Title- Postprandial Regulation of Prouroguanylin in humans of a Healthy Weight and those who are Overweight or with Obesity

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Running Title - Postprandial Regulation of Prouroguanylin

Keywords Words
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Highlights

• Mixed macronutrient meals high in fat or carbohydrates cause a delayed increase in prouroguanylin concentrations

• Fasting concentrations of prouroguanylin are suppressed in those who are overweight/ with obesity

• People who are overweight/with obesity remain sensitive to the effects of meals on prouroguanylin
Abstract

Uroguanylin is a peptide gut hormone proposed to have a role in signalling post meal satiety. Uroguanylin circulates as its pro-hormone, prouroguanylin. There has been limited investigation of the regulation of prouroguanylin by food; therefore we investigated prouroguanylin regulation following meals. In separate experiments we investigated the effects of high calorie (1451 kcal) and medium calorie (725 kcal), high fat meals, on plasma prouroguanylin concentrations. We then examined the effect of a 722.5 kcal high carbohydrate breakfast on prouroguanylin concentrations, comparing the response in healthy weight adults versus those who are overweight/with obesity. The 1451 kcal meal increased prouroguanylin concentrations, versus fasting at 60 (P<0.05), 90 (P<0.01) and 120 (P<0.001) minutes. After the 725 kcal meal hormone concentrations rose more slowly and were significant versus fasting concentrations at 120 minutes (P<0.01). The high carbohydrate breakfast 722.5 kcal, led to an initial suppression of hormone concentrations at 30 mins post meal (P<0.05) followed by an increase in concentrations until they were significant versus fasting at 120 mins (P<0.01). Participants overweight/with obesity had lower fasting prouroguanylin concentrations (P<0.05), but post meal concentrations did not differ between the groups. Our results suggest there is a delayed increase in prouroguanylin concentrations following, large and regular sized mixed macronutrient meals rich in fat or carbohydrate. Fasting levels are suppressed in people who are overweight/with obesity, but the post meal response remains intact. There may be potential to target post meal release of prouroguanylin in obesity.
1. Introduction

Hormones secreted from the gastrointestinal system (gut hormones) are essential regulators of appetite and satiety (1). Pharmacological (2) and dietary (3)(4) innovations are targeting the release of these hormones for the treatment of obesity.

Uroguanylin is a peptide ligand of the Guanylyl cyclase C receptor (GUCY2C). It is highly expressed in the proximal intestine, and in particular the enterochromaffin cells (5). However, in humans there is some debate over the cell type expressing uroguanylin; one study found expression in the human duodenum, but suggested the cells expressing uroguanylin were not enterochromaffin cells (6). In addition to the small intestine uroguanylin is also expressed in the colon (6)(7). Within the gut uroguanylin has paracrine actions, activating GUCY2C to regulate electrolyte and fluid balance (8), and uroguanylin and GUCY2C have been targeted to treat gastrointestinal disorders including, constipation (9) and irritable bowel syndrome (10). There is also evidence uroguanylin acts outside the gut to regulate electrolyte and fluid balance (8). More recently uroguanylin has been proposed as a novel appetite and body mass regulating gut hormone (11). Valentino et al.’s (11) studies suggested prouroguanylin (precursor of uroguanylin) is secreted into circulation after a meal and is processed to active uroguanylin in the hypothalamus where it signals satiety (11). In support of this hypothesis, in their study transgenic mice lacking GUCY2C receptor are hyperphagic and developed obesity (11). Subsequent studies in mice have supported some aspects of Valentino’s work and challenged others (12)(13)(14)(15)(16). A study using knockout models found loss of uroguanylin, but not GUCY2C, led to increased body mass and adiposity, and central administration of GUCYC agonists had no affect on feeding (12). While another study reported chronic central infusion of uroguanylin led to increased body mass and adiposity, but this effect was not mediated by chronic increased feeding (13). However, they did observe an acute effect of uroguanylin on feeding in the first few hours after injection. In contrast a very recent study found neither administration or upregulating the expression of either uroguanylin or prouroguanylin had any effect on feeding or glucose homeostasis (16). Yet two other studies have reported uroguanylin levels are effected by diet, leptin and obesity(14)(15). In summary, most, but not all rodent studies do
support a role for uroguanylin in the regulation of body mass, but effects on feeding and glucose homeostasis remain controversial.

