

## Baselines representing blood glucose clearance improve *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 *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 *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 *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 *in vitro* GGE release, accuracy in predicting the *in vivo* glycaemic effect from *in vitro* GGE values was greatly improved.  $GGE_{in vivo} = 0.99GGE_{in vitro} + 0.75$  ( $R^2$  0.88). Furthermore, the difference between the curves of cumulative GGE release and disposal closely mimicked *in vivo* incremental BGR curves. We conclude that valid measurement of the glycaemic impact of foods may be obtained *in vitro*, and expressed as grams of glucose equivalents per food quantity, by taking account not only of GGE release from food during *in vitro* digestion, but also of blood glucose clearance in response to the food quantity.

### Carbohydrate: Glycaemic impact: Glucose disposal: *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’<sup>(1)</sup>, and which should be measured using ‘... validated *in vitro* methodology that accurately mimics *in vivo* behaviour’<sup>(2)</sup>.

Current *in vitro* digestion methods for predicting relative glycaemic responses to foods measure carbohydrate that is rapidly available (for example, Englyst *et al.*<sup>(3)</sup>, Brighenti *et al.*<sup>(4)</sup>), or the area under the glucose release curve relative to a reference<sup>(5)</sup>. However, they cannot provide accurate or robust predictions of relative responses to whole foods varying in quantity, or accurately mimic the *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<sup>(6)</sup>, 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 *in vitro* methods that predict the relative glycaemic effect of realistic intakes of food<sup>(7)</sup>, 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<sup>(8)</sup>. *In vitro* digestion of the same foods has given us curves of cumulative carbohydrate release. With detailed *in vivo* and *in vitro* datasets for the same foods we have an opportunity to measure improvements in the predictive validity of *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 *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 *in vivo*. The aim is to develop *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 (Bürgen), 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  $\pm 0.2$  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 ( $\pm 0.2$ ), 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 at 37°C to accomplish gastric digestion. The small-intestinal phase was initiated by neutralising the gastric HCl with 2 ml of 1 M-NaHCO<sub>3</sub> and 5 ml of 0.1 M-sodium maleate buffer (pH 6)–0.2% sodium azide–1 mM-CaCl<sub>2</sub>. 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) in 0.1 M-maleate buffer (pH 6), and the pots were immediately made to the 55 ml mark with distilled water. Duplicate 1.0 ml samples were each removed to 4 ml ethanol, before ( $T = 0$ ), and at 10, 20, 30, 40, 60 and 120 min after adding the amyloglucosidase–pancreatin. After the 120 min sampling the digests were homogenised using an Omni GLH-220 homogeniser (20  $\times$  195 mm saw tooth probe, setting 3, 15 s; Omni International, Warrenton, VA, USA) to convert them into slurries. A further 0.1 ml of amyloglucosidase was added and the incubation continued for 2 h with further 1 ml samples removed to 4 ml ethanol at 180 and at 240 min from the start of digestion to determine total available carbohydrate. All digesta samples were mixed thoroughly upon adding to the ethanol, and after at least 30 min the tubes were centrifuged for 10 min at 1000 *g* at 20°C (Centrifuge Omnifuge 2.0 RS; Heraeus Sepatech, Osterade, Germany) to clarify the 80% ethanol supernatant fraction before analysis of sugars.

### 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<sup>(9)</sup>, 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 0.2 M-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<sup>(10)</sup>.

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%<sup>(11)</sup>):

