

## ORIGINAL ARTICLE

## Effects of various food ingredients on gall bladder emptying

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**BACKGROUND/OBJECTIVES:** The emptying of the gall bladder in response to feeding is pivotal for the digestion of fat, but the role of various food ingredients in contracting the gall bladder postprandially is not well understood. We hypothesized that different food ingredients, when consumed, will have a different effect on stimulating gall bladder emptying. To investigate this we designed two randomized, investigator-blind, cross-over studies in healthy subjects using magnetic resonance imaging (MRI) to measure gall bladder volumes serially and non-invasively.

**SUBJECTS/METHODS:** Study 1: exploratory study evaluating the effects of 10 different food ingredients on gall bladder emptying in eight healthy subjects. The choice of ingredients varied from common items like coffee, tea and milk to actives like curcumin and potato protease inhibitor. Study 2: mechanistic study investigating the cholecystokinin (CCK) dose response to the best performer ingredient from Study 1 in 21 healthy subjects four ways.

**RESULTS:** The largest gall bladder volume change in Study 1 was observed with fat, which therefore became the dose-response ingredient in Study 2, where the maximum % gall bladder volume change correlated well with CCK.

**CONCLUSIONS:** These serial test-retest studies showed that the fasted gall bladder volume varied remarkably between individuals and that individual day-to-day variability had wide coefficients of variation. Improved knowledge of how to stimulate bile release using food ingredients will be useful to improve *in vitro*–*in vivo* correlation of bioavailability testing of hydrophobic drugs. It could improve performance of cholesterol-lowering plant stanol and sterol products and possibly aid understanding of some cholesterol gallstone disease.

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## INTRODUCTION

The gall bladder stores and concentrates bile salts during fasting and releases them into the duodenum in response to gastric emptying of a meal.<sup>1</sup> The flow of bile into the duodenum is controlled by gall bladder muscular contraction, gall bladder tone, hepatic secretory pressure and relaxation of the sphincter of Oddi.<sup>2</sup> This process is mostly regulated by the hormone cholecystokinin (CCK) that is released by the proximal intestine in response to products of macronutrients digestion, particularly fat.<sup>3–8</sup> Although there is good knowledge of other secretory responses to dietary composition, such as pancreatic exocrine secretion, the effect of various food ingredients on gall bladder emptying has been explored to a much lesser extent. Specific food ingredients could be effective to trigger gall bladder emptying including dietary protein that stimulates CCK secretion,<sup>9,10</sup> curcumin that increases gall bladder emptying<sup>11</sup> and protease inhibitors that potentiate the CCK response to nutrients.<sup>12</sup> Widely consumed beverages such as coffee can also be effective,<sup>13</sup> but which food ingredients are most effective in contracting the gall bladder is not well understood. Increased knowledge would be desirable for many reasons. Bile secretion in the duodenum is key to lipid digestion.<sup>14,15</sup> The presence of mixed micelles of bile salts<sup>16</sup> with lipolysis products may have an impact on the performance of highly hydrophobic, low solubility (classes II and IV biopharmaceuticals drug classification system, BCS,<sup>17</sup>) compounds;<sup>18,19</sup> it also has an impact on the ability of plant stanol

and sterol products to block dietary cholesterol and the recirculation of endogenous cholesterol<sup>20,21</sup> thereby lowering levels of plasma cholesterol. Further, impaired gall bladder emptying has been linked with the pathogenesis of cholesterol gallstones.<sup>22</sup>

The main hypothesis here was that different food ingredients, when consumed, will have a different effect on stimulating gall bladder emptying. To investigate this we designed two randomized, investigator-blind, cross-over studies in healthy subjects using magnetic resonance imaging (MRI) to measure gall bladder volumes serially and non-invasively. Study 1 was an exploratory study evaluating the effects of 10 different food ingredients on gall bladder emptying in eight healthy subjects. The ingredients selected were all approved for use in the EU, a mix of technology and occasion driven food ingredients, and were chosen based on their potential ability to stimulate gall bladder emptying. Study 2 was a mechanistic study aiming at investigating the CCK dose response to the best performing ingredient from Study 1. These studies would also provide insights on normal fasting gall bladder volume ranges and their coefficients of variation.

