083.0 (11526)	0.92
IAUC	-25835.7 (33667)	-29727.1 (31040)	-29681.5 (45281)	-19646.7 (26751)	-19127.7 (15596)	0.83
PYY AUC	5547.3 (2532)	5228.9 (2093)	5612.0 (1783)	5302.7 (3333)	6960.7 (3334)	0.40
IAUC	1940.3 (1969)	1667.2 (1689)	1967.4 (1127)	1562.4 (2775)	3686.8 (1637)	**0.02**[$]
****GLP-1 AUC***	53936.0 (22849)	54163.7 (23336)	54682.2 (25970)	55141.4 (19432)	47924.0 (25586)	0.85
IAUC	16109.5 (16334)	15389.9 (12567)	13876.9 (23558)	18571.5 (9008)	18029.0 (19723)	0.74

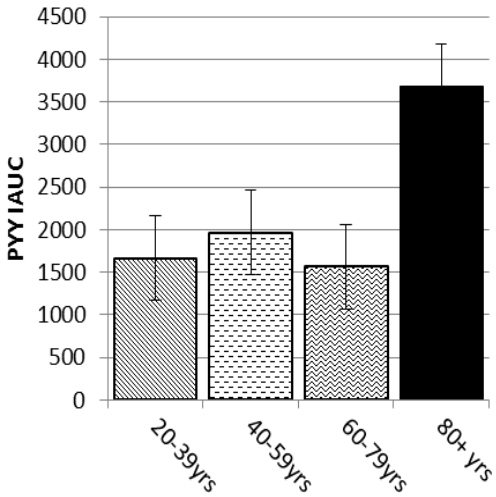
*1 value missing (40-59yrs group). IAUC measured in pmol/l/min.

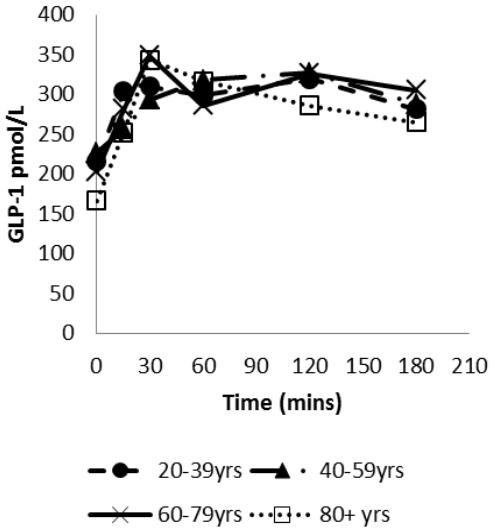
[$]PYY IAUC was significantly different between age groups, H(3)=9.69, p=0.012 (tested using Kruskal-Wallis). Step-down follow-up analysis showed that the oldest age group had significantly higher IAUC compared to the youngest; however, there were no differences between the three youngest age groups, p=0.2.

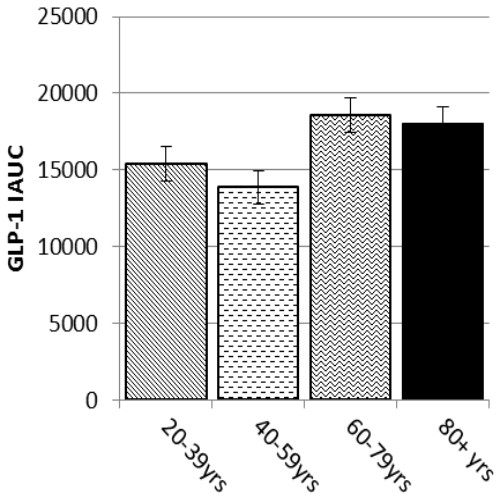
Data were not distributed consistently, some were skewed, some parametric. All are presented as mean and standard deviation and the appropriate comparative tests used.