There have been limited further studies of uroguanylin regulation in humans. Of note, a recent study (17) suggested fasting prouroguanylin concentrations are lower in people with obesity and rise following Roux-en-Y gastric bypass (RYGB), a pattern similar to is observed with glucagon like peptide -1 (GLP-1) and peptide YY (PYY) (18). In support of this another study reported an upregulation of uroguanylin mRNA following RYGB (16). A further recent study also reported female adolescents with obesity had lower fasting concentrations of prouroguanylin than those who did not have obesity, but post meal changes in prouroguanylin were similar in both groups (19). However, there has been limited work to elucidate the factors influencing the release of prouroguanylin as a post meal signal of satiety in adults. Valentino (11) and colleagues only investigated prouroguanylin release following a single large (1460 kcal) mixed macronutrient meal in healthy weight adult male volunteers, while Di Guglielmo et al. (19) studied prouroguanylin following a single meal in adolescents. To begin to understand prouroguanylin release in response to a meal we aimed to determine the effects of smaller meals more typical of the energy intake of real life single meals on circulating prouroguanylin concentrations, and to see if fasting and post meal concentrations of prouroguanylin differed between adults of a healthy weight and those who are overweight/ with obesity.
2. Methods

2.1 Ethics and Recruitment

All studies were performed according to the principles of the Declaration of Helsinki and were approved by the local research ethics committee at the University of Roehampton. Participants were recruited via posters displayed in the University of Roehampton. For all studies we recruited healthy volunteers over the age of 18. Participants with Diabetes, gastrointestinal conditions/diseases or food allergies were excluded. For studies 1 and 2 we recruited males with a BMI between 18 and 30 kg/m² to allow comparisons to the previous study (11). For study 3 we recruited both sexes with a minimum BMI of 18 kg/m² and aimed to have a similar number of participants with BMIs under and over 25 kg/m². Females who were pregnant, lactating or having given birth in the past year were excluded.

2.2 Study 1 - The effect of a large 1451 kcal meal on plasma Prouroguanylin concentrations in males

Seven healthy male participants fasted overnight (12 hours) then consumed a 1451 kcal meal of similar composition to sausage and egg breakfast meal given by Valentino et al., 2011 (11). Finger prick blood samples were taken and plasma extracted for prouroguanylin measurement (as described below) fifteen minutes before the breakfast (time 0 minutes) and at intervals up to 120 minutes after the meal (15 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes). Hunger levels were measured at all time points using a visual analogue scale.

2.3 Study 2- The effect of a 725 kcal meal on plasma Prouroguanylin concentrations in males

Seven healthy male participants fasted overnight (12 hours) then consumed a 725 kcal meal with the same components as study 2, but half the portions. Finger prick blood samples were taken at the same timepoints and plasma extracted for prouroguanylin measurement.
2.4 Study 3 - The effect of a 722.5 kcal carbohydrate rich breakfast on plasma Prouroguanilin concentrations in males and females of a healthy weight and those who are overweight/ with obesity.

Eighteen participants (10 male, 8 female) fasted overnight (12 hours) then consumed a 722.5 kcal meal. Nine of the participants (3 female and 5 male) had a BMI over 25 kg/m$^2$ and 9 under 25 kg/m$^2$. Finger prick blood samples were taken and plasma extracted for prouroguanylin measurement as described for study 1 except blood samples were only taken fifteen minutes before the breakfast (time 0 minutes) and 30 minutes, 60 minutes and 120 minutes post breakfast. These time points were chosen as the most important based on the results from studies 1 and 2. A decision was made to limit the time points for this study to minimise finger prick samples and therefore any discomfort to participants. This was both from an ethical point of view and to enhance recruitment.

2.5 Composition of meals

Contents of the meals were determined from food packaging if stated and estimated using Dietplan 7 (Forestfield Software) when not stated. Full meal contents can be found in Table 1 and 2. For Study 1 our target was to design a high calorie, unhealthy meal similar to the one used by Valentino et al. (11). However, some changes were made to accommodate for foods easily available, and regularly consumed for breakfast in the UK. The mixed macronutrient meal contained 1451 kcal and was 132 g carbohydrate (36.3% of the calories), 57 g protein (15.7% of the calories), 75 g fat (48% of the calories). The meal used in study 2 was identical to the meal in study 1 except all portions were half the size (725 kcal, 36.3%-66 g carbohydrate, 15.7%-29 g protein, 48%-3 8g fat).

