

# $^1\text{H}$ NMR spectroscopy for the *in vitro* understanding of the glycaemic index

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## Abstract

The glycaemic index (GI) characterises foods by using the incremental area under the glycaemic response curve relative to the same amount of oral glucose. Its ability to differentiate between curves of different shapes, the peak response and other aspects of the glycaemic response is contentious. The present pilot study aimed to explore the possibility of using  $^1\text{H}$  NMR spectroscopy to better understand *in vivo* digestion characteristics as reflected in the glycaemic response of carbohydrate-rich foods; such an approach might be an adjunct to the *in vivo* GI test. The glycaemic response of two types of raw wheat flour (2005 from Griffith NSW, Chara, Row 10, Plot 6:181 and store-bought Coles™ Plain Flour) and a cooked store-bought flour was tested and compared with results recorded during the *in vitro* enzymatic digestion of the wheat flour samples by glucoamylase from *Aspergillus niger* (EC 3.2.1.3) as monitored by  $^1\text{H}$  NMR spectroscopy. Comparing the digestion time courses of raw and cooked wheat starch recorded *in vitro* strongly suggests that the initial rate of glucose release *in vitro* correlates with the glycaemic spike *in vivo*. During the *in vitro* time courses, approximately four times as much glucose was released from cooked starch samples than from raw starch samples in 90 min. Monitoring enzymatic digestion of heterogeneous mixtures (food) by  $^1\text{H}$  NMR spectroscopy showcases the effectiveness of the technique in measuring glucose release and its potential use as the basis of an *in vitro* method for a better understanding of the GI.

**Key words:** Digestion kinetics: Glycaemic index: Time-resolved nuclear magnetic resonance: Carbohydrate digestion

In the past 25 years, the *in vivo* digestibility of foods has been quantified by a number of metrics, the most popular of which is the glycaemic index (GI). The GI was first defined by Jenkins *et al.*<sup>(1)</sup> as the total glycaemic response in the blood (incremental AUC of glucose concentration *v.* time above basal values) in the 2 h immediately subsequent to the consumption of a fixed amount of carbohydrate; it is expressed as a value relative to that of a standard food, normally white bread or a glucose control<sup>(1,2)</sup>. The GI of food is a measure of the rate at which the contained carbohydrate is hydrolysed in the digestive system and absorbed via active and facilitated diffusion across enterocyte membranes into the bloodstream. The immediate effects of carbohydrates on an individual's blood glucose concentration are of interest not only for nutritional guidance but the glucose concentration also has various health implications<sup>(3)</sup>. Many intrinsic and extrinsic factors affect the nature of starch and so affect the GI value of a food, including starch structure at all six levels of organisation<sup>(4)</sup> (individual branches, whole molecules, lamellar structure, granule structure, endosperm and the whole grain). These factors are gastrointestinal motility, the method of cooking and the presence of fibres, fat and proteins<sup>(5,6)</sup>. Furthermore, the subjects used to test varieties of food

must be carefully chosen, as the glycaemic response to carbohydrates varies with health status and ethnicity<sup>(7,8)</sup>.

Although widely used, both for research and commercially, the validity of these indices based on the GI as a guide for dietary design is still controversial. Sceptics question fundamental properties of this functional measure, including its reproducibility and therefore its meaning in dietetics<sup>(5,6,9,10)</sup>, as well as its relevance to diseases with a nutritional basis.

Other systems used to rank the *in vivo* digestibility of carbohydrates are glycaemic load, glycaemic response and resistant starch. The glycaemic load is a measure used as a basis for weight loss, or a parameter for diabetes control. Also controversial<sup>(6)</sup>, the glycaemic load of a food equals the product of the GI of the food multiplied by the amount of available carbohydrate in the serving consumed. As with the GI, the glycaemic load has a scale used to collect food varieties into groups suitable for certain diets. The glycaemic response is a measure of the increase in glucose concentration in the blood at any given time after the ingestion of carbohydrate and resistant starch, along with other starch fractions. Research has explored the association between a food's GI and the shape of the plasma response curve in healthy individuals.

**Abbreviations:** GI, glycaemic index; RDS, rapidly digested starch; SDS, slowly digested starch.

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Results show that multiple attributes of the glycaemic response curve could reliably predict the GI of foods<sup>(5)</sup>.

