Increased liver fat and glycogen stores after consumption of high versus low glycaemic index food: A randomized crossover study

Stephen Bawden PhD¹,² | Mary Stephenson PhD³ | Yirga Falcone¹ | Melanie Lingaya¹ | Elisabetta Ciampi PhD⁴ | Karl Hunter PhD⁴ | Frances Bligh PhD⁴ | Jörg Schirra PhD⁵ | Moira Taylor⁶ | Peter Morris PhD² | Ian Macdonald PhD⁶ | Penny Gowland PhD² | Luca Marciani PhD¹ | Guruprasad P. Aithal PhD¹

¹NIHR Nottingham Digestive Diseases Biomedical Research Unit, Nottingham University Hospitals NHS Trust and University of Nottingham, Nottingham, UK
²Physics and Astronomy, Sir Peter Mansfield Imaging Centre, University of Nottingham, Nottingham, UK
³Clinical Imaging Research Centre, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore
⁴Unilever Discover, Unilever, Colworth, UK
⁵Department of Internal Medicine II, Clinical Research Unit, Ludwig-Maximilians University, Munich, Germany
⁶School of Life Sciences, University of Nottingham, Nottingham, UK

Corresponding Author: Dr Stephen Bawden, Sir Peter Mansfield Imaging Centre, University Park, University of Nottingham, NG7 2RD, UK (stephen.bawden@nottingham.ac.uk).

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Aim: To investigate the acute and longer-term effects of low (LGI) versus high glycaemic index (HGI) diets on hepatic fat and glycogen accumulation and related blood measures in healthy volunteers.

Methods: Eight healthy men (age 20.1 ± 0.4 years, body mass index 23.0 ± 0.9 kg/m²) attended a test day before and after a 7-day macronutrient- and energy-matched HGI or LGI diet, followed by a minimum 4-week wash-out period, and then returned to repeat the intervention with the alternative diet. During test days, participants consumed either an HGI or an LGI test meal corresponding to their diet week, and liver fat [¹H magnetic resonance spectroscopy (MRS)], glycogen [¹³C MRS] and gastric content volume (MRI) were measured. Blood samples were obtained regularly throughout the test day to assess plasma glucose and insulin levels.

Results: Plasma glucose and insulin peak values and area under the curve were significantly greater after the HGI test meal compared with the LGI test meal, as expected. Hepatic glycogen concentrations increased more after the HGI test meal (P < .05) and peak levels were significantly greater after 7 days of HGI dietary intervention compared with those at the beginning of the intervention (P < .05). Liver fat fractions increased significantly after the HGI dietary intervention compared with the LGI dietary intervention (two-way repeated-measures analysis of variance P ≤ .05).

Conclusions: Compared with an LGI diet, a 1-week HGI diet increased hepatic fat and glycogen stores. This may have important clinical relevance for dietary interventions in the prevention and management of non-alcoholic fatty liver disease.

KEYWORDS
dietary intervention, fatty liver, glycaemic control, liver, randomised trial

INTRODUCTION

Shifts in eating patterns and dietary compositions are believed to be a major contributing factor to the recent rise in obesity and obesity-related problems.¹² Type 2 diabetes, for example, has been thought to be a disease of ectopic fat, and the development of non-alcoholic fatty liver disease (NAFLD) as well as non-alcoholic steatohepatitis has been considered to be a key step in its pathogenesis.³ Changes in the amount of food consumed and total energy intake influence long-term energy stores such as adipose tissue and intrahepatic triglycerides, but the specific influence of individual macronutrients on ectopic fat in general and accumulation of liver fat in particular has not been established.