The high carbohydrate breakfast used in study 3 was designed to be closer to what may have been considered traditionally to be a healthy breakfast in the UK (20), while keeping the calorie content similar to study 2. The main components were fruit (apple, banana, dried apricot), muesli and orange juice. It contained 722.5 kcal and was 134.7 g (73.9% of the calories) carbohydrate, 24.5 g protein (13.4% of the calories), 10.3 g (12.7% of the calories) fat. The main difference compared to study 2 was this meal had a much higher carbohydrate content and lower fat content. Given uroguanylin’s
reported role in regulating fluid and salt balance (21)(22)(8), it is also important to note the high

150 carbohydrate breakfast had a much lower sodium content (0.4 g versus study 1, 4.3 g and study 2, 2.15
g).

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2.6 Sample collection and Prouroguanylin measurement

Blood samples were collected using the finger prick method into Ethylenediaminetetraacetic acid
155 (EDTA) tubes, gently mixed and put on ice. The finger tip was cleaned using an alcohol swab then skin
156 punctured using a safety lance (Sarstedt, Germany), the finger was lightly pressed to start blood flow
157 (if required), then up to 0.5ml blood (per sample) was collected using a capillary EDTA tube. Before
158 processing the samples they were centrifuged at 3000g and plasma separated and stored at -20°C.
159 Samples were stored at -20°C for no longer than 8 weeks before measurement. Prouroguanylin
160 concentrations were measured using an ELISA kit from BioVendor (Human Prouroguanylin ELISA,
161 Cat. No. RD191069200R). The assay was performed according to the manufacturer’s instructions. This
162 assay has previously been validated for measurement of prouroguanylin (23). Samples from each
163 participant were measured consecutively and on the same ELISA plate. The limit of detection was 86
164 pg/ml and intra and inter- assay co-efficient of variation were 2.3% and 6.4% respectively. For study 3
165 samples from participants with healthy weights and the participants who were overweight/with obesity
166 were assayed in random order to minimise any effect of variation within the assay.

2.7 Data Analysis

For studies 1 and 2 a Repeated Measures ANOVA was used with post hoc Dunnetts test to see if there
169 was a difference between pre meal concentrations (time 0 minutes) and time points. To compare post
170 meal changes from the meals given in study 1 and 2, percentage change from baseline (time 0 minutes)
171 was calculated for each time point and comparisons made using Repeated Measures Two way ANOVA.
172 For study 3 data from all participants were analysed by Repeated Measures ANOVA post hoc Dunnetts
173 test. When data were split into two groups, data were analysed by Repeated Measures Two way
174 ANOVA with post hoc Bonferroni test. To compare data from studies 2 and 3; males from study 3 with
a BMI < 26 kg/m^2 (the maximum BMI of any participant in study 2) were selected, and for each study percentage change from baseline (time 0 minutes) was calculated for each post meal time point and comparisons made using Repeated Measures Two way ANOVA with post hoc Bonferroni test. Fasting and peak prouroguanylin concentrations from the 3 studies were analysed by independent t-test where applicable. All analysis was performed using Graphpad, Prism 6 (GraphPad Software, San Diego, CA) software. In all cases P<0.05 was considered significant.
3. Results

3.1 Study 1 - The effect of a large 1451 kcal meal on plasma Prouroguanylin concentrations in males

Seven males completed this study. The mean BMI was 23.4 kg/m\(^2\) ± 3.8 with a range between 19.8 and
24.5 kg/m\(^2\). The mean age was 23.5 years with a range between 21-35 years. Following the meal there
was a significant increase in mean plasma prouroguanylin concentrations at 60, 90 and 120 minutes
(P<0.05, 0 minutes versus 60 minutes; P<0.01, 0 minutes versus 90 minutes; P<0.001, 0 minutes versus
120 minutes) (Figure 1 A). Prouroguanylin concentrations at 15 and 30 minutes post meal were not
significantly increased versus baseline. In fact concentrations at 30 minutes appeared slightly decreased,
but this change was not significant. Feelings of hunger subsided after the meal and participants were
least hungry at 15 minutes post meal before steadily feeling hungrier with time (Figure 1 B). When
comparing the pattern of prouroguanylin concentrations and hunger levels there was no indication that
prouroguanylin concentrations increased as hunger subsided and there was not a significant correlation
between hunger and prouroguanylin concentrations.