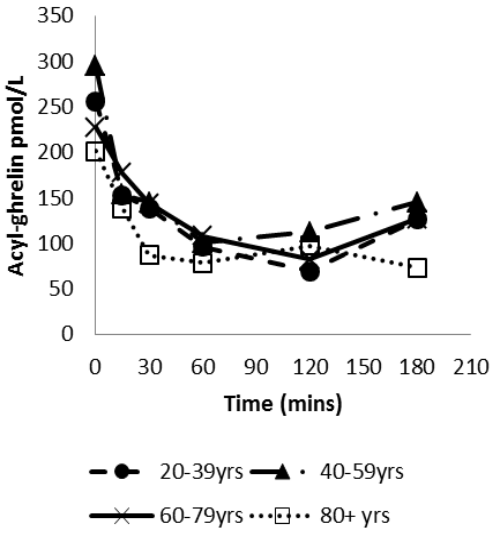
Figure 6S: Difference in PYY, GLP-1 and Acyl-ghrelin blood concentrations between oldest and younger age groups.

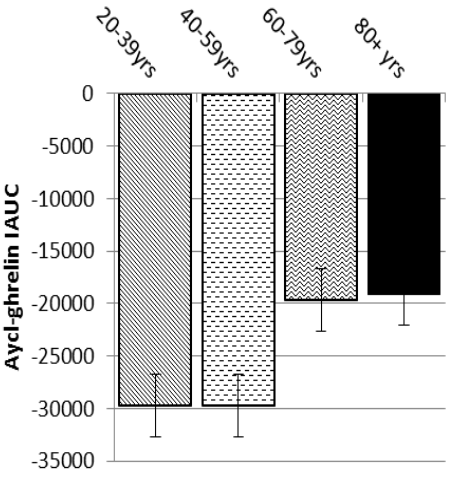












Insulin, glucose and gastric emptying measurements

Plasma insulin

Table 6S shows the data for insulin levels, each age group, both men and women (3Sa), and females only (3Sb).

Table 6Sa: Plasma insulin AUC and IAUC, in the 20-39, 40-59, 60-79 and 80+ age groups, for all patients.

Age group*	***Plasma Insulin IAUC*** Mean (SD)	***Plasma Insulin AUC*** Mean (SD)
20-39 (n=11)	4312.5 (1100.9)	6042.3 (1200.5)
40-59 (n=11)	4462.7 (1391.6)	5912.2 (1675.2)
60-79 (n=12)	4943.4 (2559.8)	6775.1 (2819.5)
80+ (n=4)	5496.8 (1691.6)	8386.2 (1885.6)

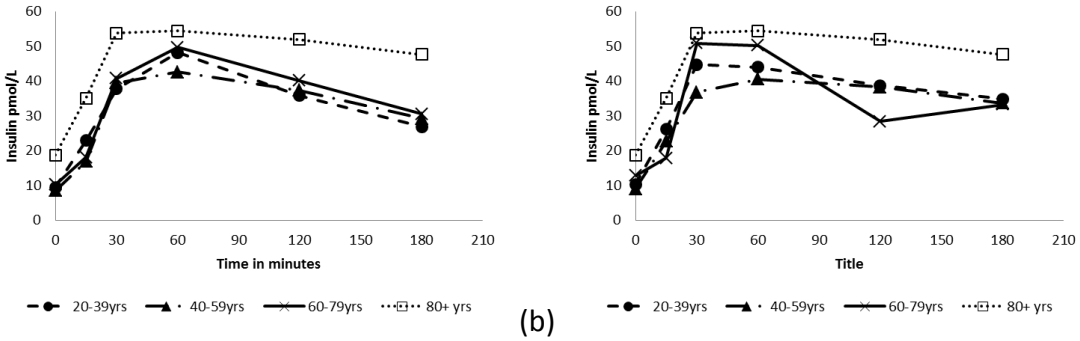
Table 3Sb: Plasma insulin AUC and IAUC, in the 20-39, 40-59, 60-79 and 80+ age groups, for females only.

Age group*	***Plasma Insulin IAUC*** Mean (SD)	***Plasma Insulin AUC*** Mean (SD)
20-39 (n=4)	4194.7 (1322.1)	6083.8 (1264.7)
40-59 (n=5)	4530.5 (955.9)	6023.5 (1123.0)
60-79 (n=4)	4770.9 (1012.5)	7074.9 (2376.6)
80+ (n=4)	5496.8 (1691.6)	8386.2 (1885.6)