For routine carbohydrate digestion analysis, *in vivo* techniques are neither time nor cost-effective. The use of *in vitro* techniques as an alternative to *in vivo* data has been widely reported over the last 30 years<sup>(2,9,11–16)</sup>. Unfortunately, *in vitro* techniques have not been applied to routine food analysis, as, currently, there is a lack of robustness, reliability and standardisation of the technology. Currently existing variation between methods can introduce significant differences in estimates of glycaemic properties<sup>(17)</sup>. However, current methods and models are capable of predicting with reasonable accuracy the glycaemic properties of carbohydrate digestion *in vivo*.

In the present study, we used NMR spectroscopy as the means whereby starch digestion by one enzyme only (glucoamylase from *Aspergillus niger*; EC 3.2.1.3) was measured *in vitro*. NMR spectroscopy has been widely applied to identify solute molecules in complex heterogeneous mixtures<sup>(18–25)</sup>. <sup>1</sup>H solution-state NMR has been used to follow the progress of physical and chemical reactions in various biological systems including cell suspensions<sup>(18–25)</sup> and starch suspensions<sup>(26,27)</sup>. Provided that the solutes monitored during the entirety of an enzymatic reaction are in free solution, and any particles in the suspensions can be kept from settling during their observation, accurate estimates can be made of product release. In the present context, we studied the formation of free glucose from various starch samples. By implication, the methodology used here could be used with more than one type of digestive enzyme and with various treatments of starch, or even using more complex food samples. Hence, the present study is presented as an initial exploration of the possibility of using <sup>1</sup>H NMR spectroscopy for an *in vitro* test of the digestion characteristics of carbohydrate-rich foods, which might be an adjunct to the *in vivo* GI test.

## Methods

The glycaemic response to two types of raw wheat flour (2005 from Griffith NSW, Chara, Row 10, Plot 6:181 and store-bought Coles™ Plain Flour) and one type of store-bought whole-wheat flour cooked as a basic pancake with water was tested and compared with respect to the response to a reference food (50 g glucose dissolved in 250 ml water tested on three separate occasions within a 3-month period for each subject). For each test, ten healthy subjects consumed a portion of food containing 50 g of digestible carbohydrate from the flour sample suspended in 250 ml water in a 10 min period following a 10–12 h fast. Finger-prick capillary blood was collected at –5 and 0 min (start of the meal) and at 15, 30, 45, 60, 90 and 120 min into 1.5 ml centrifuge tubes containing 10 U heparin. Plasma was separated after centrifugation (45 s, 7800 g) and cooled on ice until measurement on the same day. Glucose concentrations were measured in duplicate using a glucose hexokinase assay on a spectrophotometric analyser (Hitachi 912, Automatic Analyser; Hitachi). For each test, the average incremental AUC of all subjects was estimated according to the trapezoidal method and compared with the

average incremental AUC for the reference food. The area under the blood glucose fasting curve (averaging 5–12 mmol/l) was ignored. The GI was determined in all subjects, thus the GI of one flour sample was based on 640 separate glucose determinations (ten subjects, eight time points, duplicate assays, three reference foods and one test food). The test foods were randomised, although the reference food sessions were fixed at the start, middle and end of the study. There was a ‘washout’ period of at least 1 d between consecutive test sessions. The participants did not consume the same test food at the same time, the time and day which they were tested was not fixed and the order the test foods were presented to them was randomised.

Preparation of raw starch samples for *in vitro* analysis involved suspending the flour granules in the solvent described later in this section. For the *in vitro* analysis of cooked starch, a starch suspension of the same concentration (4%, w/w) was heated in a water-bath at 80°C for 30 min to mimic the production of a basic pancake that was used for *in vivo* analysis. The pancake preparation was chosen because it is in essence a simple cooked starch solution, although we cannot be sure that it provided equivalent starch evolution to the gelatinisation of granules in a water-bath.