## SUBJECTS AND METHODS

## Study design

Both studies obtained local University Medical School Research Ethics Committee approval, and all subjects gave informed written consent. The study was carried out according to Good Clinical Practice principles. All test

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products, Case Report Forms and study monitoring were provided by the sponsor.

The inclusion criteria were male or female, 18–45 years of age, healthy, with body mass index (BMI) 20–30 kg/m<sup>2</sup>. Exclusion criteria comprised pregnancy, any history of serious acute or chronic illnesses, alcohol and/or substance abuse, the presence of implant or devices in the body that are contraindicated for MRI (for example, pace makers, metal implants), use of medication that may interfere with gastrointestinal physiology and the use of sterol/stanol margarine and/or fiber supplement 30 days before and/or during the course of the study. The subjects were instructed to fast overnight for at least 8 h before the study period. On each study day, they filled in a questionnaire to confirm they followed study restrictions and eligibility.

The operator who processed the data was kept blinded to the randomization code by the investigator who dispensed the test meals and saved the image files from the MRI scanner in a blind manner. The code was broken only after data processing and blind data review were completed.

The MRI images were used to calculate the volume of the gall bladder at each specified time interval as described below, and the percentage (%) of gall bladder volume change from baseline and the area under the curve (AUC) between  $t = -5$  and  $t = 65$  min were derived.

The primary end point was the maximum % gall bladder volume change between  $t = -5$  and  $t = 65$  min by test product. This was chosen as primary end point, as it takes into account the maximum gall bladder volume change occurring over the whole postprandial period investigated and also normalizes each subject's gall bladder to the starting fasted time point on each study day, making it easier to carry out comparisons in these multiple ways studies.

The secondary end points were the gall bladder volume for each individual time point by test product, the % gall bladder at volume change at  $t = 45$  min, the AUC for gall bladder volume and the time to maximum gall bladder volume change.

## Study 1

Eight subjects were recruited and they all completed the 10-way study. They were five men and three women, 26.5 ± 8 years old with BMI 22.5 ± 0.6 kg/m<sup>2</sup>.

The subjects consumed one of 10 test products at each of the 10 test visits (one week apart from each other) based on a computer-generated randomization schedule. The products comprised emulsions, yogurt, coffee, tea and semi-skimmed milk as specified in Supplementary Table 1. At each test visit, an MRI scan of the abdomen encompassing the gall bladder was obtained at fasted baseline before consumption of the food product ( $t = -5$  min) and after that at times  $t = 5, 15, 25, 35, 45, 55$  and 65 min post ingestion of the test product, for a total of eight scans at each visit. Additional analysis investigated the correlation of maximum % gall bladder volume change between  $t = -5$  and  $t = 65$  min with fat, protein and volume characteristics of the test products.

## Study 2

Twenty-two subjects were recruited. One subject withdrew after two occasions, and hence 21 subjects completed the 4-way study. They were 11 men and 10 women, 27 ± 8 years old with body mass index (BMI) 22.5 ± 0.6 kg/m<sup>2</sup>. Four of these 21 subjects failed to give at least one blood sample (for example because a blocked cannula) so there were  $n = 17$  completed subjects available for the plasma CCK analysis.

The subjects consumed one of four test products at each of the four test visits (1 week apart from each other) based on a computer-generated randomization schedule. The products comprised milk-based, ready-to-drink beverages based on the results of Study 1. They were 150 ml milk-based drinks that contained the same amount of protein (9 g) and the same amount of plant stanol ester (2.23 g) but increasing amounts of fat (1.5 g, 3.5 g, 6.5 g, 10 g fat, respectively).

At each test visit, an intravenous cannula was inserted in a forearm vein by an experienced research nurse. A MRI scan of the abdomen encompassing the gall bladder was obtained at fasted baseline before consumption of the food product ( $t = -5$  min) and after that at times  $t = 5, 15, 25, 35, 45, 55$  and 65 min post-ingestion of the test product, for a total of eight scans at each visit. After each MRI scan, a blood sample was drawn from an intravenous cannula for CCK analysis.

