Baselines representing blood glucose clearance improve \textit{in vitro} prediction of the glycaemic impact of customarily consumed food quantities

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Glycaemic responses to foods reflect the balance between glucose loading into, and its clearance from, the blood. Current \textit{in vitro} methods for glycaemic analysis do not take into account the key role of glucose disposal. The present study aimed to develop a food intake-sensitive method for measuring the glycaemic impact of food quantities usually consumed, as the difference between release of glucose equivalents (GGE) from food during \textit{in vitro} digestion and a corresponding estimate of clearance of them from the blood. Five foods – white bread, fruit bread, muesli bar, mashed potato and chickpeas – were consumed on three occasions by twenty volunteers to provide blood glucose response (BGR) curves. GGE release during \textit{in vitro} digestion of the foods was also plotted. Glucose disposal rates estimated from downward slopes of the BGR curves allowed GGE dose-dependent cumulative glucose disposal to be calculated. By subtracting cumulative glucose disposal from cumulative \textit{in vitro} GGE release, accuracy in predicting the \textit{in vivo} glycaemic effect from \textit{in vitro} GGE values was greatly improved. 

\textbf{Carbohydrate: Glycaemic impact: Glucose disposal: \textit{In vitro} digestion}

For practical consumer and dietetic use in glycaemia management, food values are required which will ‘… communicate glycaemic response in grams per serving of food’\textsuperscript{(1)}, and which should be measured using ‘… validated \textit{in vitro} methodology that accurately mimics \textit{in vivo} behaviour’\textsuperscript{(2)}.

Current \textit{in vitro} digestion methods for predicting relative glycaemic responses to foods measure carbohydrate that is rapidly available (for example, Englyst \textit{et al.}\textsuperscript{(3)}, Brighenti \textit{et al.}\textsuperscript{(4)}), or the area under the glucose release curve relative to a reference\textsuperscript{(5)}. However, they cannot provide accurate or robust predictions of relative responses to whole foods varying in quantity, or accurately mimic the \textit{in vivo} glycaemic response, because they do not allow for the effects of homeostasis. They do not take into account the fact that glycaemic responses to foods are a net effect of the balance between blood glucose loading and blood glucose disposal (GD), which depends dynamically on the rates of both. Not allowing for blood GD, and its dependence on the amount and rate of blood glucose loading, may lead to large inaccuracies when using glycaemic impact values from experimental portions to gauge the effect of the very different and varying food quantities that are consumed in a community setting. For instance, customarily consumed food portions often have a much lower glycaemic impact than would be expected from a glycaemic load value calculated from the glycaemic index\textsuperscript{(6)}, because glycaemic index is based on a 50 g glucose reference, which is much greater than the glucose equivalents that would be consumed in most servings of foods. With the need for \textit{in vitro} methods that predict the relative glycaemic effect of realistic intakes of food\textsuperscript{(7)}, methods that take account of the effects of homeostatic responses to dose are required.

A recent well-replicated (n 20) clinical study of the glycaemic effects of five foods fed on three occasions, and glucose fed on four occasions, provided glucose response curves and clinical glycaemic glucose equivalent (GGE) values for the foods\textsuperscript{(8)}. \textit{In vitro} digestion of the same foods has given us curves of cumulative carbohydrate release. With detailed \textit{in vivo} and \textit{in vitro} datasets for the same foods we have an opportunity to measure improvements in the predictive validity of \textit{in vitro} measures when glucose homeostasis is allowed for.

In the present paper we test the hypothesis that the difference between the cumulative release of GGE during \textit{in vitro} digestion, adjusted for food intake, and the estimated cumulative disposal of them that would occur in the body will provide an accurate and intake-sensitive prediction of the relative glycaemic response \textit{in vivo}. The aim is to develop \textit{in vitro} methods that are flexible enough to accurately provide glycaemic impact values for the varying quantities of foods, of different carbohydrate composition, that are customarily consumed by humans.

**Abbreviations:** GD, glucose disposal; GGE, glycaemic glucose equivalent.

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**Materials and methods**

**Food samples**

The five test foods were all purchased from a supermarket and were white bread (Tip Top, Family Fresh Super Soft), fruit bread (Burgen), muesli bar (Mother Earth, sultana, oat and honey), instant mashed potatoes and canned chickpeas.

