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PhD in Food Science and Human Nutrition

Department of Clinical Medicine and Surgery

PhD Thesis

Evaluation of different types of pasta di Gragnano on appetite regulation and metabolic profile

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1. Introduction

High-carbohydrate foods mainly in the form of whole grains (WG) and legumes provide a higher content of dietary fiber and other nutritionally bioactive compounds compared to refined products. (Fardet et al. 2010; Okarter et al. 2010; McCrory MA et al., 2010). The presence of dietary fiber has been shown to delay gastric emptying and thereby potentially slowing glucose absorption (Weickert MO and Pfeiffer AF, 2008), affecting appetite sensation also improving glycemic response.

Among cereal products, pasta is widely consumed in Italy, particularly in the south, where it represents the main source of resistant starch (Brighenti et al., 1998). Pasta has a low glycemic index (GI) (Atkinson et al., 2008) and whole grain pasta (WGP) is a good source of dietary fiber and includes oligosaccharides, phenolics (phenolic acids, alkylresorcinols, and flavonoids), lignans, and phytic acid (Jonnalagadda SS et al., 2011; Chillo S et al., 2008; Okarter et al. 2010).

So far, many studies evaluated different types of bread but very few investigated on alternative pasta meals (Kristensen M. et al, 2010); therefore, this thesis focused on the health-promoting effect of

different type of meal based on the Italian pasta, produced in Gragnano, Naples (Italy) and classified as Protect Geographical Indication (PGI).

More specifically, in this thesis I investigated the effect of different types of pasta meals, including a mixed meal with pulses, on appetite regulation and glucose response, with special attention on the effect of WGP on satiation, satiety and food intake.

During my PhD, two research protocols have been carried out: one in Naples (Italy) and the other one in Copenhagen (Denmark), which together seek to accomplish the main objective.

1.1 Whole-grain food: Glycemic Index (GI) and Appetite

Wholegrain (WG) foods are receiving greater attention regarding their ability to reduce the risk of type 2 diabetes (T2D), cardiovascular diseases (CVD) (Ye EQ et al, 2012; Priebe et al. 2008; Wirstrom et al, 2013) and some types of cancer (Kyrø et al 2014). WG are defined as consisting of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportions as they exist in the intact caryopsis covering the *Graminaceae* family as proposed by the American Association of Cereal Chemists in 2000 (AACC, 2000).

Regular consumption of WG may potentially improve metabolic abnormalities associated with the development of T2D and CVD (Harris et al, 2010; Ye EQ et al., 2012) and affect appetite, playing a role in body weight regulation. However, a recent meta-analysis suggests a small benefit of WG foods on fat mass percentage; nevertheless, no effect was found on body weight (Pol et al., 2013).

Generally, WG foods have a lower GI than refined grain products due to a more intact botanical structure of the seeds (Liu S. 2002)although this is not always the case (Kristensen et al., 2010). The glycemic index (GI) concept was introduced by Jenkins et al. (1981) to classify

different sources of carbohydrate-rich foods to their effect on post prandial glycaemia. Low GI foods were classified as being digested and absorbed slowly compared to high GI foods, resulting in a different glycemic responses (Brouns et al., 2005). However, the GI of a single food could be affected by several factors such as the quality and quantity of carbohydrate, the industrial process, the presence of dietary fibers, the macronutrients composition, the presence of acid compounds, etc.

Thus, some studies reported that bread products with added organic acids could be useful in improving glucose tolerance to starch and in lowering postprandial glucose and insulin responses (Liljeberg et al., 1995; 1996). Improved glucose tolerance may result from the presence of organic acids that may delay gastric emptying or inhibit amylase activity and, hence, reduce the rate of carbohydrate absorption in the small intestine (Liljeberg et al., 1996).

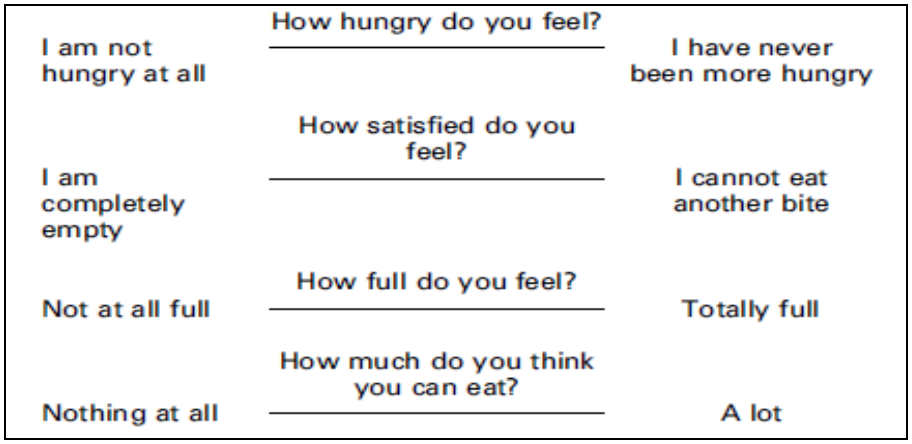
However, the mechanism underlying the beneficial effects of low GI food is not completely understood. It could be due to a decreased postprandial blood glucose response resulting from a delayed gastric emptying and/or starch digestion and absorption (Priebe et al., 2008). In addition, the lower rate of nutrient digestion and absorption, typical

of low GI food, seem to trigger signals involved in satiation and satiety.

Potentially, WG products may affect appetite with increased postprandial satiety and reduced energy intake (EI) at the subsequent meal (Mollard et al., 2014; Hartvigsen et al., 2014; Brighenti et al., 2006). WG may also affect the EI within meals by inducing satiation, i.e. process that leads to the termination of eating or intra-meal satiety (Blundell et al., 2010).This has been ascribed to their higher food volume and lower energy density resulting from the higher dietary fiber content.

The definition of appetite covers the whole field of food intake, selection, motivation and preference (Blundell et al., 2010). Appetite in humans can be measured in two ways. First, it can be assessed through the help of subjective ratings using the visual analogue scales (VAS). They are reproducible, sensitive of food components and predictive of food intake (Flint et al., 2000). VAS example was reported in **Figure 1** (Flint et al., 2000).

Figure 1



Second, appetite can be measured by *ad libitum* food intake; that is the amount of food eaten within a specific context and can be considered as a measure of appetite. The reproducibility is high (Arvaniti K et al., 2000; Gregersen et al., 2008) and the validity of this method increases when subjective appetite and palatability ratings are included.

Health-promoting effect of WG is thought to be due to different mechanisms depending on their specific properties, hormonal effects and colonic fermentation. Furthermore, WG might influence resting energy expenditure (REE), lowers fat oxidation and increases feeling of fullness (Raben et al., 1994) and the inflammation state (Nilsson et al., 2008).

1.1.1 Chemical and physical properties

The intrinsic properties of WG foods concerns the ability to bind water and form a viscous solution that delays gastric emptying and limits glucose absorption thus leading a lower blood glucose response (Weickert MO &Pffeir AF, 2008). The mechanism is that dietary fibers, especially viscous dietary fibers, induce thickening when mixed with liquids and absorb large quantities of water increasing gastric volume and prolonging gastric emptying (Kristensen M. et al., 2011). The increased stomach distension may trigger afferent vagal signals of fullness (de Graaf et al., 2004) making dietary fibers act through mechanical factors. Many studies linked ingestion of viscous dietary fibers to delayed gastric emptying (Benini et al., 1995, Bergmann et al., 1992 and Marciani et al., 2000), but not all (Shimoyama et al., 2007).

However, a recent study based on MRI observations confirmed that whole wheat bread delayed gastric emptying compared to iso-caloric refined meal due to the presence of bran and of arabinoxylan (AX) that can bind water up to 9 times more, increasing the viscosity of a meal (Marciani et al., 2013).

Stomach has long been associated with regulation of food intake through a complex, interacting network of gut regulatory peptides,

hormones, and the autonomic and enteric nervous systems (Park et al., 2005). Hence, the volume of food, rather than the energy content, is one of the most important determinant of meal size and food intake, which can affect satiation and potentially reduce the glucose absorption. Therefore, the regulation of food intake, determined by meal size and frequency of meals, was taken into account as the main factor in the maintenance of weight or the development of obesity (Steinert et al., 2012).

1.1.2 Gut hormones

The hormonal effects of WG are mediated by gut hormones, which play a critical role in relaying signals of nutritional and energy status from the gut to the central nervous system, in order to regulate appetite and food intake. Cholecystokinin (CKK), glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and peptide YY (PYY) act through distinct yet synergistic mechanisms to suppress appetite, whereas ghrelin stimulates food intake. In addition, it has been proposed another classification considering their effect on appetite as follow: CKK and GLP-1 mainly involved in the intra-meal satiety or satiation while, GIP and PYY considered as biomarker of inter-meal satiety (Blundell et al., 1991;

Halford J & Blundell J, 2000). However, the distinction between satiety and satiation and the involvement on meal termination and meal initiation or both, may not be as strict as suggested (de Graaf et al., 2004).

Below, we briefly described how fiber consumption may affect CCK, GLP-1, GIP and ghrelin in relation to appetite and glucose response.

CCK was the first gut hormone found to play a role in food intake (Liddle R.A. et al., 1985). It is mainly released in the presence of fat (i.e., long-chain free fatty acids) or protein (i.e., amino-acids) by the I cells in the duodenum and CCK levels rise within 15 minutes after meals (Liddle et at., 1985). The main mechanism by which CCK affect appetite is the delay of gastric emptying (Melton PM et al., 1992), therefore a full stomach represents a necessary condition and for this reason is considered as a biomarker of satiation. In addition, dietary fiber could modulate the CCK secretion; in fact, there is a clear evidence that the consumption of a fiber rich-meal induces higher plasma cholecystokinin levels and a longer action time (Bourdon I et al., 1999).

GLP-1 is produced from L cells in the intestine in response to nutrient intake, i.e. carbohydrate and fat (MacIntosh CG et al., 1999). GLP-1 exists in two biologically active forms: GLP-1 (7–37) and GLP-1 (7–

36) amide, the latter being the major circulating form in the blood (C. Orskov et al., 1994) and both are rapidly degraded (Kieffer TJ et al., 1995). Circulating GLP-1 levels rise at 20 to 30 minutes after meals and fall in the fasted state, but recent evidence also suggests that levels rise in anticipation of a meal (T. P. Vahl et al., 2010).

GLP-1 reduces food intake, suppresses glucagon secretion, and delays gastric emptying (D. E. Cummings and J. Overduin, 2007). GLP-1 was affected by non-digestible carbohydrate as well as by SCFAs produced by bacteria fermentation, which stimulate L cells via G-protein coupled free fatty-acid receptor 2 and enhance GLP-1 secretion (Tolhurst et al., 2012). Therefore, GLP-1 secretion could affect both satiation (delays gastric emptying) and satiety (through SCFAs, which may increase GLP-1 secretion influencing food intake). Finally, GLP-1,together with GIP, exerts a potent incretin effect stimulating insulin secretion in a glucose-dependent manner following ingestion of carbohydrate (Yabe D & Seino Y, 2011) and it is involved in the post-prandial glucose response. Both GIP and GLP-1 stimulate insulin secretion acting directly on pancreatic islets by binding to their specific receptor. Interestingly, insulinotropic actions of GLP-1 were observed when GLP-1 was infused at pharmacological

levels, while is not the case with GIP (Vilsboll et al., 2002; Mentis et al., 2011).