3.2 Study 2 - The effect of a 725 kcal meal on plasma Prouroguanylin concentrations in males

Seven males completed this study. The mean BMI was 22.6 kg/m\(^2\) ± 2.0 with a range between 20.3 and
25.1 kg/m\(^2\). The mean age was 28.9 years ± 11.2 with a range between 21-51 years. Following the meal
there was a significant increase in mean plasma Prouroguanylin concentrations at 120 minutes (P<0.01,
0 versus 120 minutes) (Figure 2 A). There were no significant changes at any other time point. When
percentage change from baseline (0 minutes) was calculated for each time point and compared to the
response seen in study 1 (1451kcal meal) (Figure 2 B), there was a significant interaction between time
and meal (P<0.05), but examining individual time points and peak level, the difference between the two
meals fell just short of being significant (Peaks, 1451 kcal 172.4%± 49.7 versus 725 kcal, 129.2%±
20.6; P= 0.055).

3.3 Study 3 - The effect of a 722.5 kcal carbohydrate rich breakfast on plasma Prouroguanylin
concentrations in males and females of a healthy weight and those who are overweight/ with obesity.
10 males and 8 females were recruited for this study. The mean BMI was 25.1 kg/m² ± 4.3 with a range between 20.5 and 39.8 kg/m². The mean age was 35.9 years ± 17.7. When data were split into BMI < 25 kg/m² (healthy weight) versus BMI >25 kg/m² (overweight and with obesity) the mean BMIs were 22.4 kg/m² ± 1.2 and 28.5 kg/m² ± 4.5 respectively. The mean ages in each group were almost identical (healthy weight 36.1±19.3 years and overweight and with obesity 35.6±15.4 years).

When all data from participants were analysed as a single group plasma prouroguanylin concentrations were significantly decreased versus fasting (0 minutes) at 30 minutes post meal, (P<0.05, 0 versus 30 minutes) and increased at 120 minutes after the meal (P<0.01, 0 versus 120 minutes) (Figure 3 A). There were no significant changes 0 versus 60 minutes. When data were split by BMI < 25 kg/m² (healthy weight) versus BMI >25 kg/m² (overweight and with obesity), fasting concentrations (time 0) were significantly lower in the group who were overweight/with obesity (P< 0.05) (Figure 3 B), but there were no differences at post meal time points. In fact when analysing the percentage change from pre meal fasting concentrations (time 0) to peak concentrations the change was similar between groups.

When data were split by sex there was no significant difference in prouroguanylin concentrations, however groups were not perfectly matched with males having a higher mean BMI (26.1 kg/m² versus 24.0 kg/m²).

To compare to study 2 we then selected males only with a BMI under 26 kg/m². The mean BMI was 24.1 kg/m² ± 2.0 with a range between 22.2 and 25.8 kg/m² (n= 9). There was no significant difference found when comparing the magnitude and profile of post meal changes (Figure 3 C).
4. Discussion

We have demonstrated that prouroguanylin concentrations are significantly increased after regular size meals (725kcal) as well as following very large meals (1451 kcal). However, we found the rise is delayed and does not follow the same pattern as self-reported hunger levels; prouroguanylin concentrations remain constant or are slightly decreased up to 30 minutes after a meal, then steadily rise, remaining at, or close to peak concentrations 120 minutes after a meal. Fasting prouroguanylin concentrations are lower in the group who were overweight/ with obesity versus those of a healthy weight, but post meal changes in hormone concentrations are similar in each group. Finally, changing the carbohydrate, fat or salt content of the meal did not appear to affect the post meal increase in hormone concentrations.

Prouroguanylin, is the prohormone and circulating form of the gut hormone uroguanylin (24)(25)(11). When we started this study a single previous study had reported prouroguanylin concentrations rise after a large meal peaking at 45 minutes post meal in healthy adult males (11). In contrast we found following a fairly similar meal concentrations did not significantly change from fasting concentrations until 60 minutes after a meal, peaking at 90 minutes and remaining at similar concentrations by 120 minutes, our final time point. The reasons for the difference in findings are currently unclear. An obvious difference between the two studies is the immunoassays used to measure prouroguanylin. We used a commercially available two site ELISA produced by Biovendor. We chose this assay as it had been previously assessed and validated for the measurement of prouroguanylin (23). Valentino et al. (11) used a one site ELISA they developed that is not commercially available. There has been limited characterisation of prouroguanylin and uroguanylin in circulation. There may be breakdown products/inactive forms of prouroguanylin or indeed multiple active forms of the hormone as is the case for several other gut hormones (26)(27)(28). Immunoassay’s can often detect multiple forms of peptide hormones, including breakdown products that have only lost one or two amino acids compare to the full hormone (28)(26)(29)(30). Thus, while we know both ELISAs detect intact prouroguanylin, we do not know if either assay is detecting other as yet unknown forms of prouroguanylin or breakdown
products. A better understanding of circulating prouroguanylin and uroguanylin and their metabolites would aid further investigation in this area.