Recently, the glycaemic index has been considered a potentially important factor influencing these conditions, and low glycaemic
index (LGI) dietary interventions have been shown to be effective in lowering total fat mass and increasing lipid utilization in patient studies.\(^4,5\) LGI foods have also been linked to more rapid recovery from previous training sessions\(^6\) and to improved satiety with less hunger between meals.\(^7\) Whilst these findings are promising and have potential clinical relevance, work is needed to investigate a wide range of factors effecting metabolic disorders. This includes both forms of energy storage in the liver, in the longer term as fats, and in the shorter term as glycogen. Gastric emptying also affects the delivery of foods to the small intestines for absorption of nutrients into the blood stream, and previous studies have shown that meal timing, volume and fibre content can affect the postprandial response.\(^8,9\)

Magnetic resonance techniques offer a unique method of investigating some of these parameters. \(^1\)H magnetic resonance spectroscopy (MRS) measurements of liver fat have been validated and used in many previous studies\(^10-12\) and \(^13\)C MRS measurements of glycogen have also been well validated\(^13,14\) and provide the only non-invasive measure of hepatic glycogen stores in vivo. Fast imaging techniques can also be used to monitor gastric emptying.\(^15,16\) These magnetic resonance measures can be obtained alongside blood samples to provide a broader picture of metabolic response.

Previous studies have focused on the acute postprandial changes alone, therefore, less is known about the longer-term effect of well controlled diets with varying glycaemic index. The aim of the present study was to investigate both the immediate and cumulative effects of varying glycaemic index on liver metabolic control in healthy volunteers by monitoring hepatic glycogen and lipid levels in vivo with MRS.\(^14,17\) Secondary outcomes were related changes in gastric content volume (GCV), blood glucose and insulin levels and subjective appetite scores.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

Eight men underwent two 7-day diet periods separated by a minimum 4-week wash-out in a randomized crossover study. The day before (visit 1) and the day after each diet period (visit 2), participants attended the Sir Peter Mansfield Imaging Centre in Nottingham for a test day. Ethical permission was obtained from the University of Nottingham Medical School Research Ethics Committee and all participants provided informed written consent before participation. This study was registered at clinicaltrials.gov: NCT02482558.

### 2.2 | Eligibility

The men were screened for eligibility (age 18-35 years, body mass index (BMI) 20–25 kg/m² and no contraindications for MRI). Participants were excluded if: they were on any special diets, weight loss programmes or strict physical training routine (defined as >5 hours of intense training per week); they were heavy drinkers (>3 units a day) or smokers; or they had any metabolic disorders or liver disease. Participants were block randomized to determine the initial intervention (high glycaemic index or LGI).

### 2.3 | Demographics

The mean age of participants was 20.1 ± 0.4 years and their mean BMI was 23.0 ± 0.9 kg/m². The mean weight of participants at the start of visit 1 was 73 ± 3 kg and at the start of visit 2 was 73 ± 3 kg.

### 2.4 | Test day

Before the test days the participants were asked to refrain from drinking alcohol and to consume the same evening meal by 21:00 hours the night before visit 1 of both diets. At the end of each dietary period the final meal was consumed before 21:00 hours on the evening before visit 2. On the morning of each test day, participants arrived fasted at the MRI centre between 07:30 and 08:00 hours, and were weighed. After fasted measurements, participants were given either an HGI or LGI test meal for breakfast (Table S1, Appendix S1) depending on their diet week, which was to be consumed within 10 minutes, followed by regular measurements for 360 minutes.

At the start of the day, participants were cannulated in the forearm and samples were taken at regular intervals throughout the day. Samples were centrifuged, frozen and stored at −80°C for analysis of plasma glucose and insulin levels (detailed methods in Appendix S1).

All MRI measurements were acquired using a Philips Achieva 3T system (Philips, Best, The Netherlands).

\(^13\)C MRS measurements of glycogen were detected with an adiabatic half passage pulse-acquire sequence (MRS bandwidth 7 kHz, TR 959 ms). Spectra were acquired using a single-loop carbon coil with proton decoupling (Pulseteq, Surrey, UK) as described previously\(^15,18,19\) (Appendix S1). Measurements were taken at the start of the day (fasted) and hourly after the test meal.