**Digestive analysis in vitro**

The food samples were provided to subjects who each took a mouthful of the size they would habitually consume, chewed until the urge to swallow, and then expectorated the mouthful into a beaker. For each food, the expectorated samples of all participants were immediately mixed in equal volumes and duplicate subsamples of the pooled material were quickly weighed into digestion pots. The chewed sample weights used were: white bread, 2.5 g; fruit bread, 2.5 g; muesli bar; 2.5 g; mashed potato, 10 g; chickpeas, 5-0 g (all ± 0.2 g).

Food samples were digested in 70 ml specimen pots inserted to their full depth in a fifteen-place aluminium heating block placed on a fifteen-place magnetic stirrer and covered with an insulating sheet. The digestion consisted of a simulated gastric digestion followed by an ileal digestion, with timed sampling during the small-intestinal phase. Water to 30 ml and 0·8 ml of 1 M-HCl were added to the sample to attain pH 2.5 (+0.2), with pH adjustment if necessary. Then 1 ml of 10% pepsin dissolved in 0·05 M-HCl was added, and the mixture stirred slowly and intermittently (15 s on, 15 s off, at 130 rpm) for 30 min to accomplish gastric digestion. The small-intestinal phase was initiated by neutralising the gastric HCl with 2 ml of 1 M-NaHCO3 and 5 ml of 0·1 M-sodium maleate buffer (pH 6)–0·2 % sodium azide–1 mM-CaCl2. Starch digestion was commenced by adding, in quick succession, 0·1 ml amyloglucosidase (catalogue no. E-AMGDF; Megazyme International, Bray, Co. Wicklow, Republic of Ireland) and 5 ml of 2·5 % pancreatin (Sigma P-7545; Sigma, St Louis, MO, USA) to convert them into slurries. A further 0·1 ml of 3,5-dinitrosalicylic acid dissolved in 1 litre of solution containing 300 g Na-K tartarate and 16 g NaOH. The fructose content of the digests was determined by the thiobarbituric acid method.

The weight of food in the chewed samples was determined by measuring available carbohydrate in the exact amount of food in the non-chewed homogenised samples. The results were then plotted as glucose equivalent release (mg) per g of unchewed sample v. time (Fig. 1).

**Converting in vitro glucose equivalents to glycaemic glucose equivalents**

In vitro GGE values were obtained by removing the fructose component of glucose equivalent (GE) (GE − fructose), and then adding back the amount of glucose that would be equivalent in its glycaemic effect to the removed fructose (0·2 fructose, as the glycaemic index of fructose is 20%): 

\[
\text{GGE (g)} = (\text{GE−fructose}) + 0.2 \text{ fructose.}
\]

**Measuring glucose equivalents released during in vitro digestion**

Sugars released during digestion were measured after an invertase + amyloglucosidase secondary digestion as glucose equivalents by a small-scale modification of the dinitrosalicylic acid colorimetric method, using glucose references. A 0.05 ml aliquot of ethanolic sample from the in vitro digestion above, or glucose standard (1 mg/ml glucose), was added to 0·25 ml of 0.2M-acetate buffer (pH 5·2) containing 1% of amyloglucosidase (catalogue no. E-AMGDF; Megazyme International) + 1% of invertase concentrate (catalogue no. 390203D; BDH, Poole, Dorset, UK) and incubated at 37°C for 10 min to complete depolymerisation to monosaccharides. Reducing sugars were then measured by adding 0·75 ml dinitrosalicylic mixture (containing a 1:1.5 mixture of 0.5 mg/ml glucose–4 M-NaOH–DNS reagent) and heated for 15 min at 95–100°C. The tubes were cooled, 4·0 ml water added, mixed, and absorbances read at 530 nm (Jenway 6100 Spectrophotometer; Jenway Ltd, Dunmow, Essex, UK). The DNS reagent consisted of 10 g of 3,5-dinitrosalicylic acid dissolved in 1 litre of solution containing 300 g Na-K tartarate and 16 g NaOH. The fructose content of the digests was determined by the thiobarbituric acid method.