Recently a study in adolescents (aged 14-17 y) has examined prouroguanylin concentrations after meals (800-1100 kcal) (19). Prouroguanylin concentrations were assayed using the same Biovendor ELISA used in our studies. The post meal changes in prouroguanylin observed were similar to our study; they reported a decrease immediately after a meal, with concentrations then rising up to their final time point, 90 minutes post meal. This suggests regulation of prouroguanylin in adolescents and adults is similar, and that the different prourguanylin assays used are the most likely cause of the discrepancy between our study and Valentino et al.’s study (11).

Although the choice of ELISA is the most logical explanation of differences between results in our study and Valentino et al.’s, there were small differences in the meals given. Meals in both studies were high calorie, high fat and sugar, unhealthy breakfasts, but the macronutrient content did differ; our study meal had higher fat (48% versus 35%) and lower carbohydrate (36% versus 54%). It may be that this difference affected the post meal response. However, given the higher carbohydrate and lower fat content of our meal in study 3 did not lead to an earlier peak in prouroguanylin concentrations we feel it is unlikely this accounts for the difference.

A final possible but less likely explanation for the differences between the two studies is the demographic of the participants. The mean BMI of the participants was almost identical between the two studies (23.2 versus, 23.4 kg/m²). The participants in Valentino et al.’s study were older, 31.7 years versus 23.5 in our study 1 (1451 kcal). However, in our study 2 the mean age was 28.9 years, yet the pattern of prouroguanylin concentrations we observed was similar, suggesting this magnitude of difference in age is unlikely to affect prouroguanylin concentrations.

A 1451 kcal meal is large and represents 58% of the recommended daily calories for men and 72.5 % for women. Before targeting the uroguanylin system for weight loss through diet or pharmacology it is important to know whether smaller meals, more typical of real life, cause release of this hormone. We
went on to show for the first time in adults that smaller 722.5-725 kcal meals lead to a significant increase in Prouroguaylin concentrations 120 minutes post meal. In study 2 (725 kcal) the meal was identical to study 1 (1451 kcal) except all portions were halved in size and both studies just included males. A similar pattern of post meal release was observed except changes did not become significant until the 120 minute time point. When comparing the percentage change from baseline concentrations in each study there was a significant interaction between time and meal with there appearing to be a greater postprandial rise following the larger meal. However, there was not a significant effect at any individual time point, and peak concentrations fell just short of being significantly different (p=0.055).

Therefore, while our results lead us to speculate that prouroguanylin concentrations, like other gut hormones (29)(31), rise or fall in proportion to the calories consumed, we cannot confirm this pattern. We acknowledge that as our two studies were separate experiments with different participants they may not be ideal in terms of design to answer this question. Instead a paired or repeated measures design where the same participants are measured following each meal is needed.

At present we do not know whether the post meal changes in prouroguanylin concentrations we observed in study one or study two are sufficient to have any physiological effect. The reported EC50 for uroguanylin at GUCY2C is 500nM (32). The mean post meal changes in concentrations we report in study one were approximately 1000 pg/ml which equates to 83 pM. This is some way below the EC50 and casts doubt on whether changes would be sufficient to affect GUCYC signalling. However, it is hypothesised prouroguanylin is processed to active uroguanylin in tissues such as the hypothalamus (11) and we do not know the uroguanylin concentrations that may accumulate in these tissues. Furthermore, circulating post meal changes of other gut hormones such as PYY (33) fall along way below the reported EC50 (34), yet they are thought to play a role in the regulation of feeding. To help us understand the significance of post meal changes in prouroguanylin, it important for future studies to establish the circulating concentrations required to elicit any potential effects on body mass or feeding.

