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This is the peer reviewed version of the following article:

Petropoulou, K. et al. (2016) Identifying crop variants with high resistant starch content to maintain healthy glucose homeostasis. *Nutrition Bulletin*, 41(4), pp. 372-377.
(doi:[10.1111/nbu.12240](https://doi.org/10.1111/nbu.12240))

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Deposited on: 10 January 2020

Title: Identifying crop variants with high resistant starch content to maintain healthy glucose homeostasis.

Running Head: Crop variants, resistant starches and β cell function

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Abstract

Identifying dietary tools that prevent disordered insulin secretion from pancreatic β cells is an attractive strategy to combat the increasing prevalence of type 2 diabetes. Dietary resistant starch has been linked to improvements in the function of β cells, possibly via increased colonic fermentation and production of short chain fatty acids (SCFA). Increasing the resistant starch content of commonly consumed foods could therefore maintain glucose homeostasis at the population level. As part of Biotechnology and Biological Sciences Research Council (BBSRC) Diet and Health Research Industry Club (DRINC) initiative we are investigating variants of *Pisum sativum* L. (pea) to identify the features of pea starch that make it resistant to digestion and available for colonic fermentation and SCFA production. Parallel *in vitro* and *in vivo*, studies are being conducted using both whole pea seeds and pea flour to facilitate a better understanding of how cells in the pea cotyledons are affected by processing and, in turn, how this influences starch digestibility. Trials in human volunteers are being used to monitor a full spectrum of short- and long-term physiological responses relevant to pancreatic β cell function and glucose homeostasis. This project is providing new insights into variants of crops that are associated with the specific types of resistant starch that provide the best protection against defects in insulin secretion and function.

Introduction

In 2013, there were over 3.2 million people in the UK with a diagnosis of type 2 diabetes and rates are expected to rise to 5 million by 2025 [1]. The increasing prevalence and economic burden of this chronic health condition calls for an urgent public health solution. Epidemiological and clinical evidence suggests that dietary changes can prevent or delay the development of type 2 diabetes [2, 3]. Nevertheless, despite national campaigns to promote healthy eating, obesity and type 2 diabetes diagnosis rates continue to escalate. An alternative dietary strategy to maintain glucose homeostasis at the population level is to improve the composition of commonly consumed foods.

Under normal physiological conditions, β cells of the pancreas secrete insulin in a controlled pulsatile manner to maintain blood glucose within a narrow homeostatic range. Insulin is released directly into the portal vein and direct measurements in humans reveal that the normal pulsatile pattern of insulin secretion varies from 4-20 min (i.e. the time interval from basal levels to peak concentration and return to basal levels) [4]. Consequently, insulin concentrations in the fasted state can range from 100-1000 pmol/L in the portal circulation and 10–30 pmol/L in the systemic circulation [4]. A plethora of research studies have shown that with sustained adverse lifestyle habits, including poor diet, type 2 diabetes develops and progresses against a background of worsening insulin resistance (reduced cellular and tissue insulin sensitivity), which ends with inadequate insulin secretion and sustained hyperglycaemia [5]. There is evidence that disruption to the normal pulsatile secretion of insulin from pancreatic β cells may be a major factor in the initial development of insulin resistance [6]. Therefore, dietary choices and behaviour can have a direct effect on β cell function but attention has focussed largely on restriction, with reductions recommended in calorie consumption, saturated fat and fibre-poor or high glycaemic index foods. Accordingly identifying more readily-acceptable dietary tools to arrest disordered insulin secretion from β cells is an important challenge to maintain healthy glucose homeostasis and prevent the natural progression of type 2 diabetes.

This project, funded by the BBSRC DRINC initiative, aims to provide a systematic basis for production of new forms of common foods that protect β cell function and maintain glucose homeostasis in ways that can be applied to the general population. **Crop Resistant Starch and Health (CRESTAR)** is a multi-partner project that examines how variants of pea seeds, high in resistant starches, can protect against defects in insulin secretion and function.

