CHAPTER 9

Results and Discussion
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The four aims of this thesis were:

1. To investigate how starchy foods can be made more slowly digestible
2. To evaluate the prediction of carbohydrate digestion and postprandial glucose response (PPG) using *in vitro* methods
3. To study the impact of the rate of starch digestion on PPG and insulin response (PPI) in humans
4. To understand the associated glucose fluxes and metabolic mechanisms related to health aspects

Carbohydrate-rich staple foods are key candidates for reducing PPG exposures, because of their frequent and consistent use. Wheat-based flatbreads and rice are the two most common carbohydrate-rich staple foods in South Asia, making them important contributors to the daily glycaemic load (GL). This GL can be lowered by slowing the digestibility of these staple foods. Slower digestion of carbohydrate-rich staple foods can also lead to delivery of more substrates to the colon, having effects on e.g. gastrointestinal hormones and insulin sensitivity (1). However, this is beyond the scope of this thesis.

In our systematic review on rice (Chapter 2) we identified 28 original articles describing the results of 32 randomized clinical trials with rice as the test food and a measure of PPG (and in some cases also PPI) as an outcome measure. Rice has been used as an example of a starch-rich product which has a wide variety in composition (physico-chemical properties (e.g. amylose/amylopectin ratio, amylose-lipid complexes) and which is processed in different ways (consumer and industrial processing), leading to a large range in GL. In our systematic rice review we found that the composition of rice together with the processing steps determine the PPG and PPI response, due to effects on gelatinization and/or retrogradation (see Figure 1). Post-harvest treatments such as (severe) parboiling can also decrease the PPG to a large extent caused by an increase in the gelatinization temperature due to the formation of retrograded starch. Home preparations such as steaming and boiling during which gelatinization takes place, can increase the PPG response, while multiple heating/cooling cycles can decrease the PPG response due the retrogradation of starch. Another major factor influencing the PPG response is processing. We saw for example that brown rice does not always give a lower PPG response than white rice, but that this is dependent on cooking time. A recent study confirmed this by showing that the level of rice polishing has no effect on glycaemic index (2). Retention of brown fibres in other food formats like bread (3) and pasta (4) did also not lead to a lower PPG. To conclude, in human clinical studies it is important that the food itself (e.g. rice type), any post-harvest processing (e.g. polished or whole-grain) and the conditions under which the food is prepared and tested (e.g. cooking time) are described in detail,
to allow for accurate replication, interpretation and comparison (e.g. in reviews or meta-analysis) of studies.

Fig 1: Relationship between rice characteristics, processing factors, physico-chemical processes and glycaemic response (+ indicated increased effect; - indicates decreased effect). This is a general figure, depending on specific processes, e.g. parboiling the effects may differ (taken from Boers et al. Br. J. Nutr. 2015; 114: 1035-1045).

Ingredients in or added to foods that can lower the PPG response include plant-derived hydrocolloids (soluble viscous fibres) (Chapter 3), a group of long chain biopolymers. These are able to lower blood glucose response due to their viscous or gelling nature under gastrointestinal conditions, which can delay gastric emptying and inhibit the propulsive and mixing effects in the intestine leading to a slower digestion (5-7) (Figure 2). The physico-chemical properties of food hydrocolloids, such as molecular weight, linearity and degree of branching, and solubility, determine viscosity under gastrointestinal conditions (5-7). A recent study suggests that the onset of coil overlap in vitro, when molecules are entangled and the solution becomes semi-dilute, may be a predictor for the reduction of PPG responses (8). Food hydrocolloids may also act via mechanisms independent of viscosity, e.g. modification of the release of digestion- and fermentation-related hormones such as GLP-1 and GIP, alteration of amylolysis, and delay of sugar absorption through reduction in rates of diffusion, interactions with the mucosa (9) or downregulation of glucose transporters (5). In the food product hydrocolloids can also coat the starch granules, resulting in a decrease in swelling and gelatinization of starch and the formation of a physical barrier to alpha-amylase (10).
Figure 2: Influence of physico-chemical properties of food hydrocolloids on physiological processes in the intestine affecting the PPG response.