Our final study recruited both sexes and examined prouroguanylin release following a mixed macronutrient high carbohydrate meal (722.5 kcals). This meal contained cereal and fruit, and although
still relatively high energy for a breakfast could be consider closer to a traditional view of a healthy
breakfast (20). In accord with the first two studies prouroguanylin concentrations were increased versus
baseline 120 minutes post meal. However, in this study hormone concentrations were suppressed versus
baseline 30 minutes after the meal. This result was not entirely unexpected as in study 1 a similar pattern
was observed but the reduction at 30 minutes was smaller and non significant. While generally in accord
with the pattern observed in the first two studies, this is a clear difference to the pattern observed by
Valentino et al. However, results were similar to those observed by Di Guglielmo et al. (19) in
adolescents. This early post meal change could relate to urogunaylin’s role within the gut regulating
fluid and electrolyte balance (8)(35) or gut motility. Based on evidence from the guanylate cyclase
agonist, Linactotide, unlike other gut hormones such as PYY (36)(37), uroguanylin is likely to speed
up rather than slow down gastrointestinal transit (38).

Study 3 also examined the effect of BMI on prouroguanylin concentrations. In accord with Rodríguez
et al. (17), the group who were overweight/ with obesity had lower fasting prouroguanylin
concentrations. However, there was not a significant difference between the groups at later time points.
In fact, in contrast to other gut hormones such as PYY (39)(29), post meal changes in prouroguanylin
were at least equal in the group who were overweight/ with obesity, with a trend towards a greater rise
in concentrations (0 versus 120 minutes) in the group who were overweight/ with obesity. This finding
is interesting as the reduced post prandial release of PYY and suppression of ghrelin in those with
obesity has been hypothesised to be important in post meal satiety (29)(31). Based on our results the
same hypothesis could not be related to prouroguanylin. Furthermore, given some rodent studies report
uroguanylin administration suppresses feeding (13)(11), the robust post prandial response observed in
our group who were overweight/with obesity may suggest potential in targeting prouroguanylin,
through diet or pharmacological activators of nutrient receptors to treat obesity. However, we are
currently still some way from knowing whether prouroguanylin is a suitable target for obesity therapies.
For example, our study suggests prouroguanylin is not involved in the initial suppression of hunger
after a meal, but it still could be involved in later feeling of satiety and this needs to be investigated.
Our results examining the effect of BMI on prouroguanylin are again comparable to those recently
reported by Di Guglielmo et al. in adolescents (19). These results are at odds with a study in rodents that suggested diet induced obesity suppressed post prandial uroguanylin secretion in mice (14). Further investigation is needed to see if there is a species difference or perhaps whether only specific diets or more severe obesity affect post meal secretion. Given its proposed role in body mass regulation it is possible the decreased fasting concentrations of prouroguanylin observed in obesity may make it harder for people to lose weight. The data from rodents (14) suggests obesity leads to low prouroguanylin concentrations, rather than being the initial cause.

Rodents studies have demonstrated uroguanylin secretion and expression are regulated by leptin (15); with uroguanylin lower following leptin administration or fasting. We would predict our healthy BMI group would have lower leptin concentrations than the group who were overweight/with obesity and this may contribute to the difference in the fasting prouroguanylin concentrations. However, higher leptin or possible leptin resistance in the adolescents and adults who were overweight/with obesity, studied by Di Guglielmo et al. (19) and us does not appear to affect post meal changes.

Some recent studies have suggested variation in the uroguanylin system between the sexes (19) (40). One study reported a negative correlation between plasma fasting prouroguanylin concentration and BMI in girls, but a positive correlation in boys (40). Another study found female but not male adolescents with obesity had lower prouroguanylin concentrations (19). In our study numbers were too small to examine the effect of BMI in each sex. Splitting our group from study 3 by sex alone suggested there was no significant difference in prouroguanylin concentrations between males and females. However, our groups weren’t ideally matched as our male group had a higher mean BMI and this may have affected the results of our comparison. It is also possible that in females prouroguanylin concentrations vary across the menstrual cycle or are affected by contraception or childbirth. We acknowledge that while we excluded those who were pregnant, breastfeeding or had recently given birth, we did not record or control for other factors relating to the female reproductive system. Further larger studies considering these factors are required to clarify whether there are differences in prouroguanylin concentrations between adult males and females.
Finally, we carried out a post-hoc analysis comparing the percentage changes from baseline following meals given in study 2 and study 3. For this we only included male subjects from study 3 within the same BMI range as subjects from study 2. The meal given in study 2, versus study 3, had much higher fat (48% versus 12.7%) and salt (2.1 grams versus 0.4 grams) content, and lower carbohydrate content (36% versus 73.9%). Despite these differences there was no statistically significant difference in the overall post meal pattern and the magnitude of the change from baseline to 120 minutes was very similar (23% vs 28%). This suggests that variation in fat, carbohydrate and salt content of the meals did not have a major influence on post prandial release. Given uroguanylin’s role in salt regulation (24)(41), it could be hypothesised, that dietary salt may affect circulating prouroguanylin concentrations. However, our findings related to dietary salt are in accord with a recent publication demonstrating dietary salt influenced urogunaylin RNA expression in the kidney (22), but not the proximal small intestine (the predicted source of circulating prouroguanylin).