$$\text{GGE (g)} = (\text{GE} - \text{fructose}) + 0.2 \text{ fructose.}$$

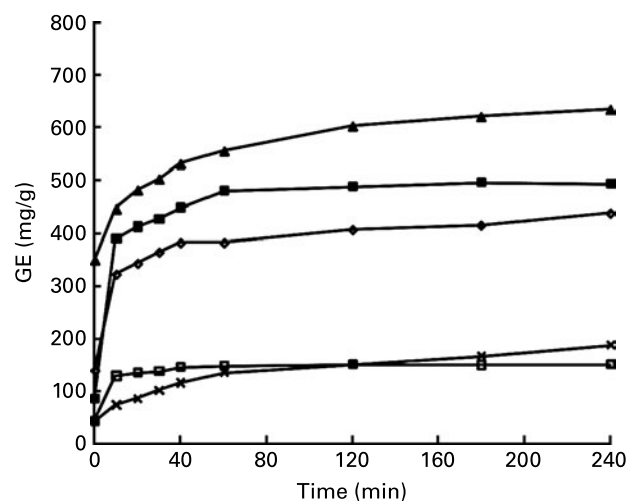


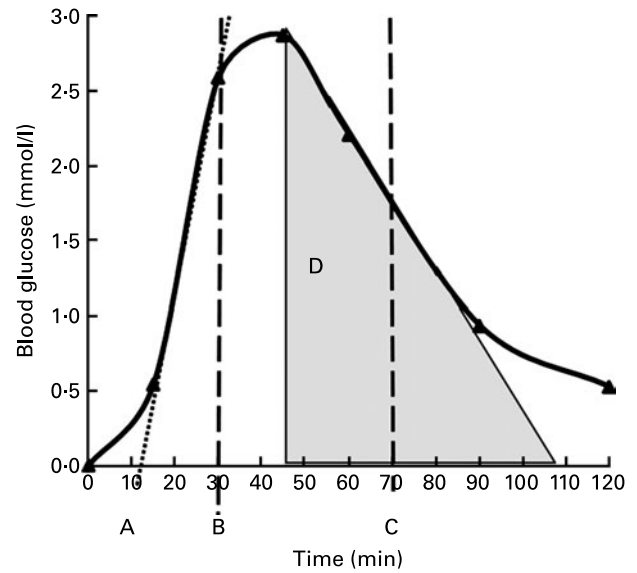
Fig. 1. Release of carbohydrate during *in vitro* digestion measured as glucose equivalents (GE) and expressed per g of unchewed food. Each point is the mean of two determinations. Precision based on mean SD for all determinations was  $\pm 8.8$  GE. Mean CV was 3.2%. ( $-\diamond-$ ), Fruit bread; ( $-\square-$ ), potato; ( $-\blacksquare-$ ), white bread; ( $-\times-$ ), chickpeas; ( $-\triangle-$ ), 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<sup>(8)</sup>. 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<sup>(12)</sup>. 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.*<sup>(8)</sup> 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



**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

(Mean values and standard deviations)

Quantity	Food intake (g)	GGE <i>in vivo</i> * (g)	
		Mean	SD
White bread			
One serving	37	12	5.9
100 g	100	28	11.7
50 g CHOAVL	118	41	9.5
Fruit loaf			
One serving	41	9	6.8
100 g	100	24.5	6.9
50 g CHOAVL	132	37.8	16.3
Muesli bar			
One bar	50	13.9	5.9
Two bars	100	23	13.8
Three bars	150	30.3	13.2
Mashed potato			
100 g	100	10.8	6.1
One serving	140	15.3	6.7
50 g CHOAVL	472	42.6	15.1
Chickpeas			
One serving	70	3.9	4.3
100 g	100	4.9	4.5
25 g CHOAVL	136	8	6.7

CHOAVL, available carbohydrate.

\* Venn *et al.*<sup>(8)</sup>.

glucose was measured. As the GGE quantity represented by the area under the whole curve had already been determined<sup>(8)</sup>, the GGE disposal rate could be calculated as:

$$\text{GD rate (GGE/min)} = ((A_D/A_{\text{total}}) \times \text{GGE}_{\text{total}})/T_h,$$

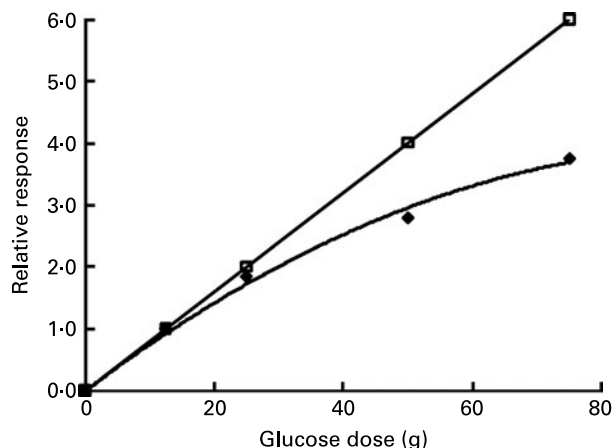
where  $A_D$  is the area under the hypotenuse,  $A_{\text{total}}$  is the total area under the blood glucose response curve,  $\text{GGE}_{\text{total}}$  is the GGE value for the total area and  $T_h$  is the time (x-axis) under the hypotenuse (Fig. 2).