GIP secretion from K cells is enhanced in response to ingestion of meals (Vollmer et al., 2008), in particular in response to fat and protein ingestion (Carr et al., 2008). GIP shares with GLP-1 its insulinotropic effect and as a result, these incretin hormones are also essential in the regulation of postprandial glycaemia (Juntunen KS et al., 2002). However, a few studies have investigated GIP response in relation to appetite (Verdich C et al., 2001, Vozzo R et al., 2005) with conflicting data, which did not support a major role for GIP in appetite regulation (de Graaf et al., 2004). Yet, GIP showed either low responses (Hagander B et al., 1984; Gatenby SJ et al., 1996) or no effects (Levitt NS et al., 1980) after fiber consumption. This result has been confirmed by a recent study in which rye kernels bread produced smaller GIP responses than refined wheat did, wheat arabinoxylan and β-glucan bread (Hartvigsen et al., 2014-b), suggesting that GIP might be not affected by fiber consumption.

Finally, ghrelin is abundantly synthesized in the fundus of the human stomach (Ariyasu H et al. 2001). It is the only known circulating orexigenic hormone that quickly decreases after the meal, increases

during fasting returning to pre-meal concentrations before the next meal is initiated (Cummings DE et al., 2001), and *it* may serve as an excellent biomarker of satiety. Plasma ghrelin concentrations in normal-weight subjects decrease after oral and intravenous administration of glucose, but the intake of an equivalent volume of water does not influence ghrelin concentrations (Shiiya T et al., 2002), which suggests that ghrelin secretion is not affected by stomach distension. Furthermore, ghrelin secretion might depend on macronutrients because it has been shown to be suppressed by carbohydrates more than by fat (Monteleone et al., 2003;Tannousdit El Khoury et al., 2006); however, this is not always the case. On the opposite, other authors *have* argued that ghrelin suppression is dependent on the energy content of the meal consumed (Callahan H et al., 2004) and not on the proportion of macronutrients. The effect of WG on ghrelin secretion is not clear. Different studies showed that whole grain foods seemed not to affect ghrelin secretion compared to refined counterpart (Hartvigsen et al., 2014a; 2014b; Giacco R et al., 2010). However, Nilsson et al., (2013) observed that serum ghrelin concentration was significantly suppressed in the morning after brown beans evening meal in comparison with the white bread.

1.1.3 Cytokines effect

Circulating concentrations of inflammatory markers, such as tumor necrosis factor (TNF)-α, IL-6, IL-10 and C - reactive protein (CRP), which are usually elevated in obese individuals, may contribute to vascular damage (Burdge GC & Calder PC, 2005) and insulin resistance (Rask-Madsen C & King GL. 2007). It has been hypothesized that a diet with a high fiber content and low glycemic index may influence markers involved in the inflammation state (Nilsson et al., 2008), but data are still conflicting.

Some epidemiological studies have demonstrated an inverse association between WG consumption and CRP in non-diabetic (Lutsey PL et al., 2007, Masters RC et al., 2010) and diabetic (Qi L et al., 2006) individuals, but not all studies are consistent (Jensen et al., 2006). Intervention studies also present mixed data regarding effects of WG on CRP and other inflammatory markers. Interestingly, WG intakes were similar or higher (Andersson A et al., 2007; Brownlee IA et al., 2010) in the studies that reported no effects compared to those reporting positive effects. It is notable that studies conducted in healthy volunteers mainly report no effect (Andersson A et al., 2007; Brownlee IA et al., 2010), whereas those conducted in non-healthy

subjects usually report positive effects (Katcher HI et al., 2008; Moazzami AA et al., 2011).

The mechanism underlying the health-benefits of WG consumption on the inflammation state might be related to the concentration of wheat polyphenols, but this is still poorly understood.

1.1.4 Colonic fermentation

Another proposed mechanism on appetite regulation is the effect of short-chain fatty acids (SCFA; acetate, propionate, and butyrate) and gases (hydrogen, methane and carbon dioxide) from fermentation of non-digestible carbohydrates by the colonic microbiota (Costabile et al., 2008; Cani et al., 2006, 2009). SCFAs are rapidly absorbed from the colonic lumen and metabolized by colonic epithelial cells, but some of them also enter the portal and peripheral circulation. Although the liver extracts about 75% of acetate, 90% of propionate, and 95% of butyrate from the portal vein, higher concentrations of SCFAs in the peripheral circulation have been observed after ingestion of non-digestible carbohydrates (Wolever et al., 2000).

Nilsson et al, (2008) observed that colonic fermentation of non-digestible carbohydrate, measured by breath hydrogen excretion, was involved in modulating satiety and may have positive implication on

glucose tolerance. The mechanism might be explained by SCFAs activities that act as a local nutrient source but can also trigger cell-specific signaling cascades by activation of the G-protein–coupled free fatty acid receptor ffar2 (grp43) and ffar3 (gpr41) affecting L cells to enhance the release of peptides such as GLP-1 and PYY (Zhou et al., 2008; Tohlurst et al., 2012). Hartvigsen et al., (2014a) showed that wheat arabinoxylan meal increased butyrate and acetate concentrations after 6 hours and reduced glucose and insulin response. Then they showed that butyrate correlates with satiety which agrees with previous results (Nilsson et al., 2008 and 2013); GLP-1 also positively correlated with both propionate and acetate, as well as with feelings of satiety and fullness, suggesting the stimulatory effect of SCFAs on GLP-1 as a possible mechanism involved in appetite regulation. Previously, Nilsson et al., 2008 observed also that GLP-1 and glucose response were inversely correlated, suggesting that low GI whole-grain foods were able to facilitate glycemic regulation not only after the acute meal, but also at the meal ingested 10 hours later, through the action of colonic fermentation, likely being enhanced by prebiotic effect of non-digestible carbohydrate.

1.1.5 Diet-induced Thermogenesis

The amount of dietary fiber in the diet may influence diet-induced thermogenesis due to an altered glycemic and insulinemic response after the meal (Scalfi et al., 1987; Raben et al., 1994). Diet-induced Thermogenesis (DIT) is the increase in Energy Expenditure (EE) engendered by the energy used in the postprandial period in the process of absorbing, metabolizing, and storing ingested nutrients (Tappy L, 1996; Westerterp KR, 2004). It is commonly expressed as percentage and together with basal metabolic rate (BMR) and activity-induced thermogenesis, is one of three component of daily energy expenditure (Westerterp KR, 2004). DIT is usually measured through indirect calorimetry and although it is the smallest component, could play a role in the development and/or maintenance of body weight.

In healthy subjects over a 24 h period, DIT represents about 10% of the total amount of energy expended (Ravussin et al, 1986). Measured DIT of the different nutrients are 0-3% for fat, 5-10% for carbohydrates and 20-30% for proteins (Ravussin et al, 1983; Thiébaud et al,1983a,b; Tappy and Jéquier, 1993). There also seems to be a line of hierarchy in macronutrient oxidation rate during post-prandial state as follow: alcohol>protein>carbohydrate>fat (Stubbs et al., 1999). However, Raben et al., 2003 showed that DIT was

increased after the alcohol and protein meals; however, the oxidation hierarchy revealed difference for alcohol compared to macronutrients, but not among proteins, carbohydrates and fats. The higher thermogenesis of proteins is due to the large amount of adenosine triphosphate (ATP) used in the postprandial period in the process of metabolizing and storing them (van Baak, 2008). This might explain the high satiating effect of proteins, suggesting DIT as one of the mechanisms that may influence appetite. Some studies on appetite sensation and thermogenesis found that DIT correlated directly with satiety and inversely with hunger ratings (Westerterp-Plantenga et al., 1999; Crovetti et al., 1997) due to protein intake. However, a recent meta-analysis on appetite and thermogenesis, found no evidence supporting an association between satiety and DIT when the protein intake ranged between 11-30% of total energy intake.

On the other hand, it has been reported hat high-fiber meal lowered the DIT of meal, influencing appetite. Scalfi et al., (1987) showed a reduced DIT after a fiber enriched meal and Raben et al., (1994) observed decreased postprandial thermogenesis and fat oxidation but increased fullness after a high-fiber meal compared with a low-fiber meal. However, DIT difference between a high-fiber meal vs. a low-fiber meal was not reflected in significant differences of post-prandial

glucose and insulin concentrations, therefore, it is unlikely that DIT was altered by the presence of the dietary fiber per se.

Finally, differences between DIT values of different macronutrients are difficult to assess and further data are required to determine any relation between DIT, dietary fiber and appetite.

1.2 Effect of pulses consumption on appetite and glucose control

Dietary pulses are the edible seeds of legumes or pod-bearing plants and include beans, chickpeas, yellow peas and lentils. Pulses intake has been related to higher-quality diets, including fiber, protein, folate, Zn, Fe and Mg, and lower intakes of saturated fat and total fat. They also contain several anti-nutrients that have been suggested to play a role in energy regulation (McCrory MA et al., 2010). In particular, they are high in fiber and provide a low energy density food (1.3 kcal/g or 5.3 kJ/g). Pulse carbohydrates are slowly digested and the GI typically range from ~29 to 48 (Jenkins et al., 1981).

Regular consumption of pulses (half cup per d) has been shown to reduce appetite and acute food intake (Wanders AJ et al., 2011; Paddon-Jones D et al., 2008). In addition, evidence from epidemiological studies has reported that legumes intake can lead to weight loss and improved glycemic control (Jenkins et al., 2012; Sievenpiper et al., 2009). Short-term studies have shown that pulses, when consumed alone, induce high satiety (Wong CL et al. 2009) and being a low glycemic food (Wong et al., 2009; Jenkins DJ et al. 1980; Nestel P et al., 2004) might have the ability to lower the plasma glucose response at a later meal (Jenkins et al., 1982).

However, pulses, at least in Southern Italy, are commonly consumed with pasta, rice and bread, but it is not clear whether the incorporation of pulses into high-carbohydrate meal influence the beneficial effects. Mollard et al. (2012) showed that pulses added to pasta contributed to earlier satiation and lower plasma glucose following the meal and after the meal, but depends on the type of pulses. Nilsson et al. (2013) showed the positive effect of an evening meal based on brown beans compared to white bread on plasma glucose, insulin and appetite sensation within 11-14 hours. Furthermore, they observed an increase of total SCFAs and breath hydrogen excretion, indicating involvement of colonic fermentation. According to a recent meta-analysis, regular consumption of dietary pulses may increase acute satiety in healthy participants. Although, this increase in satiety does not induce a detectable decrease in second meal food intake, it may not explain some of the long-term weight loss benefits associated with dietary pulse consumption (Li et al., 2014).

Hence, pulses consumed with pasta could be a valid strategy to restrain appetite and influence the metabolic state post-prandially.

1.3 Pasta di Gragnano – *Protected Geographical Indication* (PGI)

Pasta consumed in the studies of my thesis is the "Pasta di Gragnano" PGI according to the Council Regulation (EC) No 510/2006 http://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/3335.