Dietary Resistant Starch and β cell function

Dietary starches can be broadly classified as rapidly digestible, slowly digestible or resistant, depending on their digestion rate and effects on postprandial glucose and endocrine pancreas responses [7]. Resistant starch is the portion of dietary starch that cannot be digested/hydrolyzed by amylolytic enzymes, such as α -amylase, glucoamylase and sucrose-isoamylase, in the small intestine [8, 9]. Consequently they become available for fermentation by the gut microbiota [8, 9]. The main end-products/metabolites of bacterial fermentation are short chain fatty acids (SCFAs) with acetate, propionate and butyrate being the most abundant in the human colon, present in the approximate molar ratio 60:20:20 [10].

There is mounting evidence that diets rich in resistant starches can have a positive effect on glucose homeostasis and help prevent the onset of type 2 diabetes [11, 12]. Studies in humans have shown that postprandial blood glucose and insulin sensitivity improve following dietary supplementation with resistant starch, including in patients with type 2 diabetes [11-18]. In studies where participants have undergone a hyperinsulinemic-euglycemic clamp or insulin-modified frequently sampled intravenous glucose tolerance test (FSIVGTT), results confirm that resistant starch consumption improves first-phase insulin secretion, peripheral insulin resistance, and C-peptide concentrations [17, 19].

These beneficial health outcomes may be due to the actions of SCFAs. Specifically, SCFAs activate free fatty acid receptor (FFAR) 2 and FFAR3 [20-22], which are expressed on a number of tissue sites, including the colon and white adipose tissue, and generate physiological changes that would be advantageous to normal β cell function. Both FFAR2 and FFAR3 are co-expressed on

colonic L-cells [23] that synthesize and release the incretin glucagon-like peptide-1 (GLP-1) [24]. Investigations using primary colonic cell cultures have demonstrated that SCFAs trigger the release of GLP-1, primarily via the stimulation of FFAR2 [24, 25]. Following secretion into the circulation, GLP-1 binds to its receptor on the β cell, thereby stimulating insulin-release in a glucose-dependent manner [26]. In addition to this insulinotropic action, GLP-1 has been shown to preserve pancreatic β cell mass by enhancing proliferation [27] and inhibiting apoptosis [28]. Consequently, interventions that up-regulate GLP-1 secretion and function are the focus of numerous therapeutics to prevent and treat type 2 diabetes. Results from rodent studies have shown that resistant starch supplementation can stimulate GLP-1 secretion in a substantial day-long manner, independent of meal effect or changes in dietary glycaemia [16, 29]. Additionally, studies in humans have reported that increased dietary intake of fermentable starches promotes postprandial GLP-1 release [30, 31].

SCFAs that do not undergo colonic metabolism enter the systemic circulation where they interact with different organ and tissue sites. It has been demonstrated in human studies that increasing the resistant starch content of the diet raises SCFA levels in the peripheral blood [32, 33], particularly acetate. FFAR2 and FFAR3 are also expressed on pancreatic islets, and a number of studies have investigated whether SCFAs contribute to insulin secretion, with equivocal results. Both inhibitory and stimulatory effects of SCFAs on glucose stimulated insulin secretion (GSIS) have recently been reported and the conflicting data are likely due to differences in study methodology [24, 34, 35]. Additional studies are warranted to clarify the effects of SCFAs on islet function, but a direct stimulatory effect on GSIS remains a possibility.

A further possible mechanism linking SCFAs with improved β cell function is via suppression of free fatty acid (FFA) release from adipocytes. There is evidence from work *in vitro* that SCFAs inhibit lipolysis and this effect is attributed to stimulation of FFAR2 by acetate [36-38]. *In vivo* data also show that consumption of resistant starches reduces lipolysis and circulating FFA, resulting in improvements in peripheral insulin sensitivity [32]. Long-term reductions in lipolysis would also protect β cells from the lipotoxic effects of elevated FFA [39].

In summary, a potential strategy to maintain healthy glucose homeostasis is to increase the amounts of dietary resistant starch that is available for fermentation by the colonic microbiota, as the main end products (SCFAs) can potentially preserve β cell health via direct and indirect mechanisms.