All NMR spectra were acquired on a Bruker Avance III spectrometer (Bruker), equipped with a 9.4 T wide-bore vertical magnet (Oxford Instruments), operating at a radio frequency of 400.09 MHz for <sup>1</sup>H detection, using a 5 mm triple resonance inverse probe. The probe temperature was set to 37°C for all experiments. A Carr–Purcell–Meiboom–Gill pulse sequence was used with an echo time of 0.5 ms and an echo pulse train of 100 repetitions<sup>(28)</sup>. It decreased the broadness of the solvent peak as water has a short transverse relaxation time, and was coupled with a water pre-saturation pulse (power attenuation of the 100 W amplifier, 55 dB) during the relaxation delay. The 90° pulse duration was approximately 11.5 μs; and the acquisition time and relaxation delay were 8 and 2 s, respectively. For inhomogeneous starch samples, the tube was spun at a rate of 20 Hz. Each spectrum was derived from eight transients preceded by four dummy transients. The chemical shift was calibrated using the resonance from sodium 3-(trimethylsilyl)propane-1-sulfonate at 0.000 parts per million. An exponential line broadening of 1 Hz was used with no zero-filling before Fourier transformation of the free induction decay. The data were recorded and processed using TOPSPIN 2.1 (Bruker).

Digestion was carried out in 40 mmol/l of sodium acetate buffer, at pH 5.3, made up in D<sub>2</sub>O, containing 10 mmol/l of sodium 3-(trimethylsilyl)propane-1-sulfonate, an internal standard for the quantification of the concentration of reducing-end glucose residues. Before enzyme addition, a spectrum of each solution was obtained, and the integral of the –C<sub>1</sub>H resonance of the α- and β-reducing ends was subtracted from the signal recorded during the time course. α-Amylase (EC 3.2.1.1) from *Bacillus licheniformis* (Megazyme) solution (0.3 U/ml) was added to the wheat flour solutions (4%, w/w). The delay between beginning the enzymatic reaction and recording a <sup>1</sup>H NMR spectrum was precisely timed (approximately 1 min). Afterwards, up to 500 <sup>1</sup>H NMR spectra

(approximately 2 min each) were acquired sequentially, following the addition of the enzyme.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Research Integrity Group.

## Results and discussion

### *In vivo measurement of raw wheat flour glycaemic index*

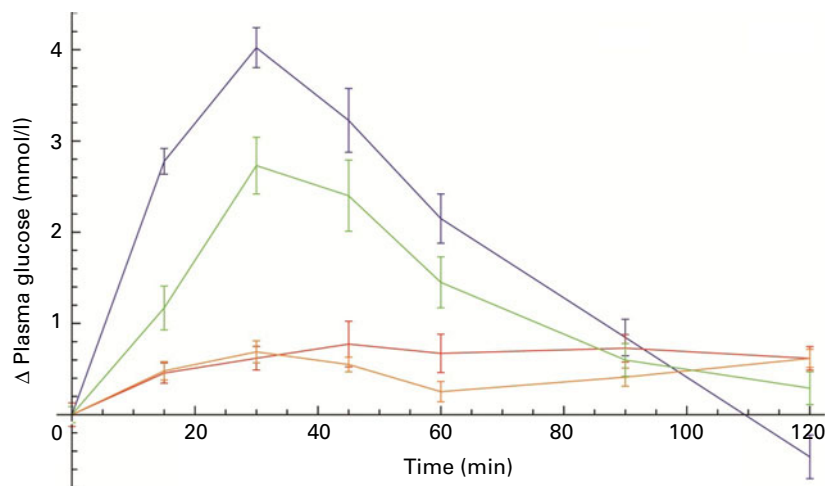
The design of the present study used starches in the form of native granules free from other constituents, allowing us to delimit better, in a multifactorial complex process, the main determinants of the physiological response. The granular integrity was confirmed by the absence of swelling or solubilisation by *in vitro* enzymatic digestion and  $^1\text{H}$  NMR. The present study clearly confirmed that starches give a weaker metabolic response when raw than when cooked.

The raw wheat starch flour samples tested *in vivo* recorded very low GI values. The wheat flour from Griffith was measured to have a GI value of 22 (SEM 3) and the store-bought plain flour a value of 20 (SEM 2). The wheat flour samples demonstrated an insignificant difference between their GI values. However, many characteristics are apparent when comparing the shape of the glycaemic response curves of raw wheat starch (Fig. 1) with foods of various GI values<sup>(5)</sup>. Similarly to other starchy foods (even when processed and gelatinised), the glycaemic response remained above the baseline for the entire 2 h period<sup>(29)</sup> (Fig. 1). Unlike foods containing simple sugars, digestive enzymes must hydrolyse starch to produce glucose, providing a relatively sustained source of glucose.