Pasta di Gragnano is a product obtained from a mixture of hard-wheat semolina with water from the local aquifer.The area of production and packaging of the product – Protected Geographical Indication PGI – Gragnano Pasta includes all the territory of Gragnano, in the Province of Naples. The traditional production process consists of the following main steps: dough and kneading, extrusion, drying, cooling and stabilization, and finally packaging.

Drying pasta is the most delicate step of the entire production cycle and it is involved in achieving a high quality product. It varies depending on the shapes of pasta and still occurs at a temperature between 40 and 80 °C for a period of timebetween 6 and 60 hours.

2. Objective of the thesis

The thesis aimed to examine the effects of different meals based on pasta PGI such as 1) refined pasta, 2) whole grain pasta, 3) lemon refined pasta and 4) pasta with brown beans,on appetite regulation and metabolic response. In addition, we paid a special attention to the effects of whole grain pasta PGI on *ad libitum* food intake both within meal and at the subsequent meal compared to refined pasta PGI. Hence, we carried out two research protocols:

- Carina project
- Pastaly project

3. Carina project: objective

Carina project aimed to investigate the metabolic effects of different types of pasta meal in order to find some nutritional strategies, using pasta di Gragnano. This study tested the hypothesis that the following alterations of a traditional pasta meal would affect appetite and glycemic response by: 1) Replacing refined pasta for wholegrain pasta, 2) Addition of acid in the form of lemon juice to refined pasta, and 3) Substituting a traditional tomato-based pasta sauce with a legume-based sauce (mixed meal). In addition to appetite sensation and glycemic response, meal-induced thermogenesis and lipid response were also measured.

3.1 Materials and Methods

3.1.1 *Subjects*

Eight healthy volunteers (4 females and 4 males) were recruited for the study. Inclusion criteria were BMI of 20-27 kg/m^2 and age 25-65 years. Exclusion criteria were: known chronic illnesses, diabetes, hypertension, hyperlipidemia, smoking, athletic physical activity, daily use of prescription medication, use of dietary supplements, allergies or food intolerances or dislikes of relevance to the composition of meals.

3.1.2 *Study design*

Four different iso-caloric lunch meals were tested in all participants who underwent one test day at least one week apart. The day before the test days subjects were instructed to follow a standardized fasting procedure, i.e. abstention from alcohol and hard physical activity. On the test days, participants consumed a standardized breakfast meal at home within 10-12 min at 8.00 am and then reached the Department of Clinical Medicine and Surgery, Clinical Nutrition Unit, at Federico II University, in Naples (Italy) at 11.00 a.m. by a calm mode of transportation. At their arrival, participants were weighed to the nearest 0.1 kg with a platform beam scale and height was measured to the nearest 0.5 cm using a wall-mounted stadiometer (at the first visit only). After 5 min rest in the supine position, REE was measured by indirect calorimetry with a canopy system for 20 minutes and hereafter, a fasting blood samples were taken and the subjects' appetite sensation was assessed using a Visual Analogue Scale (VAS) questionnaire (time point 0). Hereafter the test meal was served and consumed within 15 minutes. Test meal palatability was assessed using VAS. Blood samples and appetite sensation were measured every hour for a 4-hour period (time points 60, 120, 180 and 240). Postprandial Energy Expenditure (EE) was measured for a period of 3

hours, counted from the end of test meal, with breaks of 10 minutes each hour for blood sampling and appetite ratings.

3.1.3 *Experimental meals*

Tomato and legume based sauces were prepared by "La Fabbrica della Pasta di Gragnano", frozen and then delivered at kitchen laboratory in Naples. Tomato sauce recipe for 5 servings was as follow: 700 ml tomato puree, 30 g, 8 g garlic, 2 g fresh basil, 4 g salt and it was cooked for about 60 min. As legumes, we used brown beans Roviotti, prepared in a single batch as follow: 500 g dry beans, 60 g extra-virgin olive oil, 8 g garlic, 4 g salt and cooked for about 2 hours.

The test meals (**Table 1**) were prepared at the kitchen laboratory of Federico II University and consisted of four pasta meals as follow: 1) 100 g refined grain pasta (cooking time 7 min) with 100 g tomato sauce (RG+T); 2) 100 g WG pasta (cooking time 6 min) with 100g tomato sauce (WG+T); 3) 100 g lemon juice-supplemented refined grain pasta (cooking time 6.30 min) with 100 g tomato sauce (LRG+T); and 4) 70 g refined grain pasta with 150 g legume based sauce (RG+L).

Table 1. Macronutrient composition of the four tested meals.

	RG+T	WG+T	LRG+T	RG+L
Meal weight (g)	333	312	316	312
Meal volume (ml)	570	570	570	570
Uncooked pasta (g)	100	100	100	70
Tomato sauce (ml)	100	100	100	-
Roviotti brown beans dry (g)	-	-	-	50
Total Energy (kJ/ Kcal)	1800	1766	1800	1884
Energy from carbohydrate (%)	75,2	71,7	75,2	69
Energy from fat (%)	12,8	13,9	12,8	15
Energy from protein (%)	12	14,4	12	16
Fiber content (g)	3,8	8,1	3,4	10.5

3.1.4 *Subjective appetite and palatability measurements*

Visual analogue scales (VAS) were used to assess subjective appetite sensation (hunger, satiety, fullness, prospective food consumption, thirst and well-being) and palatability (appearance, smell, taste, off taste and overall pleasantness) of the meal test. VAS are 100 mm in length with words anchored at each end, expressing the most positive and the most negative rating (Flint et al., 2000).

3.1.5 *Meal-Induced Thermogenesis (MIT) measurement*

Resting and post-prandial EE were measured using indirect calorimetry (Vmax29, Sensor Medics, Anaheim, California)with a ventilated hood and a canopy system. The measurement was conducted under standardized conditions as described previously (Marra et al., 2007). Energy expenditure was calculated with the abbreviated Weir's formula, neglecting protein oxidation (Weirs, 1949). Pre-meal REE was determined for 30 min, and the final 20 min were considered for the analysis. In addition, the first 5 min of each postprandial EE measurement were excluded from the calculations in order to minimize the movement effects. Meal-induced thermogenesis (MIT) is the energy required for the processing and digestion of food consumed. MIT was calculated as the increase in EE over 3 h after test meals above the pre-meal REE used as baseline measure. It was expressed as both absolute value (kJ/3h) and percentage of the total energy of the test meal (Ruddick et al., 2013).

3.2.6 *Blood samples analysis*

Blood samples were drawn from an antecubital vein, before the test meal and post-prandially at different time points (60, 120, 180 and 240 min). Blood specimens were immediately centrifuged and stored at -80°C until the analysis. Triacylglycerols (TAG) and plasma glucose concentrations were measured by automated methods (ABX Pentra 400, HORIBA ABX - Rome, Italy). The assays of c-peptide and insulin serum concentrations were performed by automatic ELISA Triturus analyzer (Biomedical Diagnostics - Antwerpen, Belgium) and ELISA kits (Diasource Immunoassays S.A. - Louvain la Neuve, Belgium). For glucose, triacylglycerols, insulin and c-peptide the Coefficient of Variation (CV) was: 0.6 %, 1.7%, 5.1 % and 4.8%, respectively.

3.1.7 *Calculations and statistical analysis*

The incremental area under the curve (iAUC) was calculated as the net increment/decrement area above/below baseline value and AUC as the total area under the curve using the trapezoid model. All statistical analyses were performed using the Statistical Analysis System software package, version 9.4 (SAS Institute Inc., Cary, NC, USA). All dependent variables were controlled for homogeneity of variance and normal distribution by investigation of residual plots and normal

probability plots and histograms, respectively. If the distribution of a variable was skewed, it was log-transformed prior to analyses and back-transformed before presentation. A repeated-measures ANCOVA analysis was used to examine the effect of meal and time and the meal × time interaction term on the postprandial response in PROC MIXED, where subject was modeled as a random variable and corresponding baseline value and BMI were modeled as covariates, and sex was included as fixed variable. An ANCOVA was used to examine the effect of meal on palatability and iAUC/AUCs in PROC MIXED, where subject was modeled as a random variable and the baseline values and BMI were modeled as covariates, and sex included as a fixed variable. The meal × time interaction term was removed when $p>0.10$. Unadjusted post hoc pairwise comparisons were made for meal or for meal × time, when $p>0.10$. All data are presented as means ± standard deviations (SD) unless otherwise stated and the statistical significance level is defined as $p<0.05$, whereas tendencies are considered to be present when $p<0.10$.

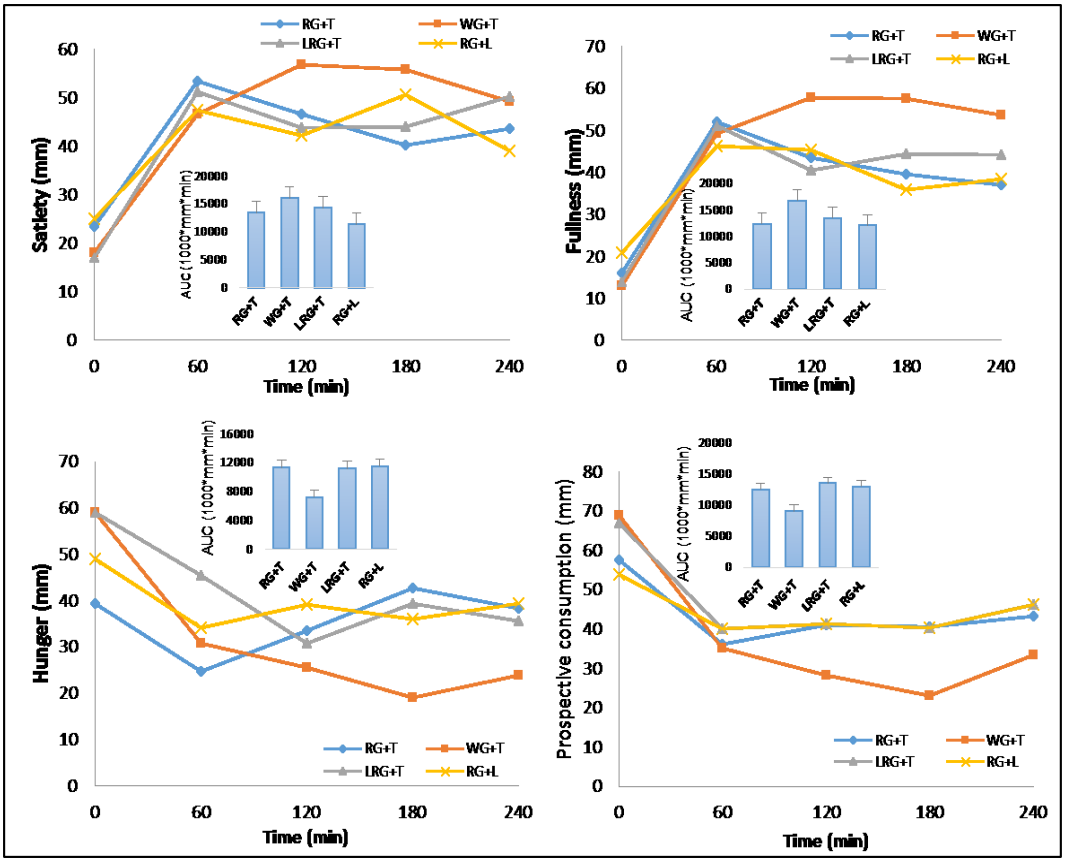
3.2 Results

All eight subjects completed the RG+T test meal, seven completed the LRG+T and WG+T meals and six completed the RG+L meal. Participants had a mean age of 39 ± 14 years, weight of 70.8 ± 11.3 kg and a mean BMI of 24.7 ± 2.7kg/m^2. Two participants dropped out due to personal reasons unrelated to the study.