This procedure gave a set of GGE disposal rates which were estimates because they represent change in the net balance between blood glucose loading and clearance, and are measured over the portion of the blood glucose response curve covering most, but not all, of the decline in blood glucose concentrations.

Because the clinically determined GGE values had used glucose references that were also subject to homeostasis the values were adjusted for the quadratic decline in the glucose reference response (Fig. 3). The adjusted GD rates were then plotted against their corresponding GGE intakes to provide a graph and equation for GD rate as a function of GGE intake (Fig. 4). The equation was used to identify GD rates with which to calculate cumulative GD baselines as GD rate × time for all fifteen food intakes.

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

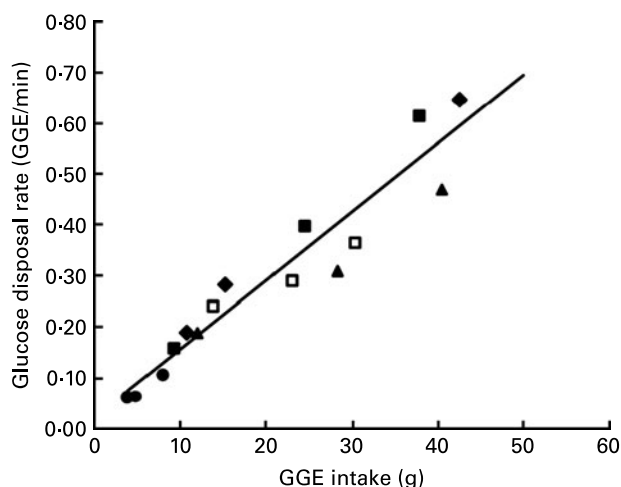
GGE released per g of food after 60 min *in vitro* digestion was multiplied by the food intake, and entered into the



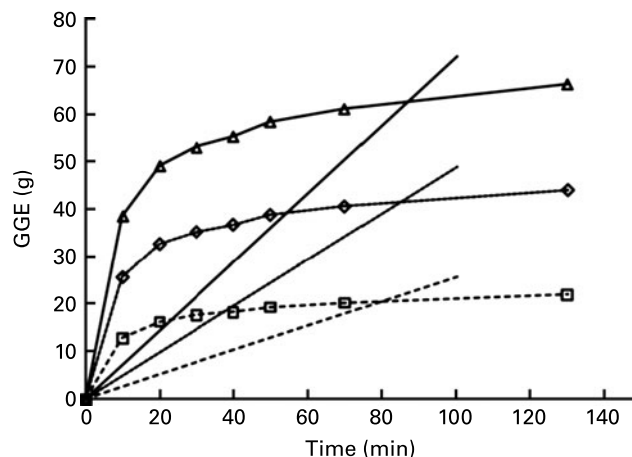
**Fig. 3.** Quadratic increase in actual blood glucose response with increasing glucose doses (—◆—), compared with a linear extrapolation from the origin through the response to 12.5 g glucose (assigned a value of 1.0) (---□---). The divergence between the curves is used as a measure of the effect of homeostasis on the blood glucose response.

equation of disposal rate *v.* GGE intake (GD rate (in GGE/min) =  $0.0135 \times \text{GGE intake (in g)} + 0.0232$ ; 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



**Fig. 4.** Glucose disposal rates measured by triangulation of the negative post-peak blood glucose response slope (Fig. 2) as a function of glycaemic glucose equivalents (GGE) ( $y = 0.0135x + 0.0232$ ;  $R^2$  0.93), after correcting for non-linearity using the ratios of the linear to quadratic responses to glucose (Fig. 3). (□), Muesli bar; (◆), potato; (▲), white bread; (●), chickpeas; (■), fruit loaf; (—), all foods.