The raw wheat samples (store bought and farm grown) had a relatively low absolute glycaemic response (the glycaemic spike), characteristic of low-GI foods (5.77 and 5.84 mmol/l, respectively). The glycaemic response curve also peaked

absolutely at 45 min with the Griffith-grown wheat flour sample. Glycaemic responses spiking at values after 30 min following consumption are attributed to foods that have a very low GI value<sup>(5)</sup>. The glycaemic spike occurred later, even though raw starch was on average digested at a significantly slower rate than cooked starch. The wheat flour appeared to contain very little rapidly digested starch (RDS), i.e. starch that is readily susceptible to enzymatic hydrolysis. The Englyst<sup>(13,30)</sup> *in vitro* procedure defines RDS as the fraction of total starch digested within 20 min under standardised *in vitro* digestion conditions, and slowly digested starch (SDS) as starch released from 20 to 120 min. In several studies, RDS has been found to be a reliable predictor of GI values<sup>(15,16)</sup>. As might be expected, starch granules in the raw wheat flour appear to contain so little RDS such that the glycaemic spike following consumption occurs after 30 min, reflecting the time lag before significant amounts of glucose are released from the SDS. Granfeldt *et al.*<sup>(14)</sup> also developed an *in vitro* procedure based on chewing to predict the metabolic response to starch in cereal and legume products. Further studies in a variety of foods comparing the amount of RDS and SDS, as defined by Englyst *et al.*, with the time course of digestion as monitored by  $^1\text{H}$  NMR spectroscopy will be necessary to define the relative merits of different *in vitro* methodologies. It is also possible that under different *in vitro* conditions, alternate time points may better reflect the peak *in vivo* glycaemic response.

The glycaemic response curves of the raw wheat flour tested also had multiple peaks that are unorthodox for glycaemic profiles of most food products<sup>(5,29)</sup>. The multiple peaks occurred because the plasma glucose response was relatively constant throughout the measured 2 h period (Fig. 1). The observed effect can be attributed to the body 'dumping' much of the ingested raw starch through the intestine without it being acted upon by hydrolytic enzymes; this would result in much of it passing through undigested.  $\alpha$ -Amylases, which first attack starch in the upper digestive tract, do not



**Fig. 1.** Incremental blood glucose profiles for 50 g glucose reference (blue), store-bought whole-wheat flour cooked as a basic pancake (green), raw wheat flour sample grown in Griffith in 2005 (red) and raw plain flour from Australia bought at a local store (orange). The plotted plasma glucose concentration ( $\Delta$  plasma glucose) is the change in plasma glucose from the average fasting concentration of the ten subjects tested (5.14 mmol/l). The error bars signify one standard deviation. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>).

hydrolyse large molecules in raw granules into many smaller  $\alpha$ -limit dextrins and linear oligomers. Hypothetically, the unsuccessful splitting of starch molecules by  $\alpha$ -amylase disrupts the action of glucosidases that are encountered more distally in the digestive tract.