**Fig. 1.** Release of carbohydrate during in vitro digestion measured as glucose equivalents (GE) and expressed per g of unchewed food. Each point is the mean of two determinations. Precision based on mean SD for all determinations was ±8·8 GE. Mean CV was 3·2%. (−●−), Fruit bread; (−○−), potato; (−■−), white bread; (−×−), chickpeas; (−□−), muesli bar.
Blood glucose responses

Blood glucose response curves were generated from background data to an earlier study for which ethics approval, subjects and methods have been described in detail (8). 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 Human Ethics Committee of the University of Otago. Written informed consent was obtained from all subjects. Foods were consumed at a serving size of 100 g and at an intake that would deliver 50 g available carbohydrate, except for chickpeas where enough was consumed to provide 25 g available carbohydrate (Table 1). Standard procedures were used, with capillary blood sampling and calculation of the incremental area under the blood glucose response curve using the triangle/trapezoid summation method described by the FAO/WHO (12). For each intake of food and glucose reference doses the blood glucose responses for all individuals at each blood sampling time were averaged and plotted against time, to give an average blood glucose response curve for each intake of each food and reference (Fig. 2). Smoothed curves were fitted to the data points using the Excel chart-plotting program and zero response at time zero was assumed. Errors associated with the blood glucose response measurements have been described in Venn et al. (8) and Table 1.

Establishing glucose disposal baselines

Rates of GD were estimated from the blood glucose response curves at each food intake by triangulation, in which the area under the linear portion of the post-peak decline in blood glucose was measured. As the GGE quantity represented curves at each food intake by triangulation, in which the area under the hypotenuse (Fig. 2). Smoothed curves were fitted to the data points using the Excel chart-plotting program and zero response at time zero was assumed. Errors associated with the blood glucose response measurements have been described in Venn et al. (8).

Fig. 2. Blood glucose responses for all individuals following the consumption of white bread at each blood sampling time averaged and plotted against time. Line A (-----): the delay in glucose loading was approximately 10 min, Line B (-----): the maximum net glucose loading rate was achieved by about 20 min. Line C (-----): an appropriate glycaemic glucose equivalent (GGE) value on which to base glucose disposal was that which had been released at 60 min. Area D (●): the glucose disposal rate was calculated as GGE loss per min from the area under the linear post-peak slope = (total GGE × triangle area/total area)/time using GGE in vivo values (Table 1). The disposal rates were then adjusted for non-linearity in the glucose standard (Fig. 3) before using them to generate lines of cumulative glucose disposal.

Table 1. Foods and food quantities used in clinical determination of glycaemic glucose equivalents (GGE) and in calculating corresponding glucose equivalents and GGE from in vitro digestion of chewed samples

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Food intake (g)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>White bread</td>
<td>One serving</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td>100 g</td>
<td>100</td>
<td>28</td>
<td>11.7</td>
</tr>
<tr>
<td>50 g CHOAVL</td>
<td>118</td>
<td>41</td>
<td>9.5</td>
</tr>
<tr>
<td>Fruit loaf</td>
<td>One serving</td>
<td>41</td>
<td>9</td>
</tr>
<tr>
<td>100 g</td>
<td>100</td>
<td>24.5</td>
<td>6.9</td>
</tr>
<tr>
<td>50 g CHOAVL</td>
<td>132</td>
<td>37.8</td>
<td>16.3</td>
</tr>
<tr>
<td>Muesli bar</td>
<td>One bar</td>
<td>50</td>
<td>13.9</td>
</tr>
<tr>
<td>Two bars</td>
<td>100</td>
<td>23</td>
<td>13.8</td>
</tr>
<tr>
<td>Three bars</td>
<td>150</td>
<td>30.3</td>
<td>13.2</td>
</tr>
<tr>
<td>Mashed potato</td>
<td>100 g</td>
<td>100</td>
<td>10.8</td>
</tr>
<tr>
<td>One serving</td>
<td>140</td>
<td>15.3</td>
<td>6.7</td>
</tr>
<tr>
<td>50 g CHOAVL</td>
<td>472</td>
<td>42.6</td>
<td>15.1</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>One serving</td>
<td>70</td>
<td>3.9</td>
</tr>
<tr>
<td>100 g</td>
<td>100</td>
<td>4.9</td>
<td>4.5</td>
</tr>
<tr>
<td>25 g CHOAVL</td>
<td>136</td>
<td>8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

CHOAVL, available carbohydrate.

Venn et al. (8).

Modelling glycaemic response as the balance between glycaemic glucose equivalent loading and glucose disposal

GGE released per g of food after 60 min in vitro digestion was multiplied by the food intake, and entered into the
equation of disposal rate \( v \), GGE intake (GD rate (in GGE/min) = 0.0135 \times \text{GGE intake (in g)} + 0.0232; \text{Fig. 4}) to obtain predicted GGE disposal rates (GGE/min) for the various food intakes (Table 1). From these predicted GD rates, baselines of cumulative GGE removal (GD rate \times time) could be plotted within the graphs of cumulative GGE release (Fig. 5).