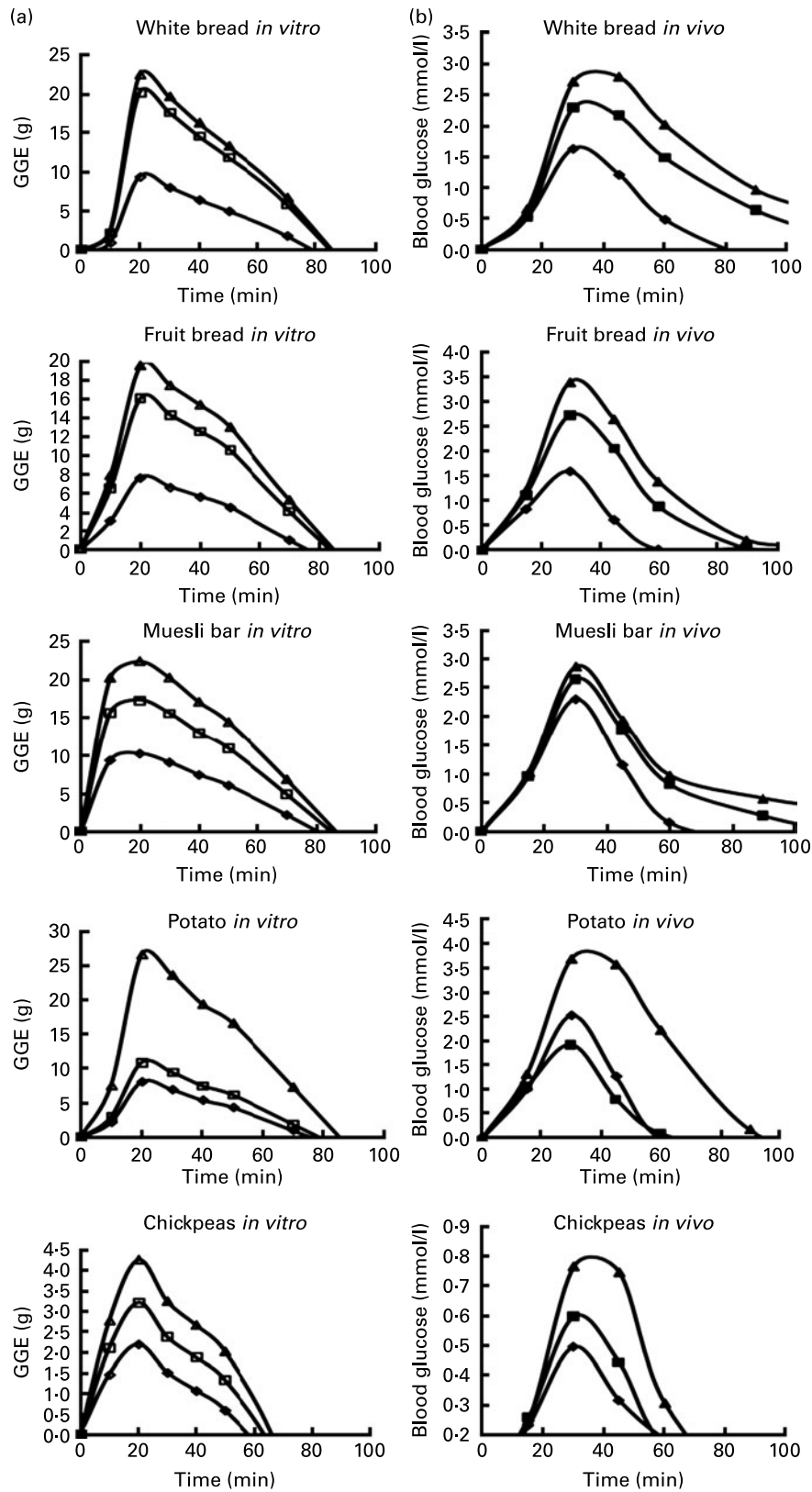
**Fig. 5.** Release of glycaemic glucose equivalents (GGE) calculated from glucose equivalents for three intakes of muesli bar (lines with markers: —△—, 150 g; ---◇---, 100 g; ····□····, 50 g), and straight lines ( $y = \text{glucose disposal (GD) rate} \times \text{time}$ ): —, GD 150 g; ---, GD 100 g; ····, GD 50 g) for cumulative GD. GD rate was calculated by substituting *in vitro* GGE release at 60 min for the given food intakes into the equation relating GGE intake to GD rate (GD rate =  $0.0135\text{GGE} + 0.0232$ ; Fig. 4).