**missing values due to haemolysed blood samples. Values (n=19) were imputed when there were no more than 2/6 values missing, the values were not from 0 or 180 time points, and the missing values were not consecutive. Values were imputed by calculating the rate of change between the two available values, multiplying by the time to the missing value, and adding on to the previous available value, using the following formula:*
Missing value= (difference between values immediately before and after)/(minutes between the two values)(minutes of missing value from the last)+value before missing one*

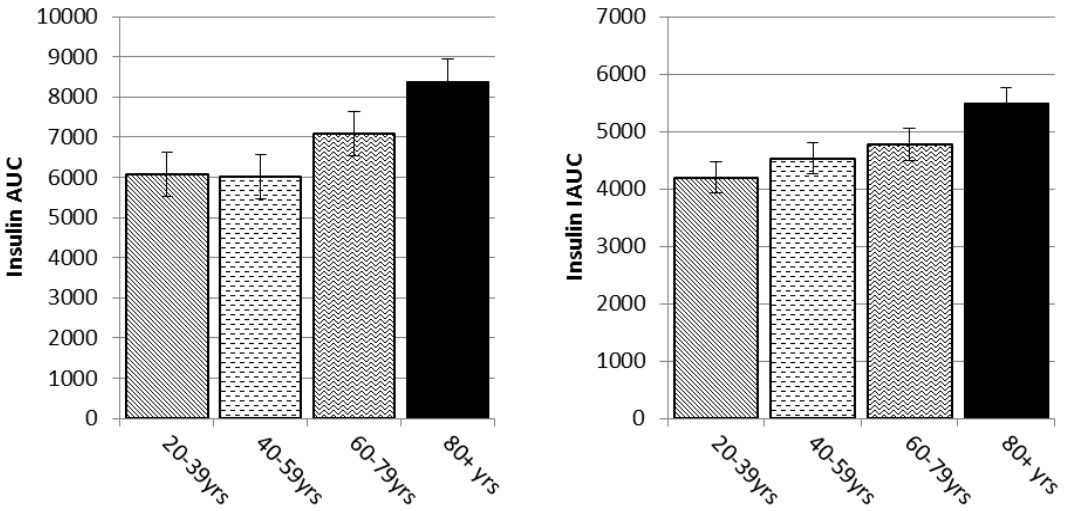
Plasma insulin concentrations at each time point can be seen in Figure 7S, and AUC and IAUC in Figure 8S ((a) all patients; (b) females only). AUC and IAUC scores for insulin increased with age but these differences did achieve significance. AUC is reported as the baseline for the older age group is higher and consequently the IAUC may mask differences. Lack of significance may be due to low numbers because of missing values.

Figure 7S: Plasma insulin concentrations over the 3 hour test meal in the 20-39, 40-59, 60-79 and 80+ age groups: (a) All patients; (b) Females only.

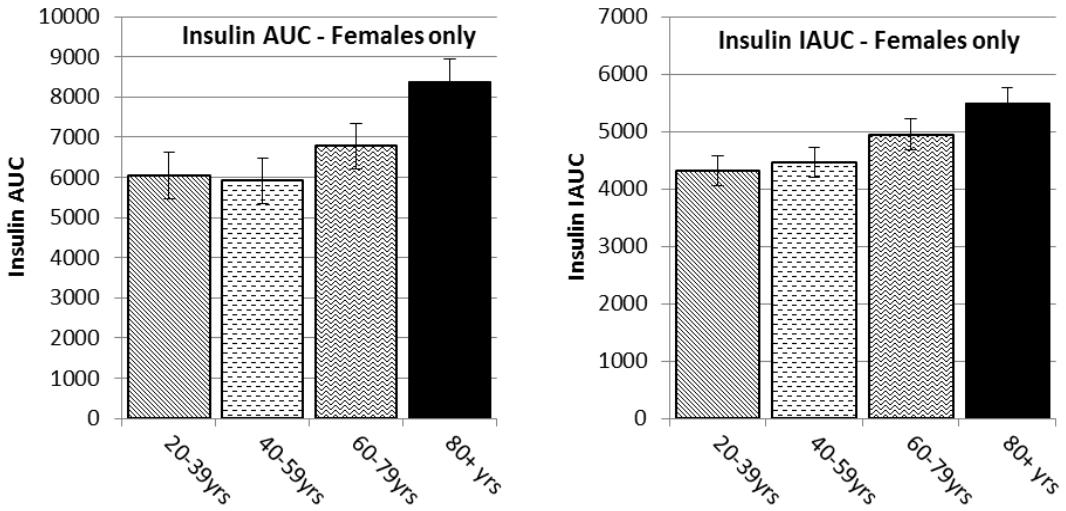


(a) (b)

Figure 8S: Plasma insulin AUC and IAUC in the 20-39, 40-59, 60-79 and 80+ age groups: (a) All patients; (b) Females only.