GD rates in response to GGE released after 60 min in vitro digestion were used to calculate cumulative GGE disposal (Fig. 5), because the linear declines in blood glucose from which GD rates were calculated were completed about 60 min after the initiation of the blood glucose response (10 min delay plus 60 min) (Figs. 2 and 6(b)). Most of the GGE release in vitro at 60 min had occurred by 20 min digestion, so the same GD baseline could be used for a two-point net GGE determination based on rapidly available carbohydrate (20 min) minus cumulative GD at 20 min, but using the GD rate for a 60 min GGE intake. From the graphs containing a curve of GGE release and a line for GD for each intake of each food (Fig. 5), values for net glycaemic loading were obtained at each time point as the difference between cumulative GGE release and cumulative GD at the time (Table 2). The values for net glycaemic loading were then divided by the same adjustment factors as had been used to compensate for the non-linear glucose reference curve (for example, Table 2) and these readjusted net glycaemic loading values were plotted against time, to provide simulated blood glucose response curves (Fig. 6(a)) that represented the adjusted areas between the curves of GGE release and GD, and which could be compared with the unadjusted blood glucose responses (Fig. 6(b)).

**Measuring net glycaemic glucose equivalent content using in vitro area between the glycaemic glucose equivalent loading and disposal curves**

The areas between the lines of cumulative in vitro GGE release and GD (Fig. 6) were measured using the triangle/trapezoid summation method described by the FAO/WHO (12) (Table 3). One slice of white bread, which contained 12.0 GGE in an area of 343.6 GGE \times \text{min}, was used as a reference of known area per GGE. The areas (A) between the in vitro GGE release and GD lines calculated for the other food quantities were thus converted to GGE values using the relationship GGE = A \times 12/343.6 (g). The values obtained were then correlated with the clinically determined GGE values for the foods (Fig. 7) and a comparison of methods was made with a Bland–Altman analysis of the in vitro and in vivo datasets.
Fig. 6. Profiles of net glycaemic loading in vitro measured as the difference between cumulative glycaemic glucose equivalent (GGE) release in vitro (a) and cumulative theoretical glucose disposal, compared with in vivo blood glucose responses (b). White bread in vitro: (–○–), 37 g; (–□–), 100 g; (–△–), 118 g. White bread in vivo: (–●–), 37 g; (–■–), 100 g; (–▲–), 118 g. Fruit bread in vitro: (–○–), 41 g; (–□–), 100 g; (–△–), 132 g. Fruit bread in vivo: (–●–), 41 g; (–■–), 100 g; (–▲–), 132 g. Muesli bar in vitro: (–○–), 50 g; (–□–), 100 g; (–△–), 150 g. Muesli bar in vivo: (–●–), 50 g; (–■–), 100 g; (–▲–), 150 g. Potato in vitro: (–○–), 100 g; (–□–), 100 g; (–△–), 472 g. Potato in vivo: (–●–), 140 g; (–■–), 100 g; (–▲–), 472 g. Chickpeas in vitro: (–○–), 70 g; (–□–), 100 g; (–△–), 136 g. Chickpeas in vivo: (–●–), 70 g; (–■–), 100 g; (–▲–), 136 g.
### Table 2. Example of spreadsheet for calculating area between the in vitro glycaemic glucose equivalents (GGE) release and glucose-disposal curves: muesli bar

<table>
<thead>
<tr>
<th>Intake (g)</th>
<th>50</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose equivalents to GGE conversion</strong></td>
<td>0·733</td>
<td>0·733</td>
<td>0·733</td>
</tr>
<tr>
<td><strong>Glucose disposal (GGE/min)</strong></td>
<td>0·256</td>
<td>0·487</td>
<td>0·719</td>
</tr>
<tr>
<td><strong>Linearising factor</strong></td>
<td>1·09</td>
<td>1·32</td>
<td>1·54</td>
</tr>
<tr>
<td><strong>Time (min)</strong></td>
<td><strong>GGE (g)</strong>*</td>
<td><strong>BL†</strong></td>
<td><strong>Diff‡</strong></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>12·8</td>
<td>2·56</td>
<td>10·3</td>
</tr>
<tr>
<td>20</td>
<td>16·3</td>
<td>5·12</td>
<td>11·2</td>
</tr>
<tr>
<td>30</td>
<td>17·6</td>
<td>7·87</td>
<td>9·9</td>
</tr>
<tr>
<td>40</td>
<td>18·4</td>
<td>10·23</td>
<td>8·1</td>
</tr>
<tr>
<td>50</td>
<td>19·4</td>
<td>12·79</td>
<td>6·6</td>
</tr>
<tr>
<td>70</td>
<td>20·3</td>
<td>17·91</td>
<td>2·4</td>
</tr>
<tr>
<td>130</td>
<td>22·0</td>
<td>33·26</td>
<td>−11·2</td>
</tr>
<tr>
<td>190</td>
<td>22·7</td>
<td>48·61</td>
<td>−25·9</td>
</tr>
<tr>
<td>250</td>
<td>23·2</td>
<td>63·96</td>
<td>−40·8</td>
</tr>
</tbody>
</table>