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<sup>(12)</sup> (Table 3). One slice of white bread, which contained 12.0 g GGE in an area of 343.6 GGE  $\times$  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  $\text{GGE} = A \times 12/343.6$  (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; (—□—), 140 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

Intake (g)	50					100					150				
Glucose equivalents to GGE conversion	0.733					0.733					0.733				
Glucose disposal (GGE/min)	0.256					0.487					0.719				
Linearising factor	1.09					1.32					1.54				
Time (min)	GGE (g)*	BL†	Diff‡	Diff adj§	iAUC	GGE (g)*	BL†	Diff‡	Diff adj§	iAUC	GGE (g)*	BL†	Diff‡	Diff adj§	iAUC
0	0	0	0	0		0	0	0	0		0	0	0	0	
10	12.8	2.56	10.3	9.4	47.0	25.6	4.87	20.8	15.7	78.6	38.4	7.19	31.3	20.3	101.5
20	16.3	5.12	11.2	10.3	98.5	32.6	9.74	22.9	17.3	165.4	49.0	14.4	34.6	22.5	213.8
30	17.6	7.67	9.9	9.1	97.0	35.2	14.62	20.6	15.6	164.8	52.8	21.6	31.3	20.3	213.9
40	18.4	10.23	8.1	7.5	82.9	36.7	19.49	17.3	13.1	143.4	55.1	28.8	26.4	17.1	187.2
50	19.4	12.79	6.6	6.1	67.7	38.8	24.36	14.5	10.9	120.1	58.2	35.9	22.3	14.5	158.0
70	20.3	17.91	2.4	2.2	82.5	40.6	34.11	6.5	4.9	158.5	60.9	50.3	10.6	6.9	213.3
130	22.0	33.26	-11.2	-10.3	5.2	44.0	63.34	-19.3	-14.6	7.5	66.1	93.4	-27.4	-17.8	8.4
190	22.7	48.61	-25.9	-23.8		45.4	92.58	-47.2	-35.8		68.1	136.6	-68.5	-44.5	
250	23.2	63.96	-40.8	-37.4		46.4	121.81	-75.4	-57.1		69.6	179.7	-110.1	-71.5	
				Total	481				Total	838				Total	1099

\* 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).

|| ΣiAUC = sum of the areas under the Diff adj × time curve between each measurement point (Fig. 5), using the triangle/trapezoid method<sup>(12)</sup>.

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

(Mean values and standard deviations)

Food...	White bread		Fruit loaf		Muesli bar		Potato		Chickpeas	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
GE release (g)										
20 min	412.6	17.7	343.5	9.3	480.6	3.4	135.5	2.8	85.8	6.2
60 min	479.4	37.3	382.5	6.3	553.6	6.1	147.5	3.4	135.1	4.7
240 min	492.9	9.4	438.4	3.5	632.7	8.9	150.0	1.5	186.5	2.2
GGE:GE ratio*	1.00		0.86		0.73		0.92		1.00	
GGE release† (g)										
20 min	411.4	17.7	295.7	8.0	352.3	2.5	124.4	2.6	85.8	6.2
60 min	477.9	37.3	329.4	5.4	405.8	4.4	135.4	3.1	135.1	4.7
240 min	491.4	9.4	377.5	3.0	463.8	6.5	137.7	1.4	186.5	2.2
Proportion released (%)										
20 min/60 min	86.1		89.8		86.8		91.9		63.5	
20 min/240 min	83.7		78.4		76.0		90.3		46.0	
60 min/240 min	97.3		87.3		87.5		98.3		72.4	

\* The GGE<sub>20</sub>:GE<sub>20</sub> ratio shows the effect of allowing for the glycaemic potency of sugars.

† GGE = GE adjusted by the glycaemic index of constituent sugars. GGE<sub>20</sub> and GGE<sub>60</sub> were values used to determine rapidly released GGE and glucose disposal rate, respectively, for a two-point *in vitro* determination of glycaemic impact.