(a)



(b)

Plasma glucose

Data on plasma glucose is shown in Table 7S (a&b), Figure 9S and Figure 10S. For the whole group glucose AUC was significantly different between age groups, H(3)=9.75, p=0.021 (tested using Kruskal-Wallis). Pairwise comparisons with adjusted p values showed that the oldest group had significantly higher AUC compared to the youngest (p=0.03), and there were no other significant differences. IAUC scores were also the highest in the 80+ age group but this did not reach significance. For females only there were no significant differences between age categories for either IAUC or AUC glucose, possibly due to the reduced number of subjects.

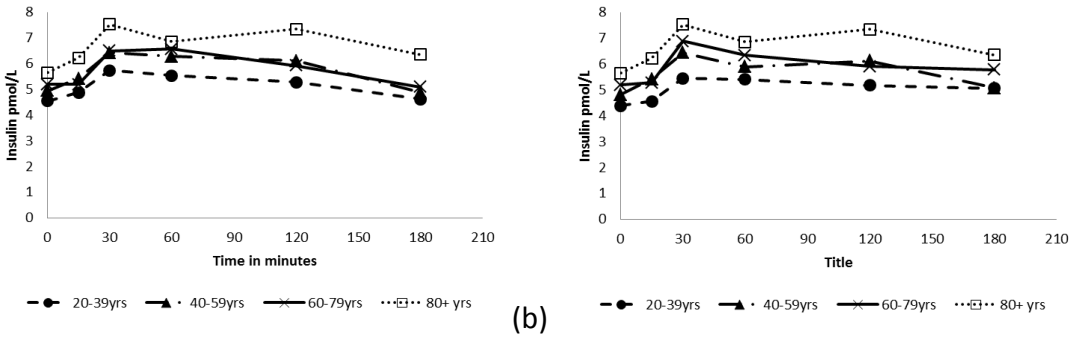
Table 7Sa: Plasma glucose AUC and IAUC, in the 20-39, 40-59, 60-79 and 80+ age groups, for all patients.

Age group	***Plasma Glucose IAUC*** Mean (SD)	***Plasma Glucose AUC*** Mean (SD)
20-39 (n=18)	121.1 (137.2)	939.6 (143.4)
40-59 (n=18)	166.8 (215.7)	1057.3 (214.6)
60-79 (n=16)	70.8 (237.9)	1077.7 (226.2)
80+ (n=6)	227.1 (113.8)	1244.1 (363.2)

Table 4Sb: Plasma glucose AUC and IAUC, in the 20-39, 40-59, 60-79 and 80+ age groups, for females only.

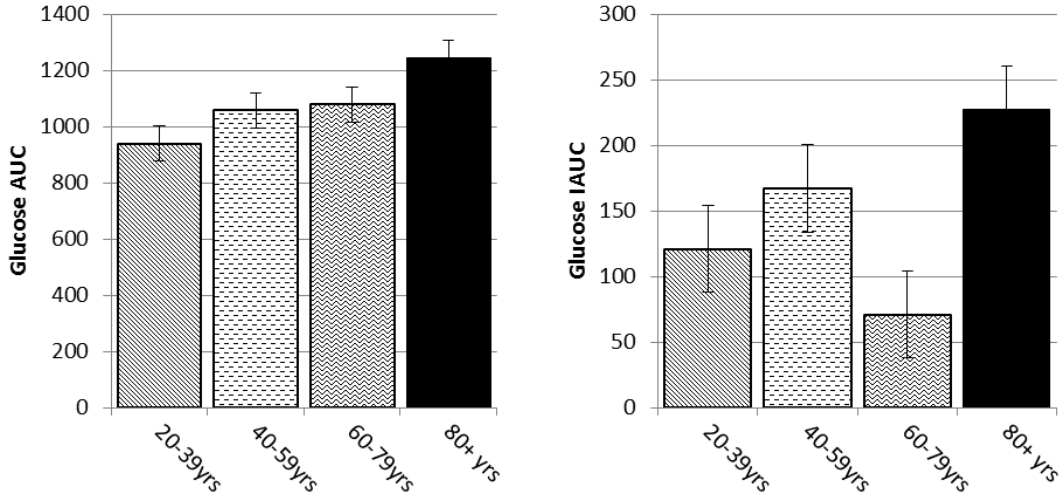
Age group	***Plasma Glucose IAUC*** Mean (SD)	***Plasma Glucose AUC*** Mean (SD)
20-39 (n=9)	121.1 (137.2)	928.8 (150.4)
40-59 (n=9)	166.8 (215.7)	1046.5 (174.6)
60-79 (n=7)	70.8 (237.9)	1113.0 (311.1)
80+ (n=6)	227.1 (113.8)	1244.1 (363.2)