* GGE = in vitro glucose equivalents/g (Fig. 1) released at each time adjusted for glycaemic potency of the contributing sugars (0·733) and multiplied by food intake.  
† BL = glucose disposal baseline = time (min) × glucose disposal factor determined from GGE release at 60 min using the equation in Fig. 4.  
‡ Diff = GGE − BL.  
§ Diff adj = Diff after non-linearity correction based on the ratio of the linear to quadratic response as a function of GGE (Fig. 3).  
‖ iAUC = sum of the areas under the Diff adj × time curve between each measurement point (Fig. 5), using the triangle/trapezoid method.  

**Notes:**
- GGE = in vitro glycaemic glucose equivalents
- BL = glucose disposal baseline
- Diff = GGE − BL
- Diff adj = Diff after non-linearity correction
- iAUC = sum of the areas under the Diff adj × time curve
All data manipulations and statistical analyses were conducted in a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA).

**Results**

**Glucose equivalent determination by in vitro digestion**

The cumulative release of sugars and starch fragments from the foods during digestion, after chewing or homogenising, and measured as glucose equivalents, is shown in Fig. 1 as glucose equivalent (mg) per g of sample. For all foods except the chickpeas most in vitro carbohydrate release had occurred within 20 min of digestion. For most foods GGE release up to 20 min of in vitro digestion also accounted for most of the release at 60 min (white bread 86·1 %; fruit bread, 89·8 %; muesli bar, 86·8 %; potato, 91·9 %; chickpeas, 63·5 %; Table 3), and at 60 min most of the total digestible (240 min) GGEs had been released (white bread, 97·3 %; fruit bread, 87·3 %; muesli bar, 87·5 %; potato, 98·3 %; chickpeas, 72·4 %; Table 2).

**Glycaemic glucose equivalent determination from glucose equivalents**

GGE values were obtained from glucose equivalent (GE) values by adjusting for the glycaemic potency of the fructose present. The need for the fructose adjustment was confirmed by the improved correlation with in vivo data that the adjustment provided:

\[
\text{GGE}_{\text{in vivo}} = 0.557\text{GE}_{20} + 3.016; \quad R^2 = 0.806,
\]

without the fructose adjustment:

\[
\text{GGE}_{\text{in vivo}} = 0.713\text{GGE}_{20} + 1.040; \quad R^2 = 0.913,
\]

with the adjustment.

**Blood glucose responses**

The all-subject mean glucose response curves are shown in Fig. 6(b) (in vivo). Most of the difference in area under the curve for different food intakes could be attributed to changes in the post-peak area. The time to peak did not differ greatly between samples and was unrelated to food intake when all foods were considered (time to peak (min) = 0·249GGE + 31·31; \(R^2 = 0.005\)), and the peak heights did not increase linearly with food intake. For most of the blood glucose response curves the area could be divided into pre- and post-peak triangles, which covered most of the area under the curve, and the decrease in blood glucose

### Table 3. Key measurement points from in vitro digestive analysis, and effects of converting glucose equivalents (GE) to glycaemic glucose equivalents (GGE)