\(^1\)H MRS measurements of liver fat were detected with a respiratory triggered point resolved spectroscopy (PRESS) sequence (bandwidth 2 kHz; TR 5 seconds) with varying TE (40, 50, 60 and 80 ms). Spectra were acquired using a 32-channel Philips XL SENSE torso coil from a 30 × 30 × 30 mm³ voxel in the lower right hepatic lobe, with and without water suppression. T2 was determined and used to correct fat-to-water ratios to determine liver fat fractions (FF\%)\(^10,20\) at start of day (fasted) and 360 minutes after test meal (Appendix S1).

The magnetic resonance images were also acquired throughout the test day and regions of interest were drawn around the content of the stomach using Analyze9 (Mayo Foundation, Rochester, MN, USA) and summed across slices to determine GCV, as described previously.\(^15,16\) GCV was therefore a combined measure of both ingested food and stomach secretion.

Visual analogue scales were completed at the same time as blood sampling to assess subjective appetite ratings using five mixed appetite questions.\(^21-23\) On day 1 (start of diet), day 4 (middle of diet) and day 7 (end of diet) participants also filled out subjective appetite ratings. The visual analogue scale methods are reported in Appendix S1.

### 2.5 | Diet week

After the test day, participants undertook a 7-day HGI or LGI diet before visit 2, and returned again after a >4-week wash-out for the
alternate diet. During the diet week, participants were provided with all the food required, as adapted from Morgan et al.24 (Table S2, Appendix S1). All food was purchased from a single supplier and given directly to participants. They were also given a booklet describing the quantities of each meal to be consumed, along with scales and a measuring jug to measure out the required ingredients for each meal. Participants recorded whether they consumed the full meal, and if not how much remained.

Before the study, participants completed the International Physical Activity Questionnaire (IPAQ) and their basal metabolic rate was calculated using the Henry-modified Schofield formula.25 This was used to scale the amount of food consumed during diet weeks to match expected energy expenditure and provide overall energy balance (no weight loss or weight gain). The energy intake and macronutrient content was matched for the HGI and LGI diets (71% carbohydrate, 14% protein, 14% fat per day). Whilst this level of carbohydrate is greater and level of fat is lower than national standards, these proportions were based on previous well-defined HGI versus LGI interventions in healthy volunteers that showed clear glycaemic differences,24 and the diet was deemed suitable for this preliminary proof-of-concept study exploring carbohydrate glycaemic index. As would be expected and is usually the case, the fibre content was greater during LGI compared with HGI (fibre ~22 g/day for HGI and ~42 g/day for LGI)24,25 and therefore the term LGI denotes a high-fibre LGI diet and HGI denotes a lower-fibre HGI index diet.

2.6 | Sample size

The exploratory nature of the present study, with few related publications, made sample size calculations difficult; however, estimates of effect size were made based on previous studies and used to determine an appropriate sample size using G*power 3.1.5.27 An a priori two-way repeated-measures F-test (analysis of variance [ANOVA]) will find a significance interaction with a power of 0.8 given an effect variance (HGI – LGI) of 2.1% and a within-group variance of 2.9% in a sample size of six subjects (effect size = 0.84). These variances were based on liver fat changes observed in a previous study,28 assuming changes only observed on HGI diet; however, there were a number of important differences in the present study, such as increased carbohydrate proportion and iso-energetic intervention, so the sample size was increased to eight participants. This sample size would also calculate a significant change of 15% hepatic glycogen using a matched-pair Student’s t-test given the variability observed in previous studies.13

2.7 | Blinding

On completion of all data acquisition, results were blinded with the help of an uninvolved colleague and analysed by the first author. Although the first author was present during scan sessions, spectroscopy data were not viewed in real time and only assessed after blinding. Blood samples were analysed by uninvolved colleagues and so were not blinded. After initial analysis a blind review meeting was held before data were unblinded. Deviations from protocol were discussed and data assessed for statistical relevance on a per-protocol basis.