Figure 9S: Plasma glucose concentrations over the 3 hour test meal in the 20-39, 40-59, 60-79 and 80+ age groups: (a) All patients; (b) Females only.

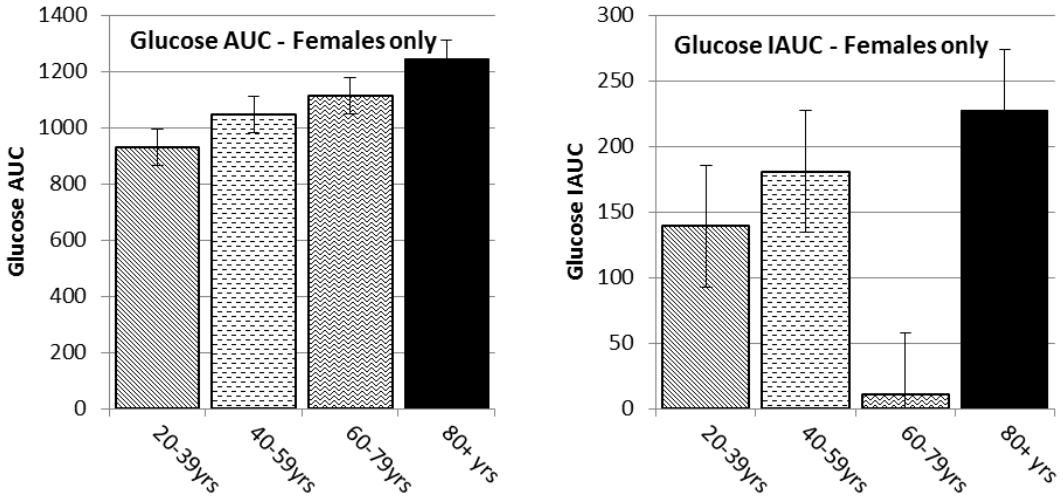


(a) (b)

607 **Figure 10S: Plasma glucose AUC and IAUC in the 20-39, 40-59, 60-79 and 80+ age groups: (a) All patients; (b) Females**
608 **only.**



609 (a)



610 (b)

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Insulin resistance

An index of fasting insulin resistance (HOMA) was measured using the following equation;

HOMA = (glucose mmol/L x Insulin mU/L) / 22.5

[where mU/L = pmol/7.5 (Raben et al.,2001)]

Data is shown in Table 8S (a&b) and Figure 11S. The Kruskall-Wallis test showed that HOMA was significantly affected by age (H(3)=8.79, p=0.032). Pairwise comparisons with adjusted p values showed that HOMA was significantly higher in the oldest compared to the youngest (p=0.037) and the 40-59yrs group (p=0.033). All other comparisons did not indicate a difference. For females only the pattern of data reminded the same by differences were not significant due to lower sample numbers.