3.2.1 *Subjective appetite sensation and palatability of test meals*

Fullness, satiety and prospective food intake ratings did not differ between meals at baseline. Hunger ratings differed between meals at baseline ($p<0.05$) with the lowest hunger ratings for RG+T (44 ± 20 mm) compared to both LRG+T (67 ± 11 mm) and WG+T (67 ± 9 mm) ($p<0.05$), but not RG+L (57 ± 21 mm). In the repeated measures ANCOVA, postprandial satiety ratings did not differ between meals ($p=0.30$), whereas ratings of fullness ($p=0.001$), hunger ($p=0.038$) and prospective food intake ($p=0.034$) did. Generally, WG+T resulted in significantly greater fullness and reduced hunger and prospective food intake ratings compared to all other meals ($p<0.02$) (**Figure 1**), whereas no differences emerged between RG+T, LRG+T and RG+L. The AUC for satiety, hunger, fullness and prospective consumption did not differ between meals.

Figure 1



Palatability ratings did not differ in term of appearance, smell, taste, off taste and overall pleasantness ($p>0.70$) among the four different meals (**Table 2**).

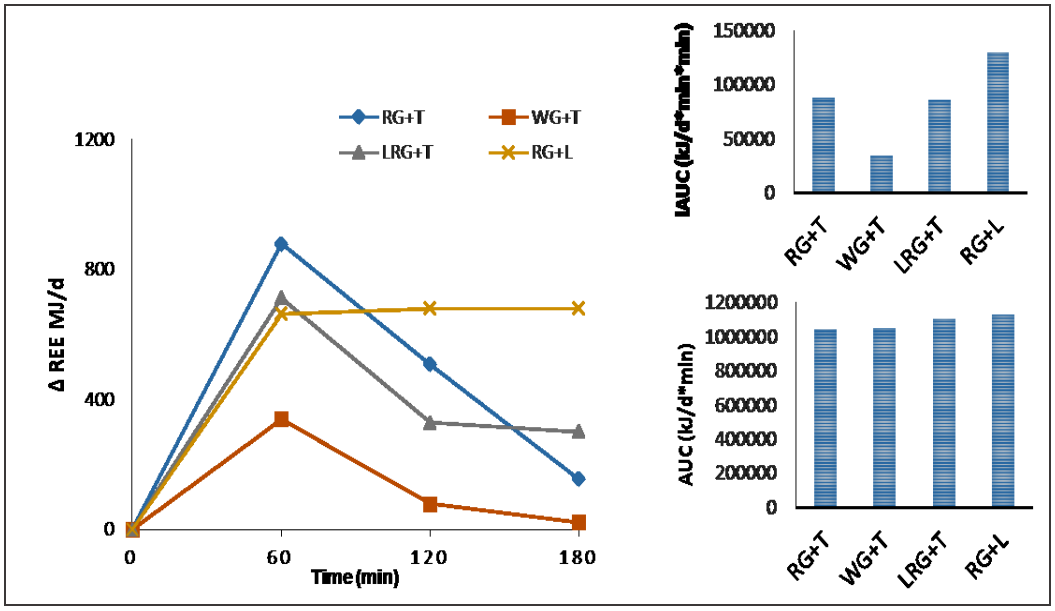
Table 2. Palatability ratings following the consumption of the four meals tested.

	Taste	Smell	Appearance	Off taste	Over-all pleasantness
RG + T	17±13	29±20	33±26	28±28	23±17
WG + T	27±20	18±18	23±17	24±24	25±25
LRG + T	24±10	23±13	33±25	20±12	22±14
RG + L	20±18	22±25	30±19	20±19	23±19

3.2.2 *Energy expenditure and MIT*

Postprandial EE increased for all the different meals as shown in **Figure 2**. Repeated measures ANCOVA showed that the post-prandial EE was affected both by meals (p=0.004) and time (p=0.002). Post hoc pair-wise analysis revealed that EE for WG+T was lower than LGR+T (p=0.02) and RG+L (p=0.0005), but not for RG+T (p=0.21). Still, post-hoc pair-wise showed that EE for WG+T was lower than EE for RG+T at time point 60 min (p=0.007) and was also reduced compared to RG+L at time point 180 min (p=0.002). The iAUC was significantly decreased for WG+T compared to RG+L (p<0.02) and slightly lowered compared to LRG +T (p=0.06).

Figure 2



MIT was calculated as the post-prandial increase of EEover 3 h after meal (**Table 3**) and resultedlower for WG+T compared to RG+L (58 ± 81 kJ vs 248 ± 188 kJ; $p<0.05$); also when expressed as percentage of the energy content of the test meal (3.2 % for WG+T vs. 13.2 % for RG+L) ($p<0.05$).

Table 3. Meal-induced Thermogenesis (MIT) of the four meals.

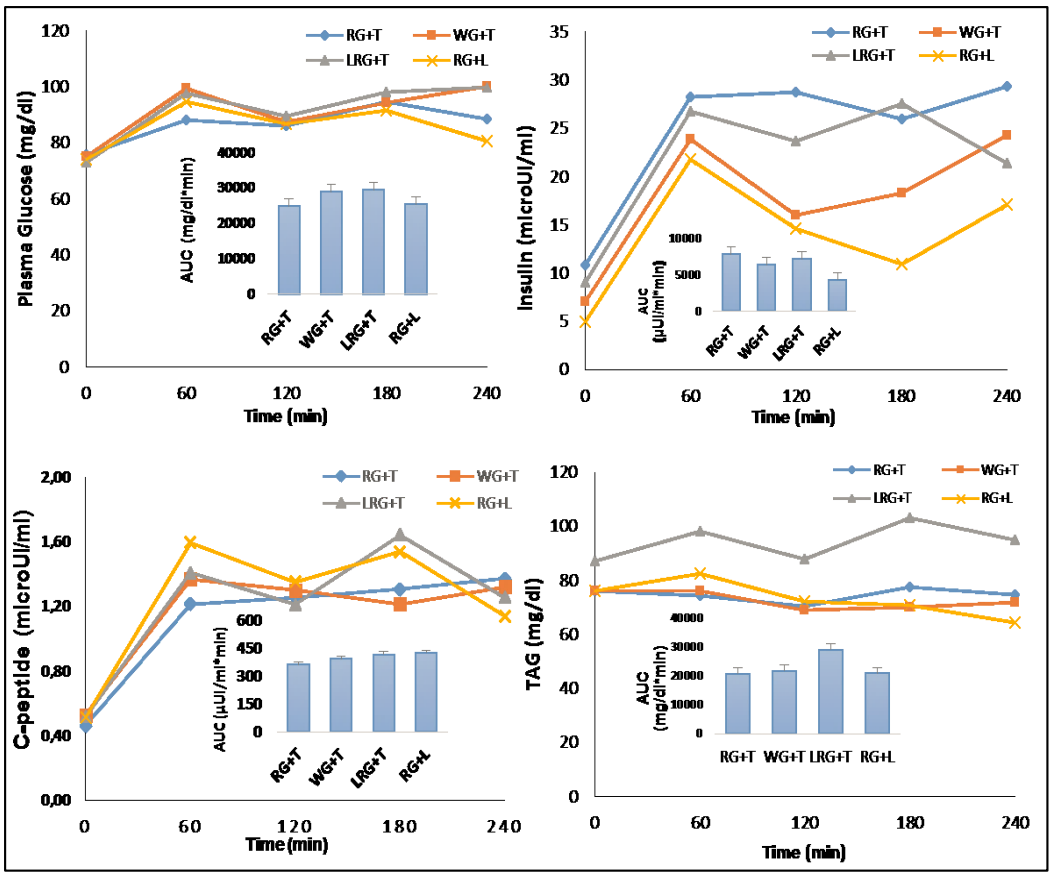
	MIT (kJ)	MIT (%)
RG+T	193 ± 197	10.7 ± 10.9
W+T	58 ± 81	3.2 ± 4.6
LRG+T	170 ± 231	9.4 ± 12.8
RG+L	248 ± 188	13.2 ± 9.9

3.2.3 *Plasma glucose, insulin, C-peptide and triacylglycerol responses*

Plasma glucose, insulin, c-peptide and triacylglycerol responses and their corresponding AUCs are shown in **Figure 3**. Glucose response was influenced by meals ($p<0.001$), in details, data revealed an overall reduced plasma glucose after RG+T compared to RG+L ($p<0.02$), WG+T ($p<0.02$) and LRF+L ($p<0.001$)**.** On the other hand, the overall post-prandial insulin response was slightly lower for both meals with the highest fiber content (WG+T and RG+L) than for the other meals (RG+T and LRF+L), although it was not significant ($p=0.60$). Likewise, c-peptide responses did not differ among meals ($p=0.23$),

but generally a higher concentration for RG+L compared to both RG+T (p=0.06) and WG+T (p=0.07) was found. The AUCs for glucose, insulin and C-peptide were not different between meals (p=0.30; p=0.40; p=0.87, respectively). Finally, plasma TAG concentration was significantly affected by different meals during post-prandial period (p=0.02). As a matter of the fact, TAG concentration increased after LRG+T compared to RG+T (p=0.02), WG+T (p=0.005) and RG+L (p=0.02) over time; nevertheless AUC was not different between meals (p=0.20).

Figure 3



3.3 Discussion

The possible influence of meals based on different type of pasta (including the addition of pulses) on post-prandial appetite, MIT and some biochemical parameters was evaluated in order to find strategies to restrain appetite and improve glucose control acutely. Our findings showed that WG+T increased fullness and lowered hunger, affecting MIT without significant effects on postprandial glycaemia.

Among the meals investigated, WG+T resulted in both significantly increased fullness and decreased hunger and prospective food consumption compared to RG+T, LRG+T and RG+L. Similar results on WG pasta PGI were observed in a randomized crossover trial conducted in Denmark by the same authors on 16 participants. On the opposite, Kristensen M. et al. (2010), showed no significant effects on subjective appetite neither with WG nor with RG pasta.

So far, few data are available on the influence of pasta's physical and chemical properties on appetite. However, some pasta properties such as the mixture semolina used for the dough and the processing cycle, in particular drying temperature, could affect its quality and protein digestibility (De Zorzi et al., 2007).

MIT has previously been suggested to be one of the mechanisms that influences appetite sensation, including satiety (Westerterp-Plantenga

MS et al., 1999). The contribution of MIT to the total daily EE is estimated to be approximately 10-15 % in healthy subject (Ravussin et al., 1986). In accordance with previous studies (Scalfi et al., 1986; Raben et al., 1994), we found a reduction of MIT after a fiber meal (WG+T). On the other hand, we observed an increased of MIT following the mixed composition meal (RG+L) where EE is held constant for 3 h without return at baseline value.