Our rice review and the chapter on food hydrocolloids underpin the conclusion that it is possible to modulate the PPG response to staple foods, and that this is influenced by factors affecting carbohydrate digestibility. Therefore, it might be useful for the food industry to have a reliable \textit{in vitro} model to predict the rate of digestion of existing and newly developed staple foods. If there are a lot of ingredients to test for PPG lowering (different fibre mixes), those without an \textit{in vitro} effect on the rate of digestion are eliminated before developing human clinical studies. The method we have applied to assess this was an \textit{in vitro} digestion assay based on the widely-used Englyst method (Chapter 4), extended by an oral digestion step, and optimization of the pH and the amount of digestive enzymes. This was specifically developed to test the relationships between the \textit{in vitro} digestion parameters and in \textit{vivo} PPG. We found that with this \textit{in vitro} model the rate of starch digestion (k) can be estimated, and in a regression model including \textit{in vitro} glucose release AUC over 120 min, the carbohydrate level and \% rapidly digestible starch, as independent variables, there was a very high correlation with \textit{in vivo} plasma glucose responses. Several other studies have found a good correlation between the \textit{in vitro} digestion of (flat)breads and the \textit{in vivo} glycaemic response (11-14); however, our study introduced a unique fibre mix of guar gum and chickpea flour. Although there is a good correlation between \textit{in vitro} digestion rate and \textit{in vivo} glycaemic responses for these starch-rich products, this needed to be
confirmed *in vivo* under real meal conditions. The reason for this is that these starch-rich products influence pancreatic and gastrointestinal hormones and gastric emptying, which are not considered in *in vitro* models. These hormones (such as e.g. GLP-1, GIP and insulin) are furthermore necessary for understanding the underlying mechanisms influencing PPG. Therefore *in vivo* studies with flatbreads with fibre mixes were executed to confirm PPG, PPI and gastrointestinal hormonal responses in Caucasian and Indian subjects.

In the first human trial (Chapter 4) we systematically tested the potential for inclusions of guar gum (GG), konjac mannan (KM) and chickpea flour (CPF) in 10 combinations (2/4/6 g GG; 2/4 g KM; 15 g CPF, and 10 or 15 g CPF plus 2 or 4 g GG) to lower the PPG response to flatbreads. Flatbreads with 6 g GG, 4 g KM, and 15 g CPF plus 2 or 4 g GG reduced PPG ≥ 30% (p<0.01), while no other combinations differed significantly from the control. For KM more was needed than expected, probably due to the low solubility of KM (15). Previous research has shown that soluble viscous fibres (psyllium, fenugreek and beta-glucan) with or without legume flour can lower the PPG (14, 16-18) and PPI (16) of flatbreads. In addition, previous studies have demonstrated that supplementation of bread with GG resulted in a substantially lower PPI (19-23).

The second human trial (Chapter 5) aimed to confirm the efficacy of the combinations of 15% CPF with 2 and 4% GG from our earlier study, and a version with 3% GG, and to further extend this work to the Indian population. The flatbreads with CPF and 3 or 4% GG significantly reduced PPG (both ≥ 15% reduction in positive incremental AUC, P<0.01) and PPI (both ≥ 28% reduction in total AUC, P<0.0001) compared with flatbreads made from control flour. We also observed that GG could partly be replaced by CPF in flatbreads to achieve reductions in PPG compared to a higher GG level alone. The reason why the combination of a viscous fibre and a legume flour in a flatbread is more effective for PPG and PPI lowering than the isolated components (16, 24) is probably that they act on different mechanisms: while GG could delay gastric emptying, and mixing in the gastrointestinal tract, CPF could have a slower or lower absorption than the wheat flour due to a higher slower digestible starch (SDS), resistant starch (RS) and amylose content (25). On the product level CPF and GG could compete for water binding and this results in less water available for and thus inhibition of starch gelatinization, resulting in formation of resistant starch and SDS (RS% was 1.6, 1.0 and 12.7% and SDS% was 41.6, 40.9 and 51.6% for control and 3% GG and 4% GG chapattis, respectively: internal Unilever R&D communication). Ekstrom et al. (19) similarly showed that a combination of GG and high amylose maize starch in a bread format gave higher RS content with increasing GG levels and a lower PGG and PPI response.
It has been hypothesised that the effect of GG on PPG is mainly determined by reducing the influx of glucose from the small intestine (26). However, the PPG is not just a reflection of starch digestibility, but is determined by different underlying glucose fluxes: the rate of appearance of exogenous glucose (RaE), endogenous glucose production (EGP) and the rate of disappearance of total glucose (RdT) (Figure 3). We therefore wanted to quantify the rates of these glucose fluxes as contributors to changes in PPG observed with these fibre mixes in wheat-based flatbreads.