2.8 | Data analysis

Methods of analysis are described in more detail in Appendix S1. Values were calculated for individual time points and hepatic glyco- gen values were also calculated as percentage baseline. The total area under curve (AUC) across the test visit was also calculated for glucose, insulin and glycogen. In addition, the glycaemic index was calculated using the area above baseline (incremental AUC) from t = 0 to t = 120 minutes from plasma glucose results. Homeostasis model assessment of insulin resistance (HOMA-IR) was also calculated from fasted glucose and insulin values using the equation: (glucose × insulin)/22.5.

2.9 | Statistical analysis

Results are reported as mean with standard error, and mean difference with standard deviation. Parametric testing was performed assuming normal distributions of lipid and glycogen in tissue, as well as postprandial hepatic glycogen and glucose response, which is reasonable given the restrictive selection criteria (healthy, male, sedentary, non-smokers).

To assess differences in the acute response between test meals, postprandial peaks, AUCs and incremental AUCs after test meals (HGI vs LGI) on visit 1 (prior to diet) were compared using a matched-pair Student’s t-test. Measurements taken across the time course on this visit were also assessed using a two-way repeated measures ANOVA and used to evaluate any significant main effect of diet (LGI vs HGI) or time of day (across the test day) and/or any significant interaction between diet and time of day.

To assess longer-term effects of the dietary intervention, differences in fasted values at each visit were compared using two-way repeated-measures ANOVA. Changes across the time course between visit 2 and visit 1 in LGI and HGI diet arms independently were also assessed using two-way repeated-measures ANOVA to evaluate any significant main effect of visit (visit 1 vs visit 2) or time of day (across the test day) and/or any significant interaction between visit and time of day.

All significant main effects were followed up by pairwise comparisons using a matched pair two-tailed Student’s t-test and significant interactions were followed up by pairwise comparisons of change from baseline values.

A Bonferroni adjustment was applied for multiple comparisons. In all cases P values < .05 were taken to indicate statistical significance. The statistical package used for analysis was SPSS version 21 for Windows (SPSS, Inc., Chicago, Illinois, USA).

3 | RESULTS

3.1 | Participant recruitment and flow

The first test day was May 13, 2013 and the final test day was October 8, 2013. One participant dropped out early, and his data
were therefore removed from analysis and one participant failed to complete the LGI diet week, therefore, his visit 2 data were excluded. For primary outcomes, this gave a sample size of n = 8 for visit 1 HGI versus LGI comparisons and n = 7 for visit 1 versus visit 2 comparisons. Other difficulties arose for secondary outcomes, such as failure to cannulate, and therefore the sample size for each analysis varied as follows: glucose, n = 5; insulin, n = 6.

3.2 | Compliance

Participants reported good compliance across the diet week (with the one exception mentioned above). According to the returned volunteer’s booklets, 98% ± 2% of meals were consumed during the HGI diet and 97% ± 3% during the LGI diet (reported energy intake was 100% ± 0% as provided for HGI and 99% ± 1% for LGI).

3.3 | Fasted values on visit 1 (prior to diet)

The HOMA-IR values were similar before both diets (HOMA-IRHGI = 1.91 ± 0.12, HOMA-IRLGI = 1.78 ± 0.05). Fasted FF% and fasted hepatic glycogen levels were also similar before both diets (FFHGI% = 1.5 ± 0.6% and FFHGI% = 1.5 ± 0.5%; P = .98; hepatic glycogenHGI = 306 ± 37 mmol/L and hepatic glycogenLGI = 290 ± 32 mmol/L; P = .67), indicating a successful wash-out period.