Macronutrient composition and energy density could have contributed to increase MIT following RG+L due to a higher protein content, which is responsible for a higher thermogenesis. The process of protein metabolism and storage used in the postprandial period requires a large amount of adenosine triphosphate (ATP). As reported by previous studies, thermogenesis derives for 20-30% from ingested proteins, 5-10% from and 0-3% from fat (vanBaak MA, 2008). It should be noted that 3 (WG+T, RG+T and LRG+T) out of 4 meals consisted mainly of pasta (single food), while RG+L consisted of pasta plus legumes (mixed food) requiring an higher amount of energy for digestion, transport and metabolism of nutrients. Hence, a longer observation time (> 3 h) should have been required to better quantify the entire EE response of RG+L; nevertheless, Ruddick-Collins at al. (2013) demonstrated that shorter measures are strongly correlated with

the 6 h measurement and therefore may provide sufficient information about the overall response.

Studies on appetite sensation and thermogenesis found that MIT directly correlated with satiety and inversely with hunger ratings (Crovetti R et al., 1998; Westerterp-Plantenga MS et al., 1999). In the present work, MIT increased following RG+L without affecting subjective appetite, in particular satiety, as reported in other studies (Raben A et al., 2003; Ravn AM et al., 2013). On the other hand, we observed a decreased MIT and an increased fullness after the WG+T meal compared with the RG+T, LRG+T and RG+L meals. This is in accordance with Raben et al., 1994, who found also that MIT and fullness are inversely correlated with fiber content (4.7 g/MJ vs 1.7 g/MJ) of different iso-energetic meals. However, MIT difference between WG+T and RG+L was not associated with significant differences in post-prandial glucose, insulin, c-peptide and triacylglycerol concentrations. Therefore, it is unlikely that MIT was altered by the presence of the dietary fiber used in this study, but this difference could be ascribed to the composition and bioavailability of macronutrients of the single food (WG+T) vs. mixed food (RG+L).

Our results indicate that the fiber content of meals did not affect blood glucose and insulin levels, in accordance with previous findings

(Kristensen et al., 2010). This inconsistency may be explained by the type of dietary fibers contained in wheat and in beans, mainly insoluble fibers that primarily increases bulk rather than form viscous solutions upon hydration in the small intestine. Also in previous studies, the glycemic response were found not to differ between refined and whole grain pasta (Kristensen et al., 2010). Moreover, data on the acute effect of pulses added to pasta on plasma glucose response compared to pasta alone showed a reduction of AUC, but brown beans were not taken into account as type of pulse (Mollard et al., 2012). However, a confounding factor could be the use of meal tests both as single food (RG+T; WG+T; LRG+T) and mixed food (RG+L) because of the quality and quantity of macronutrients can influence the digestion and absorption of nutrients (Higgins JA, 2012).

It seems that eating refined pasta with an organic acid added in the dough (LRG+T), like lemon juice, did not improve blood glucose and insulin response during post-prandial period, in contrast with previous findings with bread reported by Liljerberg et al. (1995;1996). However, it should be highlighted that pasta with lemon (LRG) was not specifically manufactured for the study aims and lemon concentration could have been not enough to positively influence the glycemic and insulin response. On the other hand, LRG+T

significantly increased post-prandial TAG compared to other meals, but this odd result could rely on intra-individual TAG variation among participants. However, a late reduction of glucose, insulin and TAG concentrations was observed for pulses (RG+L) that likely may affect these parameters at the subsequent meals over the course of the day, as reported previously (Higgins, 2012).

Palatability among the 4 type of tested meals was not different, as necessarily required for thestudy purpose, in order to avoid confounding sensations of satiety (Sorensen, L. et al., 2003), and hence palatability could not be considered a factor involved in affecting MIT (Raben et al., 1994). On the other hand, the limited sample size of the present study could have avoided to appreciate acute effects of high fiber meals, in particular of WG+T, which on the contrary positively affect MIT.

4. Evaluation of gut hormones and cytokines

We tested the hypothesis of Carina project by evaluating the acute effect of four types of meals on appetite sensation, metabolic parameters and diet-induced-thermogenesis as well as on gut hormones response and cytokines concentration in healthy participants.

The first part of the hypothesis has been developed and reported above (Chapter 3) whereas the second part on gut hormones and cytokines is briefly described in this paragraph.

4.1 Materials and Methods

The study design, the recruitment of participants and the characteristics of four meal tests have been previously reported (Chapter 3). Different gut hormones, in relation to appetite and food intake, such as Cholecystokinin (CCK), Glucagon-like Peptide-1 (GLP-1), Gastric Inhibitory Polypeptide (GIP), ghrelin and two cytokines, involved in the inflammation state, as: Tumor Necrosis Factor (TNF-α) and Interlukin-6 (IL-6) were assessed after consumption of four different pasta meals.

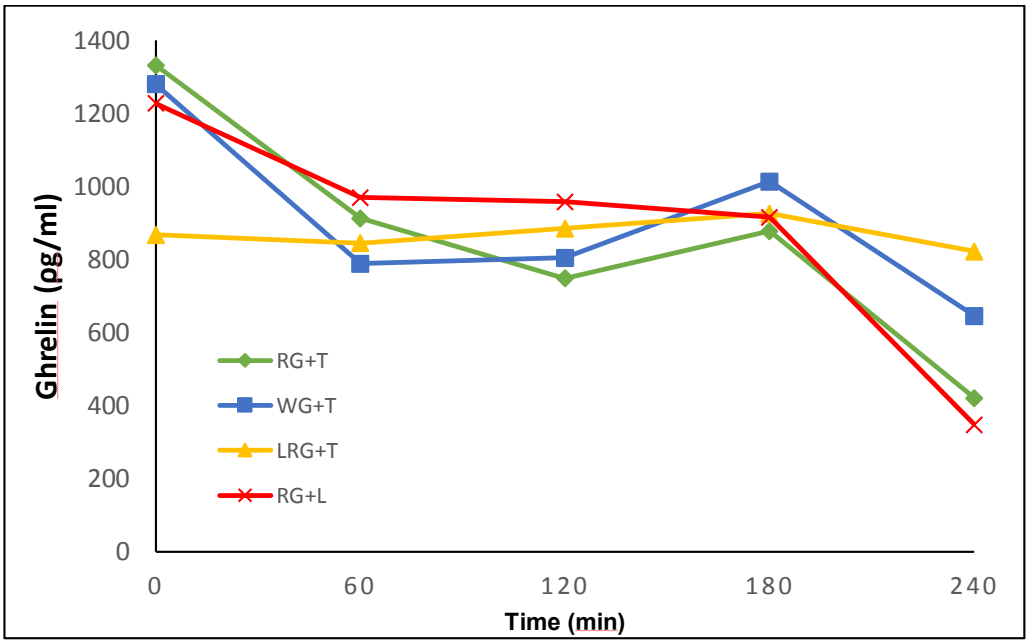
Blood samples were drawn from an antecubital vein, before the test meal and post-prandially at different time points (60, 120, 180 and

240 min). CCK was measured by an enzyme-linked immunosorbent assay (ELISA), whereas GLP-1, GIP, ghrelin, TNF-α and IL-6 were assayed by Bio-Plex suspension array system (Bio-Rad Laboratories, CA, USA).

4.2 Results

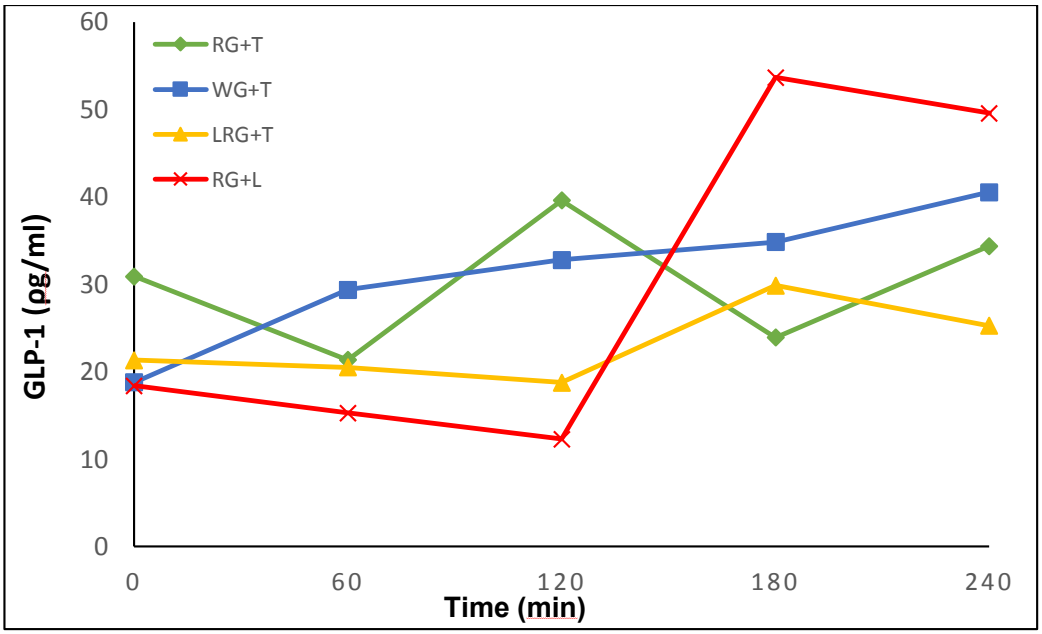
Ghrelin response is shown in **Figure 1**. We observed that ghrelin decreased after the lunch meal tests for RG+T, WG+T and RG+L ($p<0.05$), whereas as far as LRG+T did not differ from baseline to the post-prandial period. Further, ghrelin secretion was more suppressed for RG+L compared to LRG+T ($p=0.028$) at time point 240 minutes.

Figure 1



GLP-1 response did not differ among meals which consisted mainly of pasta (RG+T; WG+T and LRG+T), on the contrary it was affected by RG+L reaching a significantly higher peak compared to LRG+T (p=0.05) at time points 180 and 240 minutes (**Figure 2**).