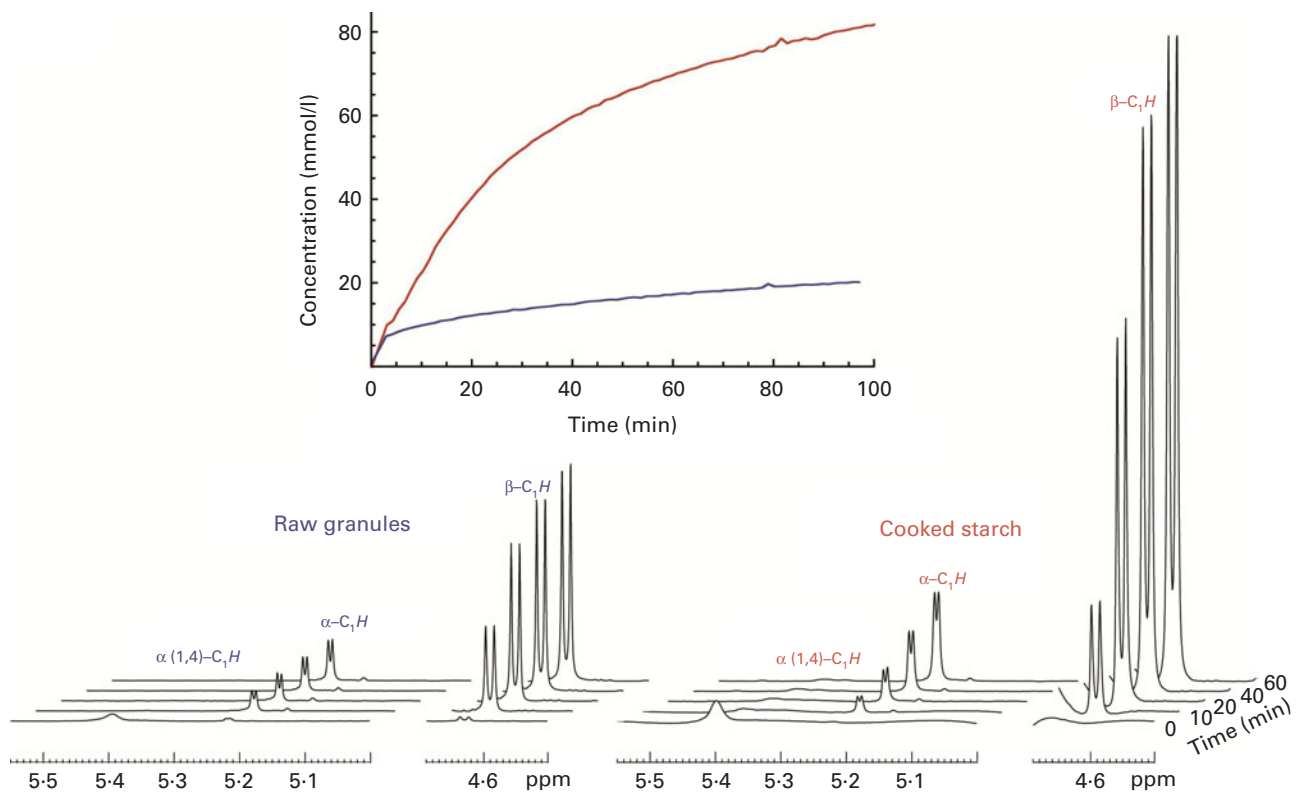
Comparing the glycaemic response curves of raw starch varieties with those of a whole-wheat flour sample that had been cooked as a basic pancake leads to many conclusions. When tested *in vivo*, cooked starch pancakes were measured to have a medium GI value of 61 (SEM 7). The absolute glycaemic response was much larger in cooked samples (7.79 mmol/l), which occurred as in many other food varieties, 30 min after consumption. Similarly to raw wheat starch samples, cooked wheat flour had a glycaemic response that remained above the baseline for the entire 2 h period of testing (Fig. 1).

### *In vitro* measurement of wheat flour digestion

As raw starch granules are not swollen in solution (in contrast to other processed and cooked starchy foods), the hydrolytic enzymes throughout the digestive system can only act on the outer surface<sup>(31–33)</sup>. The surface interactions of the core of insoluble, raw starch granules with digestive enzymes are responsible for the relatively constant production of glucose, similar to *in vitro* raw starch digestion<sup>(27)</sup>. Consequently, active transport of glucose from the gut into the bloodstream provides, over a 2 h period, a relatively constant glycaemic profile.

Although it is relatively common to use *in vitro* digestion techniques to understand further the glycaemic response of carbohydrates in the body<sup>(13–16)</sup>, comparing them with the present study is problematical as various starches, methods of enzymatic digestion and methods of glucose measurement were applied. Comparing the digestion time courses of raw and cooked wheat starch *in vitro* leads us to suggest that the percentage of starch digested during the RDS stage determines the extent of the glycaemic spike *in vivo*. During the *in vitro* time courses, four times as much glucose was released from cooked starch samples than from raw starch samples in 90 min. The initial rate of release of glucose from the RDS of both samples was  $4.8 \times 10^{-2}$  mmol/l per s. The relatively fast rate of glucose production was only maintained for 3 min in the raw starch sample, whereas in the cooked sample, the RDS stage lasted about 20 min (Fig. 2). The RDS stage took longer to complete in the cooked sample implying that a larger portion of the granule was accessible in solution resulting in relatively large amounts of glucose released.