Additional secondary end points for this study were maximum plasma CCK between  $t = 5$  min and  $t = 65$  min, plasma CCK at  $t = 45$  min and

correlation between maximum CCK levels and maximum % gall bladder volume change between  $t = 5$  min and  $t = 65$  min.

## Magnetic resonance imaging

MRI was carried out on a 1.5T Philips Achieva (Philips Healthcare, Eindhoven, The Netherlands) whole body scanner using the whole body Transmit/Receive coil. Each subject was positioned supine in the scanner. A coarse scout scan was run to locate the position of the gall bladder and plan the position of the image planes for the following data acquisition scan. Coronal, single-shot, turbo spin-echo MRI images were then acquired with spectral presaturation with inversion recovery fat suppression. This imaging sequence was heavily T2-weighted so that liquid appears very bright and the rest of the surrounding organs appear quite dark. They comprised 30 contiguous slices with no gap, echo time TE = 4.4 ms with echo spacing 4.4 ms, acquisition matrix 268 × 198 and flip angle 90° and an acquired in-plane resolution of 1.5 × 1.5 mm<sup>2</sup> and a slice thickness of 6 mm (reconstructed 0.78 × 0.78 × 6 mm<sup>3</sup>). They were acquired in a single breath-hold of 26 s. The MRI scanning schedule allowed interleaving two subjects per morning thus increasing throughput and reducing cost.

## Plasma collection and CCK assay

On arrival an 18-gauge BD Venflon Pro (Becton Dickinson Infusion Therapy, Helsingborg, Sweden) intravenous cannula was placed in a forearm vein of the subjects. 6.5-ml blood samples were collected fasted and at each postprandial scanning time point. The blood was transferred immediately into chilled blood collection tubes containing 0.3 ml ethylenediamine tetra-acetic acid and 5000 KIU aprotinin. The samples were cooled in an ice-bath immediately. The plasma was then readily separated by centrifugation for 10 min at 3000 g at 4 °C. The separated plasma was collected using a plastic pastette and transferred to clean tubes for immediate storage at -80 °C until assayed using radioimmuno assay with commercially available kits according to the manufacturer's instructions (EURIA-CCK, Euro-Diagnostica, obtained from Immunodiagnostic Systems, Ids Ltd., Tyne and Wear, UK) as described previously.<sup>8</sup>

## Data analysis, statistical methods and power of the studies

The image data set from each subject and each time point was recalled on a workstation and commercial software (Analyze 6, Biomedical Imaging Resources, Mayo Clinic, Rochester, MN, USA) was used to trace around the gall bladder a region of interest on each image slice using the 'Smart Edge' function. This function is commonly used as it searches for areas of high gradient to adjust the path of the region of interest, thus aiding the operator semi-automatedly. The gall bladder volume was then calculated by summing across all region of interests for that given subject and time point. These were tabulated and plotted in the Microsoft Excel to determine time courses and to calculate the AUC using the trapezoidal method. The % gall bladder volume change was calculated as  $(1 - \text{gall bladder volume at time } t / \text{gall bladder volume at baseline}) \times 100$  expressed in % and with time  $t$  defined as the time of imaging postprandially (that is, between 5 and 65 min).

As shown by Shapiro Wilk's test, the vast majority of the data were normally distributed and were expressed as mean ± s.e.m. Accordingly, parametric statistic tests were used to assess the significance of the effect of test products and time on the end point measures, followed by *post hoc* Dunnett's test corrected for multiple comparison comparing all test products versus the 4-ml high-fat emulsion in Study 1 and versus the least fatty sample in Study 2. As this was a cross-over study design, each subject acted as his/her own control. The CCK plasma values were not normally distributed even after log transform, and hence for these a 2-way ANOVA was not performed. Differences were being significant at  $P < 0.05$ . GraphPad Prism version 4 (GraphPad Software Inc, La Jolla, CA, USA) was used throughout.