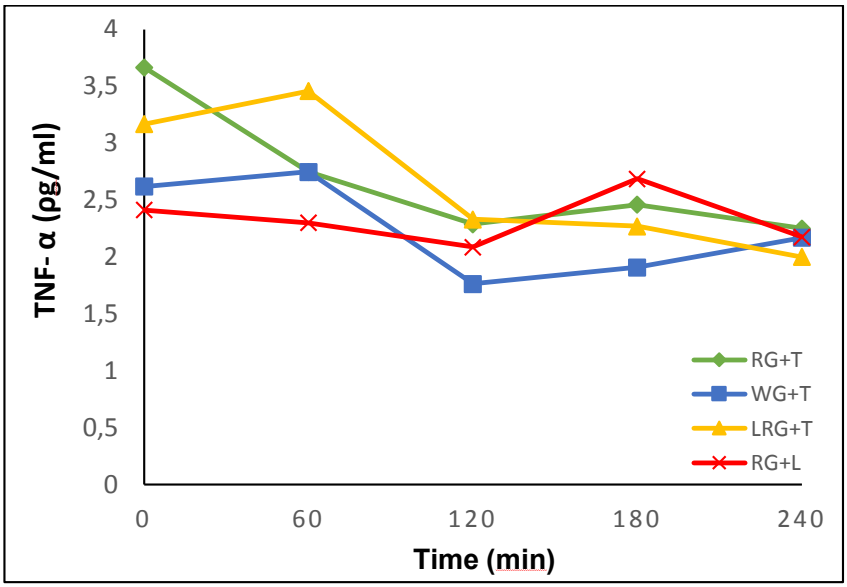
Figure 2

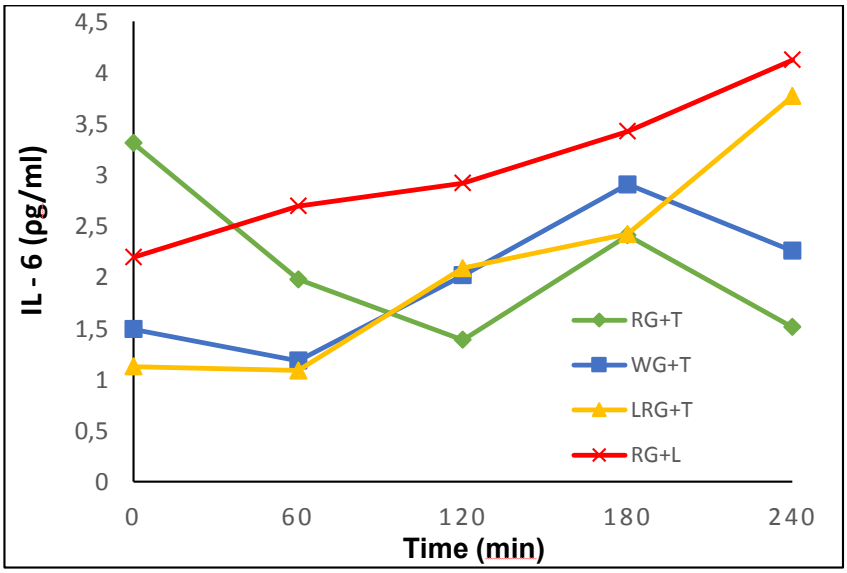


Finally, CCK response was higher both at baseline for RG+L in comparison with other meals (p=0.05) whereas, GIP secretion did not differ among meals (data not shown).

Cytokines concentration are reported in **Figure 3**, according to Friedman test we found that both TNF-α and IL-6 were not been affected by different type of meals and slightly decrease from baseline to the post-prandial period.

Figure 3


TNF- α (ρg/ml)
4
3,5
3
2,5
2
1,5
1
0,5
0
0
60
120
180
240
Time (min)
RG+T
WG+T
LRG+T
RG+L


IL - 6 (ρg/ml)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
60
120
180
240
Time (min)
RG+T
WG+T
LRG+T
RG+L

4.3 Discussion

Few studies (Giacco R et al., 2010; Hartvigsen et al., 2014 a; b; Nilsson et al., 2008; 2013) have assessed the effect of different high-carbohydrate meals on gut hormones, therefore results are still conflicting. Data from different trails (Hartvigsen et al. 2014 a; b; Giacco R et al., 2010) showed no difference in ghrelin concentration among meals with different fiber content. In contrast, Nilsson et al., (2013) observed that ghrelin was less suppressed in the morning after white bread in comparison with brown beans evening meal, suggesting that fiber intake could have influenced ghrelin. Similar results were seen in the present work, where serum ghrelin following RG+L (with more fiber content) was more suppressed compared to LRG+T (with less fiber content) at time point 240 minutes, nevertheless, ghrelin concentration did not influence the feeling of hunger measured by VAS.

On the other hand, GLP-1 response was slightly increased for RG+L in the late post-prandial phase (180-240 minutes) due to the presence of dietary fiber that might act increasing fermentation and formation of SCFA. However, GLP-1 secretion was not associated with an increase of satiety or fullness sensation after RG+L consumption.

Similarly, Nilsson et al. (2008) reported an increase in GLP-1 secretion at the standardized breakfast after the evening meal based on brown beans in comparison with white bread, but they also showed that breath hydrogen was positively correlated with feeling of satiety. In contrast, other studies did not show an increase of GLP-1 response between meals with different fiber content (Hartvigsen et al., 2014).

Likewise, GIP secretion was not affected by meals as reported by previous studies (Levitt NS et al., 1980; Giacco et al., 2010) and as observed in the present study. Finally, CCK response did not differ among meals and at different time points in contrast with a previous study (Bourdon et al., 1999) which showed that the consumption of a fiber rich-meal induces higher plasma cholecystokinin levels and a longer action time.

Regarding the inflammation state, meals based on pasta (RG+T, WG+T, LRG+T, RG+L) did not influence the cytokines response, which remained unaltered or slightly reduced post-prandially. This acute effect is in contrast with a high-fat meal that promotes acute post-prandial inflammation (Herieka & Erridge, 2014).

Data following the WG-enriched diet on the inflammation state are still unclear. Some studies (Ampatzoglou et al., 2014; Brownlee et al., 2010) did not observed an improvement in CVD markers after a WG-

enriched diet in people who usually did not consume whole grain. While, a recent study (Vitagliano et al., 2015) showed that WG wheat consumption for 8 weeks reduced TNF-a concentration and increased circulating dihydroferulic acid, a component of plant cell wall such as arabinoxylan. Therefore, WG consumption could positively affect some markers involved in the inflammation state in the long-term.

However, it has to be highlighted that Carina study presents some limitations that do not allow a complete interpretation of the results obtained with gut hormones and cytokines response. First, we missed the sample collection for the evaluation of the early secretion of both GLP-1 (within 30 min) and CCK (within 15 min) that might have altered the interpretation of the overall response. Second, the small sample size may have affected the results with determination of a type II error. Finally, RG+T, WG+T and LRG+T slightly differed, in fact, WG+T provided only 4 grams of fiber more compared to RG+T and LRG+T, so we did not expect to see any relevant differences, in contrast with RG+L which was a mixed meal.

5.4 Conclusion

In conclusion, RG+L decreases ghrelin and slightly increases GLP-1 secretion in the late post-prandial phase, nevertheless no effects were found on appetite sensation. This result could be related to a slight difference in macronutrient composition i.e. protein and fiber content and energy density which might have modulated gut hormones differently. Cytokines did not differ among meals, but tended not to change from baseline until time point 240 minutes.

5. Pastaly project: Objective

Following CARINA study, Pastaly project aimed to extend the effect of whole grain pasta compared to refined pasta on appetite sensation and energy intake (EI) both within meal and at the subsequent meal, focusing on satiation in overweight/obese patients, that to our knowledge has not been yet described in the literature for at-risk population.

The present study was carried out at the department of Nutrition Exercise and Sport (NEXS), Faculty of Life Science, University of Copenhagen, and tested the hypothesis that whole grain pasta (WGP) would induce early satiation and promote post-prandial satiety resulting in lowered *ad libitum* EI compared to refined grain pasta (RGP). Furthermore, colonic fermentation using breath hydrogen excretion as a proxy measure was assessed in the postprandial phase.

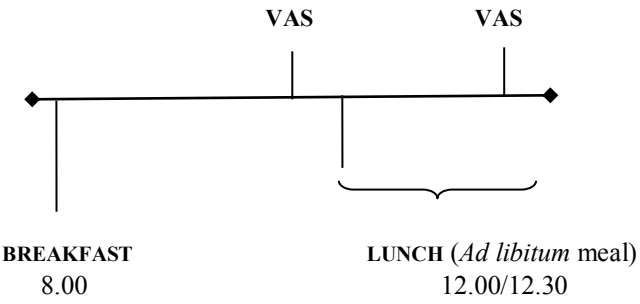
As primary endpoint, we assessed the WGP effect on *ad libitum* EI both within meal and at the subsequent meal, whereas the evaluation of hydrogen excretion and subjective appetite measurement were observed as secondary endpoints.

5.1 Study Design

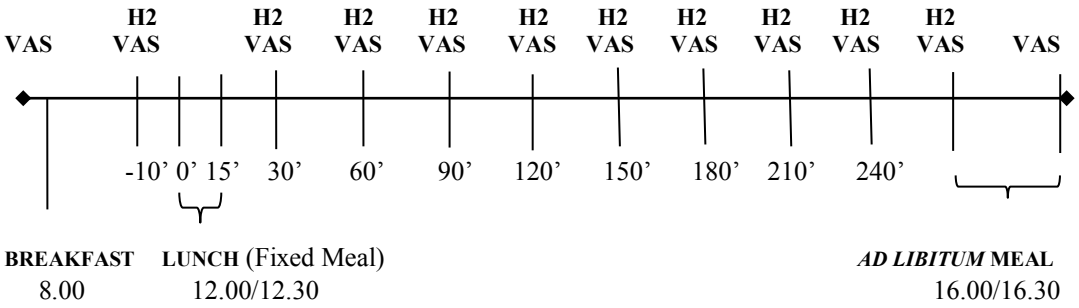
Two different iso-caloric lunch meals and two different *ad libitum* lunch meals were tested in two different sub-studies, study A and B, in a crossover manner in order to assess the EI both within meal (sub-study A) and at the subsequent meal (sub-study B). The outline of the two sub-studies are shown in **Figure 1**. All participants attended all 4 sessions, separated by at least three days wash out. During the last 24 hours before each test day, participants were instructed to consume a low-fiber diet, to avoid alcohol and hard physical activity, and to have dinner no later than 10 pm. In addition, participants were asked to fill in a food diary where they noted which food items they consumed during the day in order to evaluate their compliance at low-fiber diet. On the test day, participants consumed a standardized breakfast at home (8.00 a.m.) The breakfast meal consisted of 25 g of cornflakes, 150 g of skimmed yogurt and 15 g of raisins (1 MJ; 1.43 g fiber), and was provided by the department. From breakfast until 11.00 a.m., the participants were allowed to drink 500 mL of tap water (including 1 cup of coffee or tea). They were instructed to come to the department at 11.45 a.m. by a calm mode of transportation.

Figure 1

Sub-study A



Sub-study B



5.1.1 *Study A*

On the test days of the fixed meal test, participants consumed a standardized breakfast at home at 8.00 within 10-12 min, then they got to the Department of Nutrition, Exercise and Sports at 11.45 a.m. by a calm mode of transportation. The day before, they had followed a standardized fasting procedure, i.e. abstention from alcohol and hard physical activity and fasting for at least 10 hours prior to the breakfast. Participants were instructed to follow a low-DF diet and fill in a food diary to ensure compliance at standardized breakfast. From breakfast until 1 h before lunch, the participants were allowed to drink 500 mL

of tap water (including 1 cup of coffee or tea). The fixed test meal was served at 12.00 and consumed within 10-15 min. Appetite was assessed using a visual analogue scale (VAS) and fermentation by breath hydrogen was measured before the meal and every 30 minutes for the following 240 minutes (counted from start of the fixed lunch). After 120 minutes, 250 mL of water was served. After 240 minutes, an *ad libitum* meal was served in order to assess energy intake.