3.4 | Glycaemic and insulinaemic response to diets

Acute changes in plasma glucose and insulin in response to HGI and LGI test meals on visit 1 (prior to diet) are shown in Figure 1A and B. Plasma glucose rose significantly more after the HGI compared with the LGI test meal (P < .01). The postprandial insulin AUC was significantly greater after the HGI compared with the LGI test meal (INSULINHGI – INSULINLGI = 19 ± 3 IU/1 h; P < .05). There was no significant change in HOMA-IR on visit 2 versus visit 1 for either diet (ΔHOMA-IRHGI = 0.42 ± 0.93; ΔHOMA-IRLGI = 0.13 ± 0.43) and there were no significant differences in the glucose and insulin response to the test meal between visit 1 and visit 2.

3.5 | Study outcomes

3.5.1 | Effect of dietary intervention on liver fat fraction

There was a significant interaction between diet and visit for fasted FF% (P ≤ .05), with mean values increasing after the HGI dietary intervention and decreasing after the LGI dietary intervention (ΔFFHGI% = 1.3% ± 2.0% and ΔFFLGI% = −0.4% ± 0.7%). In the LGI arm, the main effect of diet on FF% was significant, and a subsequent pairwise comparison showed a significant reduction in liver lipids at t = 360 minutes on visit 2 compared with visit 1 (FFLGI% visit 2 – visit 1 = 0.4 ± 0.1; P ≤ .001), as shown in Figure 2.

3.5.2 | Acute effect of test meal on hepatic glycogen

The main effect of test meal on postprandial glycogen concentration was significant on visit 1 (prior to diet), with values increasing from fasted concentrations for the first 180 minutes and then beginning to decline until the end of the test day, as shown in Figure 3A (P ≤ .01). In contrast, after the HGI test meal, hepatic glycogen concentrations increased from fasted levels throughout all of the visit, but the main effect of test meal on glycogen concentration did not reach significance because of increased inter-subject variability. The coefficient of variation (CV) after consumption was significantly greater during the HGI visit compared with the LGI visit (CVHGI = 48%; CVLGI = 20%; P ≤ .001). There was no significant interaction between test meal and time of day.

3.5.3 | Longer-term effect of dietary intervention on hepatic glycogen

Figure 3B shows the postprandial changes in hepatic glycogen on visit 2. There was no significant increase after either test meal, and no significant change from visit 1 to visit 2. Figure 3D, E and F shows changes in hepatic glycogen for fasted, postprandial peak and AUC.

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**FIGURE 1** A, Plasma glucose (n = 5) and B, plasma insulin (n = 6) results on visit 1 for high (▲) and low (●) glycaemic index test days. Values are means, with standard error of the means represented by vertical bars. *P < .05 between diets, †P < .005 between diets using matched pair Student’s t-test.
between visit 2 and visit 1 for HGI and LGI diets. There was no significant change in fasted glycogen stores between visit 1 and visit 2 (Figure 3C), but the main effect of diet on peak glycogen concentration was significant (P ≤ .05), with mean HGI values greater than LGI (Figure 3D). A subsequent pairwise comparison showed HGI peak glycogen concentration at visit 2 was significantly greater than at visit 1 (P = .04). The effect sizes of LGI diet on fasted glycogen and peak glycogen values were small (0.06 and 0.38, respectively), whereas the effect sizes of HGI diet on fasted glycogen and peak glycogen values were moderate to large (0.67 and 1.15, respectively).

The main effect of diet on hepatic glycogen AUC was also significant, with mean HGI AUC greater than mean LGI AUC (P < .05), as shown in Figure 3E.

3.5.5 | Longer-term effects of dietary intervention on gastric content volume
Visit 1 and visit 2 GCVs are shown in Figure 4. In the HGI arm, the main effect of diet on GCV was significant (P < .03) and a subsequent pairwise comparison showed GCVs were significantly greater on HGI visit 2 compared with HGI visit 1 at t = 20 minutes (P ≤ .05), 140 minutes (P ≤ .05) and 200 minutes (P < .05). In the LGI arm the main effect of diet on GCV was not significant. There was also no significant interaction between diet and visit.

3.5.6 | Subjective Appetite Rating
VAS results are reported in Appendix S1. Changes in appetite across the test day are shown in Figure S1.