A widely-accepted way to measure postprandial glucose fluxes is the dual stable isotope technique, in which $^{13}$C-labelled starch in combination with a deuterium glucose infusion is used to differentiate between the different glucose fluxes. In a study using $^{13}$C-labelled starch in flatbreads with guar gum (2 and 4% GG) and CPF (15%) (treatment arms designated GG2 and GG4), GG4 significantly reduced 4-h AUC values for the RaE, RdT and EGP by 11, 14 and 64% respectively, whereas GG2 showed minor effects (Chapter 6). Other studies with soluble fibres have reported a reduction in RaE for the first 2h, while the effect on EGP was mixed (27, 28). Nazare et al. (27) found that the addition of 5g beta-glucan to a polenta meal resulted in an 18% reduction in RaE during the first 2h, after which this effect was reversed. In their study the EGP was significantly more inhibited after the polenta and beta-glucan meal compared to the polenta meal alone, while there was no significant difference between meals with respect to RdT (27). Battilana et al. (28) gave a group of 10 healthy volunteers either an isoenergetic diet containing 0 or 8.9g/day added beta-glucan for 3 days. On the third day, the diet was administered as fractioned meals ingested every hour for 9 h. The RaE for the first 2 h on the third day was 21% lower with the addition of beta-glucan, while the EGP was similar for both diets (28). In that study (28) though it cannot be ruled out that fermentation of the dietary fibres also played a role. There

Figure 3: Underlying glucose fluxes of PPG (adapted from slides of Marion G. Priebe, UMCG Groningen, The Netherlands)
are no dual or triple isotope studies which have studied the effect of legume flour alone on glucose fluxes.

In our study with GG (Chapter 6), we found that an apparent slower rate of digestion due to GG does not only have an effect on the RaE but also on the RdT. To establish the presence and magnitude of these effects and their relationship to PPG and PPI across studies, we executed a systematic review (Chapter 7) including trials with carbohydrate-rich products varying in digestion rate between treatment and control (i.e. treatments likely to induce differences in RaE). The aims of this systematic review were to quantify the effect of a change in the RaE on PPG and PPI as a primary objective, and secondarily to quantify the effect of a change in the RaE on EGP and RdT. The systematic search resulted in 12 articles with 17 comparisons satisfying the inclusion criteria. Results of the weighted linear regression analysis revealed that a 10% reduction in RaE was associated with a reduction of 7% (95% CI 2 to 12%) in PPG, of 8% (95% CI 2 to 13%) in PPI and of 11% (95% CI 4 to 17%) in RdT. This meta-regression analysis confirms that reducing exogenous glucose influx from carbohydrate rich meals has substantial effects on PPG and PPI across a range of different carbohydrate-rich meals, and may therefore be a good dietary approach for reducing PPG and PPI. However, it is important to note that all of the studies were executed in healthy persons and therefore we do not know if the same relationships would be seen in people with (pre-)diabetes.

Given that a slower rate of digestion does not only have an effect on the RaE, but also on RdT and EGP, we would like to know whether reducing the RaE also affects tissue glucose metabolism (formation of metabolites) and EGP. Our working hypothesis was that slowing the RaE would also delay the EGP and appearance rates of $^{13}$C glucose metabolites. In the final part of the research (Chapter 8), tracer-based metabolomics was applied to samples from the preceding study of glucose fluxes (Chapter 6), to measure the fluxes of exogenous glucose metabolites. The aims were 1) to monitor the dynamics of several $^{13}$C labelled metabolites derived from the $^{13}$C-labeled wheat flour, and 2) to find out whether the delay in glucose influx propagate to changes in downstream glucose metabolites. The appearance rates of these metabolites were determined from a kinetic model. In this metabolomics study guar gum dose-dependently delayed the formation of the glycolysis-derived triple-labelled metabolites lactate and alanine. The effect is less pronounced in the TCA cycle (-related) dual-labelled metabolites citrate and glutamate. At this moment it is difficult to relate these metabolic observations to specific (pathophysiological) mechanisms.

**Implications**
We have found that variation PPG responses induced by a lower rate of starch digestion is not only influenced by the RaE (as expected), but also by glucose disposal.
The third flux (EGP) does not seem to be a major source of differences between treatments. Is it preferable to influence RaE or RdT for lowering the PPG response? Glucose tissue uptake can be increased e.g. by increasing the insulin response. This is not preferable because a high insulin response is detrimental, while a lower PPI response may be beneficial for health in the short and longer term (29-32). In the acute postprandial period, a lower insulin response prevents hypoglycaemia and increases in free fatty acids and stress hormone concentrations (29-31). In the longer term, regular consumption of diets that generate a low PPI response is assumed to preserve pancreatic beta-cell function owing to lower strain on the beta-cells, especially in individuals with impaired first-phase insulin response (32). A high insulin response on a regular basis can lead to higher glycaemic variability, which is positively associated with micro- (33) and macrovascular complications in T2DM patients (34). Lowering the PPG response by slowing the influx of glucose therefore seems a preferred approach, and one which this thesis confirms as feasible.