<table>
<thead>
<tr>
<th>Food</th>
<th>White bread</th>
<th>Fruit loaf</th>
<th>Muesli bar</th>
<th>Potato</th>
<th>Chickpeas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>GE release (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>412·6</td>
<td>17·7</td>
<td>343·5</td>
<td>9·3</td>
<td>480·6</td>
</tr>
<tr>
<td>60 min</td>
<td>479·4</td>
<td>37·3</td>
<td>382·5</td>
<td>6·3</td>
<td>553·6</td>
</tr>
<tr>
<td>240 min</td>
<td>492·9</td>
<td>9·4</td>
<td>438·4</td>
<td>3·5</td>
<td>632·7</td>
</tr>
<tr>
<td>GGE/GE ratio*</td>
<td>1·00</td>
<td>0·96</td>
<td>0·73</td>
<td>0·92</td>
<td>1·00</td>
</tr>
<tr>
<td>GGE release† (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>411·4</td>
<td>17·7</td>
<td>295·7</td>
<td>8·0</td>
<td>352·3</td>
</tr>
<tr>
<td>60 min</td>
<td>477·9</td>
<td>37·3</td>
<td>329·4</td>
<td>5·4</td>
<td>405·8</td>
</tr>
<tr>
<td>240 min</td>
<td>491·4</td>
<td>9·4</td>
<td>377·5</td>
<td>3·0</td>
<td>463·8</td>
</tr>
<tr>
<td>Proportion released (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min/60 min</td>
<td>86·1</td>
<td>89·8</td>
<td>86·8</td>
<td>91·9</td>
<td>63·5</td>
</tr>
<tr>
<td>20 min/240 min</td>
<td>83·7</td>
<td>78·4</td>
<td>76·0</td>
<td>90·3</td>
<td>46·0</td>
</tr>
<tr>
<td>60 min/240 min</td>
<td>97·3</td>
<td>87·3</td>
<td>87·5</td>
<td>98·3</td>
<td>72·4</td>
</tr>
</tbody>
</table>

*The GGE20:GE20 ratio shows the effect of allowing for the glycaemic potency of sugars.
†GE = GE adjusted by the glycaemic index of constituent sugars. GGE20 and GGE60 were values used to determine rapidly released GGE and glucose disposal rate, respectively, for a two-point in vitro determination of glycaemic impact.
corrections was almost linear from the peak to baseline, which allowed triangulation to be used to determine a GD rate.

The maximum rate of net glycaemic loading in vivo was between the 15 and 30 min blood glucose measurements for all intakes, and extrapolating the line from the 15 and 30 min readings back to the x-axis showed a delay in response of about 10 min from consuming food (Fig. 2). Therefore, the curves of GGE release from different food quantities based on in vitro analysis (for example, Fig. 5 for muesli bar) were moved 10 min along the x-axis relative to the original in vitro curves (Fig. 1), so that the start of in vitro carbohydrate release and in vivo blood glucose loading coincided. In vitro GGE release at 20 min then coincided with the 30 min upper end of the linear increase in blood glucose, which closely preceded the blood glucose response peak for all foods except chickpeas (Figs. 2 and 6(b)).

**Corrections for dose–response non-linearity**

The blood glucose responses to 25, 50 and 75 g glucose, relative to the response to 12.5 g glucose (assigned a value of 1.0), yielded a quadratic curve, which diverged substantially from a line through zero and 1.0 (Fig. 3). To remove the influence of homeostasis on the glucose reference the blood glucose response curve was multiplied by the ratio of the linear curve (unaffected by homeostasis) to the quadratic curve (resulting from homeostasis).

**Glucose disposal rates**

The rate of GD was calculated from a post-peak triangle in which the hypotenuse was a section of the linear slope of decreasing blood glucose concentrations (Fig. 2). The GD rates, calculated as GGE/min and multiplied by the linear: quadratic adjustment in the reference, when plotted against GGE intakes gave a line (Fig. 4) with the equation:

\[
GD = 0.0135GGE + 0.0232 \text{ (GGE/min)},
\]

which allowed the GGE disposal rate for a given GGE intake to be calculated. Because the slopes from which the GD rates were calculated all finished at 60 min in vitro, when most of the digestion of the foods was complete and not very different from the 20 min value (Table 2), were used in the calculation.

**Modelling the interaction of glycaemic loading and glucose disposal**

The in vitro digestion curves for GGE/g sample (Fig. 1) curves of glucose equivalents adjusted to GGE values by allowing for glycaemic potency of the constituent monomers) were multiplied by the food quantities consumed (Table 1), to give curves of theoretical GGE yield per portion consumed. As an example, the curves for muesli bar GGE intakes are shown in Fig. 5 along with a line of cumulative GD for each portion consumed, the latter based on the GD rates appropriate for the GGE intake in each portion consumed (Fig. 4), multiplied by time. The results in Fig. 5 show how the slope of GD increases with the GGE dose consumed. Subtracting values for cumulative GGE disposal at each time from the values for cumulative digestive release of GGE at the same time gave the net GGE loading at the time (Table 2). When these values for net in vitro GGE loading at each time were plotted against time, curves showing how much cumulative GGE loading was exceeding cumulative GGE disposal were obtained that mimicked in vivo blood glucose responses to different foods and food intakes (Fig. 6).