5.1.2 *Study B*

On the test days of *ad libitum* meal, participants are asked to follow a standardized fasting procedures like the Study A and to arrive at the Department of Nutrition, Exercise and Sports at 11.45. In the morning they will consume a standardized breakfast at home at 8.00 and they are allowed to drink a total amount of 500 mL of water (including 1 cup of coffee or tea) until 1 h before lunch. Appetite will be measured with VAS. At lunch about at 12.00 an *ad libitum* test meal will be served, in order to assess voluntary energy intake.

5.2 Composition of test meals

Test meals comprised both fixed and *ad libitum* lunch. The fixed meal was made with 100 g RGP or WGP with 100 mL of tomato sauce. The *ad libitum* meal consisted of 300 g RGP or WGP with 300 mL of tomato sauce. The tomato sauce was prepared following an Italian recipe: 700 g tomato puree, 100g of water, 30 g extra-virgin olive oil, 8 g garlic, 2 g fresh basil, 4 g salt and 1.75 g sugar and it was cooked for about 60 min (~1.9 MJ, 7% E from protein, 57.3% E from fat and 35.6% E from carbohydrate). Cooking time for RGP and WGP was 6 min and 5 min, respectively. Macronutrient composition and dietary fibre content are reported in **Table 1**. The Italian pasta company La Fabbrica della Pasta di Gragnano s.r.l. (Gragnano, Italy) provided pasta, whereas tomato sauce was prepared at the department in one batch.

Table 1. Composition of both *ad libitum* (A) and fixed (B) lunch meals.

	WGP A	RGP A	WGP B	RGP B
Energy (MJ)	5.7	5.7	1.9	1.9
- Carbohydrate (E%)	71.7	75.2	71.7	75.2
- Fat (E%)	14.9	13.8	14.9	13.8
- Protein (E%)	13.5	11	13.5	11
Dietary fiber (g)	24.6	11.4	8.2	3.8

WGP=whole grain pasta, RGP=refined grain pasta, A=sub-study A, B=sub-study B.

5.3 Study participants

A total of 16 participants (8 men and 8 women) were recruited for the study.

5.3.1 In- and exclusion criteria

Inclusion criteria are:

- Overweigth to obese (> 25 BMI ≤ 40 kg/m^2)
- 25-65 years of age

Exclusion criteria are:

- Smoking
- Regular intake of pre- or probiotics supplements
- Daily use of prescription medication (except for hypertension medications, oral contraceptives and H.R.T.)
- Participation in other intervention studies
- Non-adherence to the protocol or lack of cooperation
- Antibiotics up to 3 months before study start
- Pregnancy or lactation

5.3.2 *Recruitment of participants*

Sixteen participants were recruited for the study via internet postings, advertising in the Copenhagen area and mailing lists within the department. Participants were sought via the press, announcements, www.forsøgsperson.dk and participants who have previously

participated in trials at the Department of Nutrition, Exercise and Sports and who have agreed to be contacted for further studies were invited to participate. If the participants wanted more information, they should contact the trial site.

5.3.3 *Screening visit*

When the participants had signed the informed consent form, they underwent a screening procedure. Here, body weight and height, food frequency questionnaire to assess specific habitual fiber intake, were measured. These procedures did not require fasting, and thus could take place in conjunction with the information meeting if the participant wishes.

5.3.4 *Randomization*

The 16 participants were randomly assigned to the sequence of WGP and RGP meals within each sub-study, as well as the order of sub-study A and B using a web-based program (http://www.randomization.com), and the randomization was stratified according to sex.

5.4 Measurements

5.4.1 Habitual dietary fiber intake

At the screening visit, participants were asked to fill in a FFQ in order to estimate their habitual fiber intake. In the FFQ, participants stated portion size and frequency for their intake of fiber-rich food items during the last month. The FFQ was developed for Danish population where rye bread is the major contributor of dietary fiber intake (Vulhom et al., 2014).

5.4.2 *Subjective appetite and palatability measurements*

Visual analogue scales (VAS) were used to assess subjective appetite sensation (hunger, satiety, fullness, prospective food consumption, thirst and well-being) and palatability (physical appearance, smell, taste, off taste and overall pleasantness) of the meal test. They consisted of a scale, which was 100 mm in length with words anchored at each end, expressing the most positive and the most negative rating (Flint et al., 2000). VAS are made as small booklets showing only one question at a time and subjects were not allowed to discuss or compare their ratings with each other.

5.4.3 *Colonic fermentation measurements*

Breath hydrogen excretion is an index of colonic fermentation and was measured by the Gastro+ Gastrolyzer® (Bedfont Scientific Ldt). Participants were instructed to inhale deeply, hold their breath for 15 sec and then exhale at a steady pace into the cardboard mouthpiece of the device until their lungs felt empty.

5.4.4 *Ad libitum energy intake measurements*

To assess *ad libitum* energy intake, a large homogenous meal was served. To ensure reproducibility, any deviations in volume of the meal during preparation were adjusted with water. The participants were instructed to eat until comfortably satiated each time and were not allowed to read, talk or listen to music etc. while eating the *ad libitum* meal.

All foods that had not been eaten, whether in the plate or in the boiling pan, were weighed back and the amount of food and energy consumed was calculated.

5.4.5 *Sample size, calculations and statistical analyses*

Based on Gregersen*et a*l. (2008) a difference of 1000 kJ in *ad libitum* EI would be detected with $\alpha=0.05$ and a power of 80% using 8 subjects in a paired design. Similarly, 8 participants were necessary to detect a 10 mm difference in VAS (Flint et al., 2000). We recruited 16

participants for this study to allow smaller effect sizes to become significant as a difference in EI of less than 1000 kJ is considered relevant.

The area under the curve (AUC) was calculated as the total area above zero for hunger, satiety, fullness, prospective food consumption, whereas the incremental area under the curve (iAUC) as the net incremental above baseline was calculated for breath hydrogen excretions, both using the trapezoid model.

All statistical analyses were performed using the Statistical Analysis System software package, version 9.4 (SAS Institute Inc., Cary, NC, USA). All dependent variables were controlled for homogeneity of variance and normal distribution by investigation of residual plots and normal probability plots and histograms, respectively. If the distribution of a variable was skewed, it was log-transformed prior to analyses and back-transformed before presentation.

An ANCOVA was used to examine the effect of meal on EI (both within meal and subsequent meal), palatability and AUCs, where subject was modeled as a random variable and the baseline values and BMI were modeled as covariates, and sex included as a fixed variable as well as the sex × meal interaction term.

In sub-study B, a repeated-measures ANCOVA analysis was used to examine the effect of meal and time and the meal × time interaction term on the postprandial response of appetite measures and breath hydrogen, where subject was modelled as a random variable and corresponding baseline value and BMI were modelled as covariates, and sex was included as fixed variable as well as the sex × meal interaction term. Interaction terms were removed when $p>0.10$. Unadjusted post hoc pairwise comparisons were made where appropriate. All data are presented as means ± standard deviations (SD) unless otherwise stated and the statistical significance level is defined as $p < 0.05$, whereas differences are considered to represent tendencies when $0.05> p <0.10$.

5.5 Results

Sixteen subjects (9 women and 7 men) with a mean age of 44 ± 10 years and an average BMI of 30.1 ± 2.8 kg/m^2 participated in the study. Fifteensubjects completed all 4 test days as one dropped out due to personal reasons, thus only data from 15 subjects are included. Habitual dietary fiber intake obtained from FFQ was higher for women compared to men (36.5 ±15.0 g vs. 21.2 ± 13.5 g; $p<0.05$). Participants complied well with low fiber diet as reported by food diaries.

5.5.1 *Ad libitum energy intake*

In study A, no differences were found in the *ad libitum* EI within meal (WGP: 2625 ±1183 kJ vs. RGP: 2952 ± 612 kJ; p=0.23). No sex × meal was observed (p=0.35), but there was a trend to difference between sexes (p=0.08), thus sex-stratified analyses were performed. Among female subjects, we observed a lower EI within meal (WGP: 2200 ± 881 kJ vs. RGP: 2762 ± 627 kJ; p=0.14), whereas no effect was seen among men (WGP=3111 ± 1358 vs. RGP=3170 ± 558 kJ; p=0.88).

In sub-study B, *ad libitum*EI at the subsequent meal did not differ between meals (WGP= 2287 ± 820 kJ vs. RGP =2499 ± 1014 kJ;

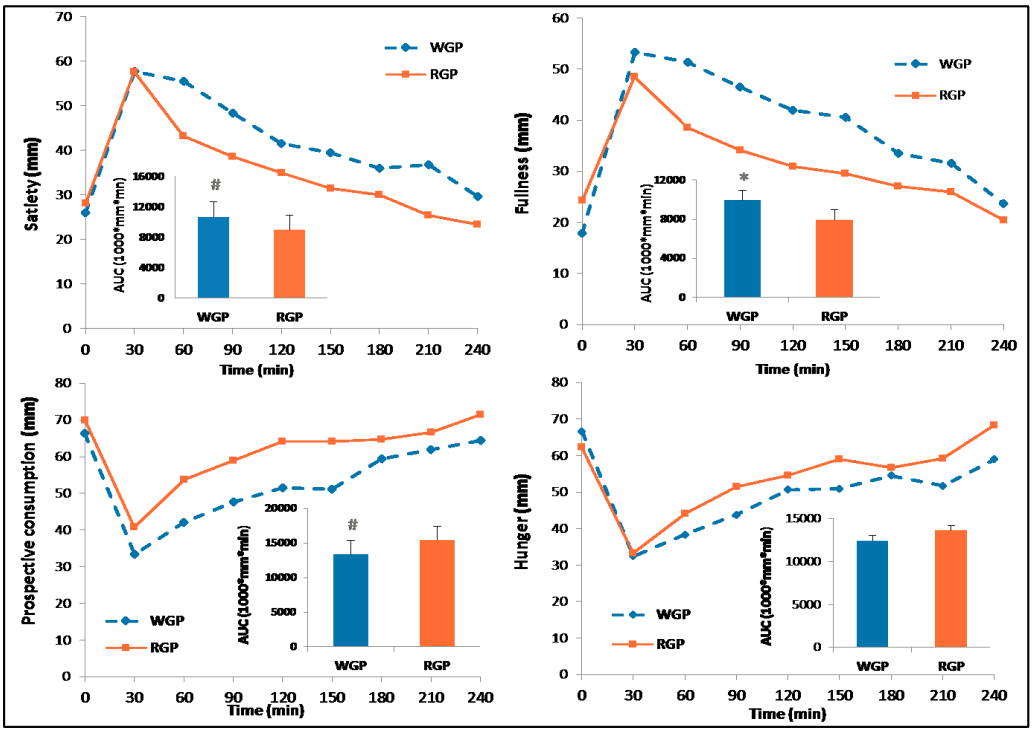
p=0.12).However, a tendency for a sex × meal interaction was seen (p=0.07). In the sex-stratified analyses, 18 % lower EI following WGP consumption (WGP=2255 ± 1057 kJ) was seen compared to RGP consumption (RGP=2757 ± 1274 kJ) ($p<0.05$) among men, whereas no effect was seen among women (WGP=2020 ± 966 kJ vs. RGP=2057± 1023 kJ; p=0.82).