The fraction of the granule consisting of RDS determines the amount of glucose rapidly absorbed into the blood that is subsequently removed relatively quickly via insulin-dependent GLUT in the tissues of healthy subjects. Large portions of RDS produce a considerable glycaemic spike in subjects which the body compensates for, causing, in a relatively short period, the glucose concentration in the blood to drop below the normal fasting value. On the other hand, small portions of RDS (as seen in raw granules) produce only a small



**Fig. 2.** <sup>1</sup>H NMR (400-13 MHz) spectra displaying the time dependence of the digestion of raw wheat flour starch (4%, w/w; blue) and wheat flour starch that had been thermally treated at 66°C for 30 min (red), after the addition of glucoamylase (*EC* 3.2.1.3; 1.2 U/ml) at 25°C. ppm, Parts per million. Inset: the kinetic time courses of the production of glucose from the digestions. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>).



glycaemic response in subjects, thus failing to trigger a considerable release of insulin, causing the concentration of glucose in the blood to maintain a relatively constant value.

Both cooked and raw starch granules produce glucose at a slow rate during the later SDS stage (Fig. 2), as the enzymatic activity is limited by acting on the insoluble core during this stage of starch hydrolysis. The rate for the cooked starch was nonetheless 3.3 times that from the uncooked starch. However, the most prominent difference between the time courses of raw and cooked starch granules was the amount of starch digested during the initial 20 min of the reaction (the portion of RDS).

During the quasi-steady-state stage (slowly digested starch stage) of digestion, both cooked and raw granules yielded glucose at a relatively slower rate as observed by  $^1\text{H}$  NMR (Fig. 2). During the later stages of digestion of raw granules, glucose is absorbed into the bloodstream from the gut at a slow rate, and it is removed by tissues from the blood at approximately the same slow rate. The similarity of these rates causes the almost constant glycaemic profile that is observed in subjects *in vivo* following ingestion of raw granules (Fig. 1). However, a rapid increase in blood glucose causes a subsequent rapid uptake of glucose by tissues in the body (as in cooked starch samples; Fig. 1). Hence, during the SDS stage of cooked starch samples, when glucose production in the gut is still more than three times faster than when compared with raw granules, the concentration of blood glucose decreases at a significant rate. Consequently, a 'glycaemic spike' is produced post-cooked starch ingestion rather than a constant glycaemic profile produced post-raw granule digestion (Fig. 1).

### Conclusions

The shape of the glycaemic profile from the digestion of raw wheat starch granules was explored and compared with typically consumed food products of various GI values. After ingestion of food, it is considered healthy for humans to have a sustained level of glucose in the blood. These levels provide perceptions of satiety and obviate hypoglycaemic episodes. However, the notion that a low-GI food has a glycaemic profile with a uniquely long tail, or a sustained extended glucose profile, is incorrect<sup>(5)</sup>. Although an SDS or oligosaccharide may constitute a slow-release form of energy, this does not mean that a low-GI food produces a sustained glucose response in the blood plasma. On the other hand, digestion of raw wheat starch does constitute a carbohydrate source with a low GI value and a sustained glucose response.

The experiments conducted *in vitro* on raw granule digestion attribute the sustained blood glucose response observed *in vivo* to two factors. (1) Raw granules contain a large portion of starch that contributes more glucose moieties than the same weight of sugars such as fructose, or as the disaccharides sucrose and lactose that contain an amount of glucose equal to fructose and galactose, respectively. (2) The largely impenetrable granule architecture of the starch granule hinders the enzyme-mediated digestive process, thus delaying

the release of small oligomers and, subsequently, glucose. Hence, the raw starch granule engenders a postprandial state that is considered 'healthy' for humans<sup>(34–36)</sup>.

Monitoring enzymatic digestion of heterogeneous (food) mixtures by  $^1\text{H}$  NMR spectroscopy, as presented here, showcases the effectiveness of the technique in measuring glucose release and its potential use as the basis of an *in vitro* predictor of the GI.

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