**Measuring net glycaemic glucose equivalent content using in vitro area between the curves**

Incremental areas under the in vitro net GGE release curves (incremental area under the curve GE20 in vitro; iAUCGE20 in vivo) accurately predicted the in vivo responses (Fig. 7) over the range of food intakes (Table 4) \((y = 0.999x + 0.65; R^2 = 0.88)\). The correlation was much closer if the muesli bar alone was excluded from the analysis, but for the muesli bar alone the correlation was an almost perfect function of GGE intake, although the slope of the relationship showed that the in vitro analysis gave higher GGE values than the in vivo analysis. A Bland–Altman analysis\(^{(13)}\) showed that for the whole dataset the in vitro and in vivo methods corresponded well (Table 4).

**Table 4. Relationship between glycaemic glucose equivalents (GGE) determined in vivo* (y) and GGE determined in vitro (x) either from the area between the in vitro GGE release and glucose disposal lines, or from carbohydrate release at 20 min**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Correlation equation</th>
<th>Bland–Altman equation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>y</td>
<td>R²</td>
</tr>
<tr>
<td>GGE measured as area between lines of release and glucose disposal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All foods</td>
<td>0.999x + 0.65</td>
<td>0.88</td>
</tr>
<tr>
<td>Muesli bar excluded</td>
<td>1.103x − 1.532</td>
<td>0.97</td>
</tr>
<tr>
<td>Muesli bar alone</td>
<td>0.606x + 2.843</td>
<td>0.99</td>
</tr>
<tr>
<td>Two-point analysis based on GE20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GE20</td>
<td>0.557x + 3.006</td>
<td>0.81</td>
</tr>
<tr>
<td>GGE20</td>
<td>0.713x + 1.040</td>
<td>0.91</td>
</tr>
<tr>
<td>GGE20 − glucose disposal</td>
<td>1.01x + 2.603</td>
<td>0.93</td>
</tr>
</tbody>
</table>

GE20, glucose equivalents released after 20 min in vitro digestion.

* Venn et al.\(^{(10)}\).
**Two-point analysis of net glycaemic glucose equivalents**

A two-point analysis of GGE was achieved using the values for GGE release at 20 min, multiplying by food intake, and subtracting the cumulative GD that would have occurred at 20 min.

\[
\text{Net GGE}_{20} = \text{GGE}_{20} - 20(0.0135\text{GGE}_{20} + 0.0232) \text{ (g)}.
\]

Correlations of GGE determined by the two-point analysis with GGE in vitro are given in Table 4.

**Discussion**

The present study has addressed the challenge of finding a way to express relative glycaemic impact of foods on a grams per consumed quantity of food basis, using a validated in vitro method that mimics the human blood glucose response to foods\(^{(1)}\). The ultimate goal is to be able to make relevant comparisons between foods and food intakes in terms of the relative effects of amounts that are customarily consumed. Until this goal is achieved it will be difficult for health professionals, food developers and consumers to make adequate comparisons between alternatives. The principles involved apply generally to the marketing and use of functional foods in promoting health\(^{(14)}\).

Our approach in the present study was to combine a highly replicated set of clinically measured responses to multiple intakes of several foods with in vitro analysis in which a number of factors had been addressed to improve physiological relevance:

1. The samples were chewed before in vitro analysis because of the considerable impact of the physical state of food on digestion\(^{(15,16)}\).
2. The amylolytic phase of digestion was within the gastric–colonic transit time of about 4 h.
3. The contribution of all carbohydrates to the glycaemic response was taken into account by expressing glycaemic impact in terms of grams of GGE rather than in terms of measured glucose.
4. Account was taken of the effect of non-linearity of the glucose dose–blood glucose response relationship on the glucose reference values.
5. Most importantly, in the context of the present paper, baselines were used to represent dose-dependent blood glucose clearance in response to blood glucose loadings from the fifteen food intakes considered.

The model presented here can be regarded as a first iteration and as a test of feasibility in which there were a number of approximations and assumptions, the dataset was small, and the fit between the in vitro (Fig. 6(a)) and in vivo (Fig. 6(b)) response profiles was far from exact. Nonetheless, despite its imperfections, the study has shown that when in vitro digestive analysis uses baselines to represent blood glucose clearance, it is able to mimic in vivo glycaemic responses, and to greatly improve the validity and accuracy with which they are predicted in vitro. The approach has intrinsic validity because it is based on a simulation of the balanced physiological processes known to occur in glucose homeostasis – blood glucose loading and disposal. As key processes in the glycaemic response, both need to be part of any ‘validated in vitro methodology that accurately mimics in vivo behaviour’\(^{(2)}\).