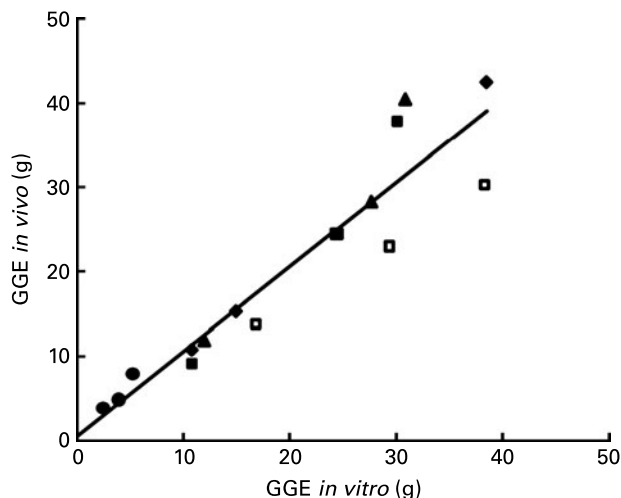
### Statistical analysis

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



**Fig. 7.** Correlation between glycaemic glucose equivalent (GGE) content of foods measured as area under the blood glucose response curve (*in vivo*) v. GGE as the area between the lines of GGE release during *in vitro* digestion and glucose disposal (Fig. 5). (□), Muesli bar; (◆), potato; (▲), white bread; (●), chickpeas; (■), fruit loaf; (—), all foods. For white bread, fruit bread, potato, chickpeas,  $y = 1.156x - 0.54$  ( $R^2$  0.96); for muesli bar,  $y = 0.761x + 0.99$  ( $R^2$  0.99); for all foods,  $y = 0.999x + 0.65$  ( $R^2$  0.88).

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:

$GGE_{in vivo} = 0.557GGE_{20 in vitro} + 3.016$ ;  $R^2$  0.806, without the fructose adjustment;

$GGE_{in vivo} = 0.713GGE_{20 in vitro} + 1.040$ ;  $R^2$  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

concentrations 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:

$$\text{GD rate} = 0.0135\text{GGE} + 0.0232 (\text{GGE}/\text{min}),$$

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 between 40 and 90 min, the GGE quantities released 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<sub>*in vitro*</sub>; iAUC<sub>*in vitro*</sub>) 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 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<sup>(13)</sup> 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

Samples	Correlation equation		Bland–Altman equation	
	<i>y</i>	<i>R</i> <sup>2</sup>	<i>y</i>	<i>R</i> <sup>2</sup>
GGE measured as area between lines of release and glucose disposal				
All foods	$0.999x + 0.65$	0.88	$-0.065x + 0.681$	0.032
Muesli bar excluded	$1.103x - 1.532$	0.97	–	–
Muesli bar alone	$0.606x + 2.843$	0.99	–	–
Two-point analysis based on GE <sub>20</sub>				
GE <sub>20</sub>	$0.557x + 3.006$	0.81	$0.491x - 1.89$	0.56
GGE <sub>20</sub>	$0.713x + 1.040$	0.91	$0.297x - 0.32$	0.51
GGE <sub>20</sub> – glucose disposal	$1.01x + 2.603$	0.93	$-0.048x - 1.86$	0.03

GE<sub>20</sub>, glucose equivalents released after 20 min *in vitro* digestion.

\* Venn *et al.* (8).



### 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<sup>(1)</sup>. 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<sup>(14)</sup>.

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<sup>(15,16)</sup>.
- (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’<sup>(2)</sup>.

Further research is required to address some of the approximations made in the present study. For instance, the GD baselines used in the present model 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<sup>(17)</sup>. 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<sup>(18,19)</sup>, 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<sup>(20)</sup>, or acid<sup>(21)</sup>, 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<sup>(3,22)</sup>.

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<sup>(11)</sup>. Similarly, the glucose dose–blood glucose response relationship has been published several times<sup>(8,17,18,23–25)</sup> and the results, when normalised, show it to be a highly reproducible expression of human physiology<sup>(6)</sup>.

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<sup>(26)</sup>.

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<sup>(27,28)</sup>. 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<sup>(14)</sup>. 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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