Even in the absence of an effect on PPG, lowering the rate of glucose influx seems beneficial, because this could lower the PPI response (29, 30) and liver fat accumulation (35). A lower RaE influences the PPI by affecting the entero-insular axis, first by influencing the incretin response (GIP and GLP-1). In many studies there is a correlation between a lower RaE and a lower GIP response (36-39). While looking for a biological meaning of a low GIP response it seems that there are mainly animal data and some preliminary human data in the literature, suggesting that a low GIP response is beneficial. In a high-fat fed obese mouse model it has been shown that antagonism of GIP significantly reduced body weight (40, 41). This suggested that there is an association, between the influx of glucose, GIP release and body weight control, at least in mice. In a recent cross-sectional study there was a positive association between levels of postprandial GIP and visceral fat in men, independent of insulin (42). Strong parallels exist with the beneficial metabolic effects of Roux-en-Y gastric bypass in obese, insulin-resistant humans that surgically ablates GIP-secreting K cells (43). There is also evidence that GIP plays a role in inflammation (44). The association between the RaE and the GLP-1 response is less clear, but the lower RaE potentially shifts glucose absorption distally in the intestine, leading to a later GLP-1 response (39). Another study showed a lower GLP-1 response after consumption of kernel bread compared to wheat flour bread (45), while there was not a difference in RaE between the treatments. A lower incretin response results in a lower insulin response, leading to a diminished glucose disposal. Another positive aspect of a lower RaE could be that it influences liver fat accumulation, as a study showed that healthy volunteers after a low GI diet had lower liver fat fractions than those after a high GI diet (35). However, fermentation of dietary fibres can also lead to lower liver fat accumulation and it is hard to disentangle the effects of the slow influx of glucose from the fermentation of resistant starch/dietary fibres (46).
Another way of lowering the PPG, without increasing the PPI, is by increasing the glucose disposal by improving insulin sensitivity. Sustained intake of some fibres may benefit insulin sensitivity (1). However, this is beyond the scope of this thesis.

Who could benefit from lowering glucose influx? It seems likely to be beneficial for the general population, but particularly for people with insulin resistance (e.g. people with overweight and obesity, sedentary and older people). In addition, some ethnic groups e.g. South-Asian people are in general more insulin resistant compared to Caucasians with the same BMI (47). In these subjects the PPG response will remain high compared to the response in more insulin sensitive subjects, or this will be compensated by a higher PPI response (48). A slower glucose influx can avoid these compensatory mechanisms and contribute to normoglycaemia. People who regain weight after weight loss, will also benefit from a lower RaE, due to a lower insulin response and the fact that after weight loss insulin sensitivity increases especially in adipose tissue compared with skeletal muscle. This has been implicated in a greater metabolic tendency for fat storage (49).

The golden standard for measuring the RaE is the dual stable isotope technique; however, this technique is labor-intensive and relatively expensive, especially if an intrinsically labelled substrate must be grown specifically for the research. Other clinical methods (based on metabolomic analyses of co-ingested tracers) could be an alternative for intrinsically labelled substrates. However, alternative *in vitro* digestion methods would be preferred by the food industry to study the rate of influx of glucose. In a recent meta-analysis it was shown that the SDS content in cereal products (measured by the *in vitro* digestion method) had a strong correlation with the RaE (50).

In summary, it is possible to lower the PPG response to carbohydrate-rich staple products, for example by introducing a fibre mix with a viscous fibre and legume flour. In addition, by choosing the right cultivar (e.g. high amylose rice) and processing steps (e.g. parboiling rice, short-cooking time, cooking in combination with cooling steps) one can substantially decrease the PPG response. The best way to lower the PPG response is by decreasing the rate of influx of glucose, which evokes a change in hormonal responses such as lower GIP and insulin response, which are also beneficial for health.

**Future perspectives**

This thesis shows that by slowing the influx of glucose it is possible to change the hormonal profile, glucose kinetics and metabolites down-stream to a healthier profile. The food industry has many solutions to slow the influx of glucose from staple foods either by choosing the cultivar of the starch-containing plant or introducing specific ingredients or processing conditions. To put this in a wider public health context, it would be recommended not only to study starch-rich products in isolation, but also in a total meal concept. Another recommendation is to characterize the starch or fibre...
product very precisely (e.g. name of cultivar) and specify the exact processing conditions to be able to faithfully replicate and compare studies. In addition, it would be useful to include in future studies also analyses of other glucose-modulating hormones such as amylin, peptide-c and glucagon, as well as a broader profile of metabolites, to get more insights in the mechanism of action and prediction of potential health impacts.
REFERENCES