4 | DISCUSSION
4.1 | Glycaemic response
The immediate glycaemic responses were as expected, and blood glucose levels were in strong agreement with those observed in the study by Morgan et al.,24 confirming a variation in glycaemic index as intended. Plasma insulin responses were also as expected,29 with greater plasma glucose levels prompting increased insulin secretion. There was no change in fasting insulin resistance after the diet week (HOMA-IR), which was not surprising given the short intervention

![Liver fat fractions (FF%) at fasted state and end of day (t = 360 minutes) on visit 1 and visit 2 for HGI (■) and LGI (□) dietary interventions (n = 7). Values are means, with standard error of the means represented by vertical bars. *P < .05 between diets using a two way repeat measures ANOVA; †P < .05 FF% at t = 360 minutes on visit 2 compared with visit 1 using matched pair Student’s t-test.](image)

![Hepatic glycogen concentration (% baseline) across the time course on A, visit 1 (n = 8) and B, visit 2 (n = 7) for HGI (visit 1 = ▲, visit 2 = △) and LGI (visit 1 = ■, visit 2 = □) test days; C, D, and E, are fasted, postprandial peak and AUC respectively (n = 7). Values are means, with standard error of the means represented by vertical bars. *P < .05 between visits using matched pair Student’s t-test; †P < .05 significant main effect of diet using two-way repeated-measures ANOVA.](image)
period. Changes in liver fat are expected to precede insulin resistance, and future studies should explore the longer-term impact of HGI and LGI diets on insulin sensitivity.

4.2 | Liver fat fraction

Results from $^1$H MRS were striking and of high clinical relevance. Hepatic fat fractions increased after 1 week of HGI diet and decreased after LGI, suggesting that reducing dietary glycaemic index has the potential to provide long-term health benefits in the prevention and management of NAFLD, obesity and type 2 diabetes.

Previous HGI versus LGI dietary intervention studies have not controlled for macronutrient content or total energy intake and energy balance; as such the present study provides new evidence that glycaemic index and/or fibre content plays an important role in ectopic fat deposition independent of nutritional composition. In a recent cross-sectional analysis, Valtuena et al.\textsuperscript{30} reported a strong correlation between steatosis grading and dietary glycaemic index specifically. Whilst the smaller sample size of the present study limits its direct applicability to the general population, it does provide preliminary data that support the findings of this previous study,\textsuperscript{30} and suggests that glycaemic index is indeed associated with liver lipid storage, even under iso-energetic conditions.

A recent four-way trial comparing glycaemic index (high vs low) and carbohydrate content (65% vs 50%) during a period of weight gain found significant increases in liver fat after consumption of a high carbohydrate diet but no association with glycaemic index\textsuperscript{31}; however, in this study the refeeding phase included excess energy, whereas the present study used a dietary intervention that provided no energy surplus or deficit in participants and also had a greater proportion of carbohydrates. Further studies should explore whether the significant effects of glycaemic index found in the present study are driven by the increased carbohydrate consumption and how this relates to excess energy intake. These results indicate the potential importance of type of carbohydrate consumed in the prevention of metabolic disorders; for example, in people with prediabetes. Whilst excess energy intake will provide the most significant contribution to fat deposition and metabolic dysfunction,\textsuperscript{32} the glycaemic index should also be seen as relevant.