5.5.2 *Subjective appetite sensation and palatability*

In sub-study A, no significant differences in satiety ($p<0.48$), fullness (0.70), hunger (p=0.99) and prospective food consumption (p=0.99) were found immediately after the *ad libitum* meal between WGP and RGP. The same was the case for thirst (p=0.34) and as well as comfort (p=0.18) after completion of the meal.

In a repeated measures ANCOVA, WGP in sub-study B resulted in an increased sensation of both satiety and fullness feelings ($p<0.001$), and lower ratings of both hunger and prospective food consumption ($p<0.001$) compared to RGP (**Figure 2**). Furthermore a strong effect of WGP on AUCs for satiety ($p<0.05$), fullness ($p<0.007$) and prospective consumption ($p<0.05$) was found, but not for hunger (p=0.17).

Figure 2



Thirst did not differ between meals (p=0.17), whereas comfort ratings were generally higher after WGP consumptions (mean rating 72 ± 17 mm) compared to RGP (62 ± 25 mm) (p <0.001). Also, we found an effect of WGP on AUCs for comfort (p<0.05) not for thirst (p=0.56) (data not shown).

In a paired t-test, subjective palatability ratings (appearance, smell, taste, off taste and overall pleasantness) did not differ between WGP and RGP in either sub-study. Only off taste was more pronounced for WGP compared to RGP in sub-study A (p=0.08) (**Table 2**).

Table 2. Palatability ratings of both*ad libitum* (A) and fixed (B) lunch meals.

	Taste	Smell	Appearance	Off taste*	Over-all pleasantness
WGP A	58±20	58±19	49±20	31±18[a]	53±20
RGP A	62±21	63±20	53±21	19±21[b]	55±20
WGP B	58±18	59±14	55±19	22±20	54±20
RGP B	58±19	57±17	51±19	19±15	52±22

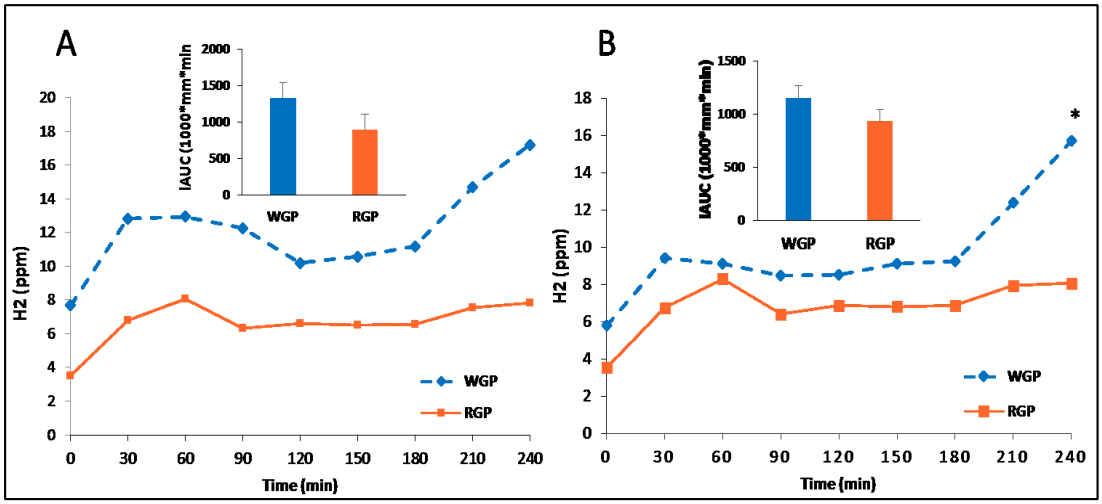
Data are presented as mean ± SD (n=15). * Different letters in the same row indicate p=0.08.

5.5.3 *Breath hydrogen excretion*

Generally, WGP produced higher hydrogen excretions than the RGP; however, as one participant had very high baseline breath hydrogen levels, which greatly contributed to this difference between diets, we conducted the statistical analyses both with and without this participant. In repeated measures ANCOVA including all 15 subjects, no difference in breath hydrogen excretion between meals was evident ($p=0.11$). However, if this atypical participant was excluded, a marked difference was seen ($p<0.01$). Similarly, iAUC for breath hydrogen excretion did not differ between meals when all participants were included ($p=0.68$), but after this one participant was excluded, the difference in breath hydrogen excretion between meals was more pronounced, although it still did not reach significance ($p=0.15$)

(**Figure 4**). There was no difference between the sexes in breath hydrogen excretion patterns (data not shown).

Figure 4



5.6 Discussion

We hypothesized that WG foods would induce early satiation and promote post-prandial satiety lowering *ad libitum* EI, affecting both appetite sensation and colonic fermentation. However, we were not able to substantiate this hypothesis entirely; WGP positively enhanced satiety, but not the ad libitum EI, although it reached a positive tendency when sex is included in the analysis. In order to evaluate satiation and satiety the study has been split up in A and B, respectively.

To our knowledge, this is the first study on the effect of an ad libitum WG meal on satiation (sub-study A) in overweight/obese patients. WGP consumption did not show an overall effect on satiation; nevertheless, we found a difference between sexes, where a lower, albeit not significant, ad libitum EI after WGP compared to RGP was seen among women ($p=0.14$). We hypothesized that WGP affects satiation due to increased viscosity and volume of meal by delaying gastric emptying. This is in accordance with a previous study where whole meal bread formed a homogenous bolus in the stomach compared to refined counterpart and the presence of arabinoxylan (AX), that can bind water up to 9 times more, increasing viscosity of

meal and prolonging stomach distension (Marciani et al., 2013). Also, similar results were reported by Steinert et al., (2011) who showed that early satiation in the stomach was primarily affected by gastric distension. However, no significant differences in subjective appetite were found immediately after the meal as well as among palatability ratings. Only off taste was slightly more pronounced for WGP compared to RGP (P=0.08) and this might have affected the EI within meal (Sørensen et al., 2003).

In sub-study B, we found that WGP improved subjective appetite and increased breath hydrogen excretions, whereas no overall effect on the *ad libitum* EI at subsequent meal was seen. Similar results have also been observed in another study by our group (Cioffi et al., in preparation) using the same pasta, whereas no differences on subjective appetite between WGP and RGP were found in another study comparing WGP and RGP (Kristensen et al. 2010). In that study, a large amount of cheese was served with the meal, and the meal was served in the morning with a large amount of cheese. This may have affected the study outcome as a hot pasta meal is not usually eaten for breakfast. However, only few studies on pasta have been conducted, and differences according to the meal composition due to wheat quality and pasta processing cycle, where drying temperature is

crucial for the quality and protein digestibility of pasta (De Zorzi et al., 2007), may also play a role in the physiological effects of the different pasta.

Although the positive effect on satiety did not result in a significant overall reduction in *ad libitum* EI at the subsequent meal, where male participants had a lower ad libitum EI following WGP ($p<0.05$). The test meals used in sub-study B only differed in fiber content by ~ 4.5 g of a total meal weight of 223 g. It is therefore interesting that this low amount of dietary fiber was sufficient to affect subjective appetite sensations and decrease *ad libitum* EI, although only in men. We speculate that this effect may be more pronounced if the dietary fiber products were given to a group of participants with a low habitual dietary fiber intake, although adjusting for habitual fiber intake in the present study did not affect the results. Finally, no difference was found in palatability ratings between meals WGP and RGP.

The effects both on satiation and satiety have been investigated also by Mollard et al. (2012) in healthy men. They observed an early satiation, i.e. lower *ad libitum* EI within meal, following consumption of pulses added to refined pasta, especially lentils. However, *ad libitum* meals tested by Mollard et al., (2012) were homogenized with

a food processor and provided a higher energy intake (6.3 MJ vs 5.7 MJ) and therefore, some relevant factors such as meal composition, meal volume and consistence, which may influence gastric distension, differed from our study. In contrast with our findings, no effects were found neither on EI at the subsequent meal nor on subjective appetite after pulses consumption, likely due to a different study design where the first meal was *ad libitum*, not fixed, and this might have influenced the effect on EI at the later meal.

Regarding colonic fermentation, one could speculate that *ad libitum* EI at the subsequent meal might have been affected by the increased colonic fermentation, indicated by greater breath hydrogen concentrations after the WGP meal. This early increase in breath hydrogen excretion after the WGP meal is somewhat surprising as in previous studies (Nilsson et al., 2008; Hartvigsen et al., 2014) breath hydrogen first increased at later time points (> 4 h). Potentially the gastric emptying following a pasta meal is faster than after a meal consisting of boiled kernels used in these studies. This would give rise to an earlier rise in breath hydrogen excretion. Breath hydrogen excretion has been found to be negatively correlated with *ad libitum* EI (Rosen et al., 2011). Furthermore, after AX consumption breath hydrogen excretion has been reported to correlate positively with

plasma butyrate and further, the latter correlated positively with satiety sensation (Hartivigsen et al., 2014). Therefore, colonic fermentation might be the link between WG intake and satiety, but further investigations in different groups of patients are required to establish this direct relationship.

The overall numerical difference between WGP and RGP was small both within meal (~328 kJ) and at the subsequent meal (~212 kJ) and the study was underpowered to detect such a difference in *ad libitum* EI (Gregersen et al., 2008). Also, we did not take the menstrual cycle of the female participants into account, which may be regarded as a limitation. It is surprising that the observed sex differences go in opposite directions for the two studies. It can be speculated that sex-specific different behavioral aspects come into play in the short (study A) vs. long (study B) study designs, or these differences may be due to chance. Cornier et al., (2010) showed with functional Magnetic Resonance Imaging (fMRI) that there are important sex-based differences in the appetitive responses to food. They observed that women had a heightened satiety response to meals as compared to men, and men are more likely to overeat during *ad libitum* feeding, but this is in contrast with the results from the long study.

6. Overall Conclusion

Whole-grain pasta di Gragnano increased feeling of satiety, fullness and decreased hunger sensation compared to other types of pasta analyzed in the thesis. WG pasta influenced MIT and increased fullness, suggesting a relation between appetite and meal-induced thermogenesis. However, it is unlikely that fiber content of meal might have affected the MIT due to the missing acute effect in the post-prandial metabolic profile and gut hormones response. On the other hand, positive is the overall effect of meals on cytokines concentration in comparison with a high fat-meal that induces acute post-prandial inflammation.

In addition, WG pasta resulted in an early increase in breath hydrogen excretion compared to refined pasta, suggesting that colonic fermentation might be a possible link between satiety and WGP intake. Overall, no effects of WGP on *ad libitum* EI both within meal and at the subsequent meal might was seen, but some sex differences were seen which needs further investigation.

In conclusion, the results strongly showed the beneficial effect of WG pasta on appetite, nevertheless, further investigation are needed in order to better define the mechanism of actions that linked the WG

consumption with higher satiety compared to refined pasta products. As no differences were observed on subjective palatability, neither in Italy nor in Denmark, WG pasta can be promoted for a more favorable effect on the control of appetite sensation.

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