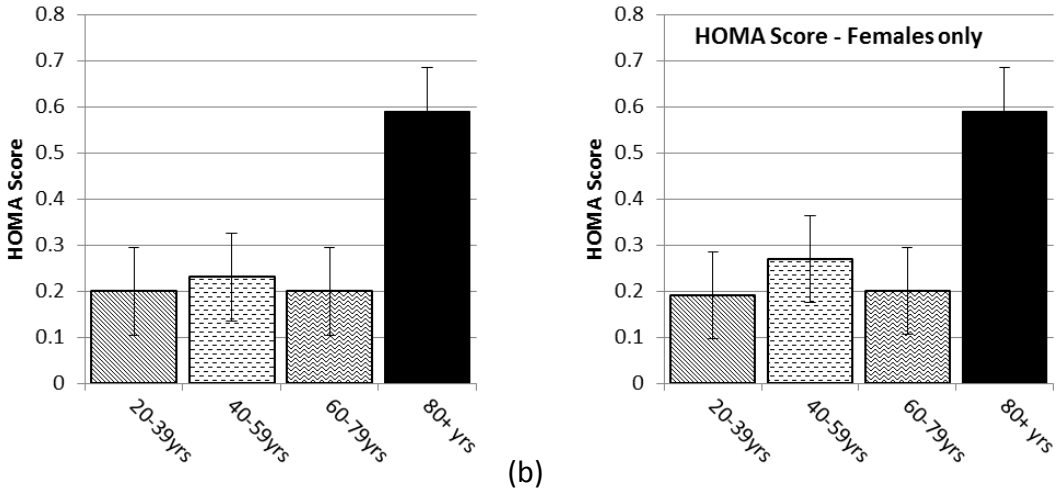
Table 8Sa: HOMA in the 20-39, 40-59, 60-79 and 80+ age groups, for all patients.

Age group	***HOMA*** Median (IQ Range)
20-39 (n=17)	0.20 (0.16 – 0.34)
40-59 (n=16)	0.23 (0.15 – 0.36)
60-79 (n=11)	0.20 (0.16 – 0.38)
80+ (n=6)	0.59 (0.46 – 0.80)

Table 5Sb: HOMA in the 20-39, 40-59, 60-79 and 80+ age groups, for females only.

Age group	HOMA Median (IQ Range)
20-39 (n=8)	0.19 (0.13-0.41)
40-59 (n=8)	0.27 (0.11-0.36)
60-79 (n=3)	0.20 (0.13-0.66)
80+ (n=6)	0.59 (0.46-0.80)

Figure 11S: HOMA scores in the 20-39, 40-59, 60-79 and 80+ age groups: (a) All patients; (b) Females only.



(a) (b)

Paracetamol method- gastric emptying

The time to peak concentration for paracetamol was used as a measurement of gastric emptying. Data is shown in Table 9S (a&b). Peak paracetamol concentrations did not change with age, no significance was detected between the four age groups for peak paracetamol concentrations in either all patients or females only.

Table 9Sa: Peak paracetamol in the 20-39, 40-59, 60-79 and 80+ age groups, for all patients.

Age group	***Time to peak paracetamol concentration*** Median (IQ Range)
20-39 (n=18)	150 (26.3 – 180.0)
40-59 (n=18)	180 (120.0 – 180.0)
60-79 (n=16)	180 (60.0 – 180.0)
80+ (n=6)	180 (97.5 – 180.0)

Table 6Sb: Peak paracetamol in the 20-39, 40-59, 60-79 and 80+ age groups, for females only.

Age group	***Time to peak paracetamol concentration*** Median (IQ Range)
20-39 (n=9)	180 (75.0-180.0)
40-59 (n=9)	180 (150.0-180.0)
60-79 (n=7)	180 (120.0-180.0)
80+ (n=6)	180 (97.5-180.0)

Energy intake from ad libitum meal

Table 10S shows the energy intake at the *ad libitum* meal by age group for all subjects (men and women), which was significantly different ($p<0.001$). Post hoc analysis (Hochberg) showed differences at the 0.05 level between the youngest and two oldest age groups (20-39yrs vs 60-79 yrs & 80+ yrs). Figure 12S shows the association with age when energy intake is controlled for lean mass; showing a negative correlation between energy intake (kcal)/lean body mass (kg) ($p<0.001$, $r=-0.57$) and age.

Table 10S: Energy intake from ad libitum meal (men and women)

Age group	Energy intake (kcal) at *ad libitum* meal (sd)
20-39 yrs	1018.9 (352.3)
40-59 yrs	801.1 (275.3)
60-79 yrs	638.7 (302.2)
80+ yrs	415.62 (177.4)

Figure 12S: Association between age and energy intake (kcal)/kg lean mass from the *ad libitum* meal (men and women).