Further research is required to address some of the approximations made in the present study. For instance, the GD baselines used in the present study were straight lines, yet insulin release is in response to blood glucose loading, which changes during digestion. Therefore, non-linear GD baselines that track glycaemic loading may give more accurate measures of net effects on blood glucose\(^{(17)}\). Also, using the same in vitro digestion curve to create curves for different intakes of a food (Fig. 5) does not allow for possibly more protracted digestion as food intakes increase. The uniformity of the blood glucose response curves (Fig. 6(b)) suggests that this did not greatly influence the results, except perhaps in the case of muesli bars.

The need to build factors into the glycaemic analysis to allow for effects on glycaemic response that are additional to glycaemic carbohydrate intake, such as effects on gastric emptying, should also be considered. A number of studies have suggested that in most foods at normal intakes macronutrient composition has little effect on glycaemic index determination\(^{(18,19)}\), and in the present study the relationship between GGE intake and GD for all foods was approximately linear after adjusting for curvature in the glucose reference (Fig. 4). However, others have shown that fat\(^{(20)}\), or acid\(^{(21)}\), added to a starchy meal causes a large depression of glycaemic response. In the present study the in vitro—in vivo relationship for the muesli bar differed from that of the other foods (Table 4). While the proportional responses to changing intakes of muesli bar were very close for the in vitro and in vivo determinations, the in vitro values were greater than the in vivo. This further suggests that for some food formats additional factors to be included in the analysis of the in vitro results may improve accuracy.

The present paper has shown the importance of taking into account homeostasis and, that by including it in in vitro glycaemic analysis, human blood glucose responses can be mimicked. For routine application, however, a much abbreviated two-point in vitro analysis may be used, in which cumulative GD at 20 min is subtracted from rapidly released (20 min) GGE, and it provides an almost equally accurate prediction of in vivo responses (JA Monro & S Mishra, unpublished results). As the maximum glycaemic loading occurred about 20 min after the initiation of the postprandial blood glucose surge, at about 30 min from food consumption (Figs. 2 and 6(b)), a 20 min in vitro sampling time appears to be appropriate to measure rapidly available carbohydrate as an indicator of the glycaemic loading responsible for the glycaemic response\(^{(3,22)}\).

For the experimental approach presented here to be generally applicable as a dose-sensitive in vitro method for predicting the GGE contribution of foods, the relative values that it uses must be generally true. In fact, they are becoming universally established. The relative glycaemic potency of common food mono- and disaccharides required to convert glucose equivalents to GGE values has been measured in numerous studies\(^{(11)}\). Similarly, the glucose dose–blood glucose response relationship has been published several times\(^{(18,17,18,23–25)}\) and the results, when normalised, show it to be a highly reproducible expression of human physiology\(^{(26)}\).
And assuming that most of the non-linearity in the relationship between glucose dose and glycaemic response is due to increasing GD with increasing glycaemic loading, rates of GD are also likely to be universal when expressed relatively, as in the present study. This view is supported by results from a separate study of the *in vitro* digestion of eighty-three foods, in which relative GD rates from the present study gave values for net GGE *in vitro* that predicted GGE *in vivo* as accurately as in the present study (26).

Because the GD rates, being a function of GGE intake, were based on responses relative to glucose they should be largely immune to effects of individual fluctuations within groups, which introduce enormous variability into clinical determinations of glycaemic response (27–28). Nonetheless, as the range of foods used in the present study was small, and experimental groups are likely to differ in insulin sensitivity, further research is required to firmly establish that relative GD rates are universally applicable and may be used to provide the same GGE values for foods in different population groups. However, if groups that differ in glucose tolerance, such as different age groups, require different GD baselines, such ‘customised’ baselines could be useful in matching food choices to physiology.

GGE has been defined as a ‘virtual food component’ because, rather than being a tangible food component *per se*, it expresses a food effect, but as grams per consumed quantity of food (14). Being in the same weight format as a nutrient, it can easily be used in conjunction with other nutrient data to represent a net glycaemic effect of a whole food, to enable comparisons between foods for glycaemic control. It is, therefore, important that a way is found to allow for the homeostatic modulation of glycaemic responses in response to varying intakes and compositions of foods and meals. The present study has suggested that use of a dose-dependent glucose-disposal factor may overcome the problem, by enabling GGE to be based on a net effect that mimics the human blood glucose response, and is able to be expressed as grams per given quantity of food.

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References