4.3 | Glycogen

As far as the authors are aware, the present study showed for the first time increased hepatic glycogen storage after an HGI breakfast compared with an iso-energetic LGI breakfast. During the visit prior to the diet, the increase in mean absolute glycogen levels after the HGI test meal accounted for 25% of the ingested intake of carbohydrates, in strong agreement with the literature.\textsuperscript{33,34} In contrast to this, the peak LGI hepatic glycogen response was lower and declined from 180 minutes. Similar findings have been reported in muscle in a number of studies\textsuperscript{35,36} in which HGI test meals prompted a greater storage of muscle glycogen. This relationship may be attributable to increased insulin levels driving an increased rate of glycogenesis and these effects may differ in patient populations, such as people with insulin resistance or obesity.\textsuperscript{13C} MRS provides a powerful non-invasive method for monitoring these effects in future studies and provides useful insight into metabolic diseases. Related to this finding was the observation of increased peak glycogen levels on the visit after the 7-day diet, which was only significant after the HGI intervention, although this may be attributable to the larger proportion of carbohydrates in the dietary intervention consumed compared with the standard UK diet. Whilst previous studies have shown longitudinal glycogen MRS measurements have considerable variability,\textsuperscript{20} there was a large effect size in fasted and peak measures after the HGI diet. This may be accounted for by the increased postprandial glycogen levels from the evening HGI meal before visit 2. Greater glycogen stores at the start of the day would seem beneficial to individuals who need a sustained postprandial energy release, for example, athletes or other physically active individuals, but have the potential to be broken down through glycogenolysis and enter lipogenesis for longer-term energy stores in more sedentary individuals. The significantly greater CV after the HGI compared with the LGI test meal also indicates a more variable glycogen response to HGI food in healthy individuals and may be relevant to the prevention or treatment of patients with glycogen storage disease.

4.4 | Gastric content volume

The present study also showed evidence of changes in postprandial GCV after the diet week, although these could be attributable to either changes in gastric emptying or gastric secretion that were not
distinguished in the present study. During the visit before the diet week, gastric content was greater for LGI compared with HGI despite meal volumes being matched, which may be a result of slowed gastric emptying during LGI as a result of increased fibre content; however, during visit 2 this was reversed and gastric content was significantly smaller for LGI visit 2 compared with LGI visit 1. Further work is needed to establish whether these changes are an adaptive effect of the dietary interventions.

There were a number of limitations with this study. First, the study group was small; given the multifactorial nature of the study, it would have been preferable to have allowed more for non-compliance and cannulation difficulties while calculating sample size. Whilst 8 participants could be analysed for the proposed primary outcomes, problems with blood samples and incomplete response to survey limited our ability to assess some of the secondary outcomes. Secondly, it was difficult to account for the effect of the variation in fibre content between diets and this cannot be excluded as a factor independent of glycaemic index that influenced some of the outcomes. In addition, obtaining information about the eating habits of participants before entry into the study would have allowed the investigators to compare more directly changes seen in both diets rather than assuming that intake reflected average UK dietary intakes. This could also be used to exclude those with unusual eating habits or to normalize intake in a pre-diet period. Thirdly, we recruited young healthy white men with the intention of limiting metabolic and hormonal variability and to improve statistical power given the small sample size; however, this limits the generalizability of our findings and further work should explore if the results can be extrapolated to a wider population.

In conclusion, this study provides preliminary data that suggest that iso-energetic HGI diets compared with LGI diets lead to significant accumulations of liver fat without changes in body weight; therefore, LGI high-fibre foods offer significant health benefits in reducing FF% compared with HGI foods, and should be considered in dietary interventions in NAFLD, obesity and related metabolic disorders. Future studies should explore the impact of glycaemic index over a longer period, and also in patients with obesity or metabolic syndromes to assess whether the findings of the present study can be used in the prevention and management of these conditions.

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Conflict of interest

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K Hunter, E Ciampi and F Bligh were employees at Unilever at the time of the study.

None of the other authors had conflicts of interest to declare.

Author contributions

S.B, M.S, E.C, K.H, M.T, P.M, I.M, P.G., and L.M did the study conception and design. S.B, M.S did the task of acquisition of data. Analysis and interpretation of data were done by S.B, M.S, Y.F, M.L, K.H, F.B, M.T, I.M, P.G, L.M, and G.A.

The drafting of manuscript was done by S.B. Critical revisions were done by S.B, K.H, F.B, J.S, M.T, I.M, P.G, L.M, and G.A.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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