Increasing Dietary Intake of Resistant Starch: The CRESTAR Project

Whilst many fruits and vegetables contain resistant starch, UK diets generally contain low amounts. Resistant starch is thought to make up approximately 5% [40] of total starch intake, but the actual resistant starch availability to the colon is difficult to estimate for various reasons, such as food processing and preparation, as well as gut physiology and digestibility. The most significant structural aspect that influences starch digestibility is the level of crystallinity within the starch granule [41]. Starches are semi-crystalline and form roughly spherical granules in plant tissues [42]. The shape and size of granules depends on the ratio between amylose and amylopectin and, specifically, how these two molecules are organized within the granule [42]. Amylose has a degree of polymerization up to 6,000 and a linear structure [43]. Starches with linear, long chains have a greater tendency to form crystalline structures that are resistant to enzymatic digestion [41]. A higher content of amylopectin has the opposite effect: it increases the digestibility of the starches, due to the large surface area. Amylopectin has a degree of polymerization up to 2×10^6 and is highly branched [43]. Raw potatoes, unripe bananas, and some legumes are high in resistant starches [44]. Accordingly, a higher resistant starch content is found in naturally occurring variants of crops, including the amylose-extender variants of maize and the *r* variants of pea. [45, 46]. These variants have lower starch branching enzyme (SBE) or starch synthase (SS) activity, which results in higher amounts of amylose and thus resistant starch content. Most of the starches consumed in westernized diets are in a gelatinized form. Gelatinization is the process whereby the cooking of starch granules in water makes them swell and thus opens up the structure of the starch granule making it more accessible to digestive

enzymes[42]. Starch from crops lacking specific SBE or SS remains more crystalline even after cooking, thus preventing swelling of the starch granule and amylose leaching [42].

The CRESTAR project will determine the features of pea starches that make them resistant to digestion and available for colonic fermentation. Pea seeds are being studied as they are a commonly consumed food item in the UK and there is a range of available variants known to contain different types of resistant starches. The first aim of the project is to identify the features of the starch matrix structure that make it optimal for resistance to enzymatic digestion, by exploiting the variation that exists in pea seeds. Selected pea seeds are tested to study the effects of cooking and simulated oral-gastro-duodenal digestion on the structure of the pea tissue and starch granules using both whole pea seeds and pea flour. This is important as it facilitates a better understanding of how the food matrix (principally cells in the pea cotyledons) is affected by processing and in turn how this influences the starch digestibility. The Dynamic Gastric Model (DGM) [47] is used to examine how the starch microstructure controls the rate and extent of *in vitro* digestibility. Digested samples are then fermented in a model colon to mimic the physiological conditions of the human large intestine [48]. In parallel, selected genotypes of pea are being used in human volunteer studies to monitor a full spectrum of short- and long-term physiological responses relevant to pancreatic β cell function and glucose homeostasis. Initial studies examine *in vivo* digestibility, using enteral tubes that allow samples from the GI tract to be collected following consumption of the selected whole pea seeds and pea flour. Microscopy and metabolomics assessment provides a measure of the impact of initial digestion on the breakdown of the different peas. A second acute study will provide information on whether the resistant starch from pea genotypes lacking specific SBE or SS is effective in the production of SCFAs in the colon. Pea plants have been grown in a $^{13}\text{CO}_2$ enriched environment to intrinsically label the starch within their seeds. This labelling, with stable (non-radioactive) isotopes allows the non-invasive tracking of starch digestion and fermentation and the direct measurement of the rate of appearance of molecules that reflect the complete utilization of starch *in vivo*. The measurement of ^{13}C glucose and ^{13}C SCFA output in blood and urine samples post-ingestion determines the extent to which the resistant pea starch is digested and fermented. Finally, a

randomized controlled trial will provide information on beta cell function following long-term supplementation with the selected pea variants, by measuring pulsatile secretion of insulin and glucose handling in intravenous and oral glucose tolerance tests.

In summary, the CRESTAR project is providing knowledge to underpin a systematic basis for incorporating resistant starches into common foods with the aim to protect β cell function and glucose homeostasis for the general population. This project will provide new insights into the relationship between resistant starch and susceptibility to type 2 diabetes. Importantly it will inform crop breeders about which variants of crops are associated with the specific types of starch that provide the best protection against defects in insulin secretion and function. Furthermore, the project is expected to impact on the versatility of the pea crop, by investigating the use of pea flour, which can be incorporated into commonly consumed foods. The accrued knowledge will underpin further research on increasing the resistant starch content of more widely consumed foods such as those derived from wheat and maize.

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