4.1 Conclusion

This is the first study to examine prouroguanylin release in adults of a healthy weight and those who are overweight/with obesity following large and medium sized meals. The results suggest that immediately post meal prouroguanylin concentrations remain stable or decrease, then increase steadily to above fasting concentrations, remaining at, or close to peak concentrations 120 minutes after ingestion of a meal. Fasting concentrations are lower in those who are overweight/with obesity, but the magnitude of the post meal rise of the hormone is similar. These results are in accord with recent observations in healthy weight and adolescents with obesity (19). Our study suggests total calorie content of a meal may influence prouroguanylin concentrations, but variation of fat, carbohydrate and salt content do not appear to have a major effect. However, these were examined using a post-hoc analysis of different experiments, so need to be confirmed by further studies. Overall our study has increased understanding of the regulation of prouroguanylin and may help assess whether it is a viable target for obesity therapies.
Declaration of interests statement- MP, HW, DH, HAHN and SR have no conflicts of interest to declare.

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Authors Contributions- MP conceived study idea. All authors were involved in the design of the studies. MP, HW, DH, HAHN performed the studies and analysed the data. MP and SR supervised the studies and data analysis. All authors contributed to the writing of the manuscript.

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**Figure Legends**

**Figure 1.** Plasma prouroguanylin concentrations A, and visual analogue hunger scores, B, from healthy males (n=7) pre (0 minutes) and post a 1451 kcal. Data presented are the mean with error bar representing the standard deviations. Repeated ANOVA was used with post hoc Dunnett’s test to see if there was a difference between individual time points (* =P < 0.05 versus pre meal, 0 minutes, ** = P<0.01 versus pre meal, 0 minutes.)

**Figure 2. A.** Plasma prouroguanylin concentrations from healthy males (n=7) pre and post a 725 kcal. Data presented are the mean with error bar representing the standard deviations. Repeated ANOVA was used with post hoc Dunnett’s test to see if there was a difference between individual time points (** =P<0.01 versus 0 minutes.). B. Comparison of the percentage change from fasting (Time =0) at each post meal time point, following the 725 kcal meal versus the 1451 kcal meal (n=7 for each study).

**Figure 3.** Plasma prouroguanylin concentrations following a 722.5 kcal high carbohydrate meal, from a mixed sex group including, 9 healthy weight adults and 9 overweight or with obesity. Data presented are the mean with error bar representing the standard deviations. A Includes all participants as a single group. Repeated ANOVA was used with post hoc Dunnett’s test to see if there was a difference between individual time points (* =P < 0.05 versus 0 minutes, ** = P<0.01 versus 0 minutes.). B Shows the data split into the participants with a healthy BMI (n=9) and those who were overweight or with obesity (n=9). Fasting concentrations alone were significantly different between groups (* =P<0.05). But there was no overall effect of BMI group when data were analysed by two way ANOVA of repeated measures. C Study 2 versus Study 3, Males BMI under 26 kg /m² only. Comparison of the percentage change from fasting (Time =0) at each post meal time point, following the 722.5 kcal high carbohydrate meal (study 3) versus the 725 kcal high fat meal (study 2) (n=7-9).
Figure 1.

A

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B

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Not hungry at all

Hunger score  mm
Figure 2

A

B

**
Figure 3.

A

B.
C.

![Graph showing Prouroguanylin % of baseline over time for study 2 high fat and study 3 high carbohydrate. The x-axis represents time in minutes (0, 30, 60, 90, 120) and the y-axis represents Prouroguanylin % of baseline (0 to 160). The graph includes error bars for each data point.]

- **study 3 high carbohydrate**
- **study 2 high fat**