Study 1 was an exploratory study, and hence a power calculation of sample size was not available. On the basis of the results of Study 1 in which the semi-skimmed milk drink achieved a mean maximum % gall bladder volume change of 41% with an s.d. of 9%, it should be possible to detect a difference in maximum gall bladder volume change of 15% with  $P < 0.05$  and a 90% power using  $n = 22$  subjects.

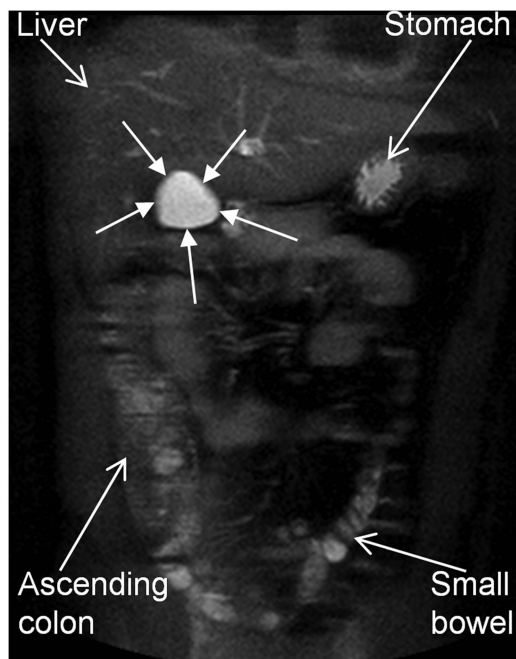
## RESULTS

The study procedures were well tolerated and all subjects completed the studies, but one subject in Study 2 withdrew after two study days for personal reasons. Imaging subjects with an MRI scanner in-built body coil was very rapid, requiring no positioning of additional abdominal receivers on the subjects or parallel imaging calibration scans, while providing images of good quality for gall bladder volume measurements as shown by an example in Figure 1.

### Study 1

The primary end point was the maximum % gall bladder volume change between  $t = -5$  and  $t = 65$  min. This was significantly different between test products as shown in Figure 2 ( $P < 0.0001$ ). The 20 ml high-fat emulsion yielded the largest gall bladder volume change (42%), and this was significantly larger than for all the other test products ( $P < 0.05$ ) apart from the 250 ml semi-skimmed milk (41% volume change,  $P > 0.05$ ) and the 4 ml high-fat emulsion (27% volume change,  $P > 0.05$ ). Comparing drinks with equal volumes showed that the semi-skimmed milk achieved a significantly higher maximum % gall bladder volume change than coffee (22%) and tea (18%) ( $P < 0.05$  for both). All data are summarized in Supplementary Table 1.

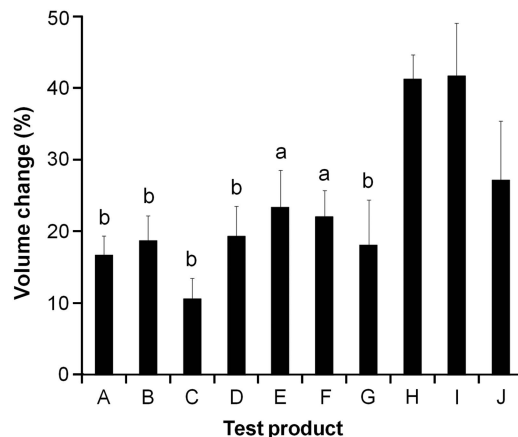
Looking at the other outcomes, there was a significant effect on gall bladder volumes by meal ( $P < 0.0001$ ). There was a significant effect of meal type on gall bladder emptying as measured by AUC of the volume curves ( $P < 0.0001$ ) and also as % gall bladder volume change at  $t = 45$  min ( $P < 0.0006$ ). The maximum % gall bladder volume change correlated significantly with fat content ( $r = 0.80$ ,  $P < 0.0052$ ) but not with protein content ( $r = 0.41$ ,  $P < 0.2$ ) or drink volume ( $r = 0.13$ ,  $P < 0.7$ ). The slope of the line to the maximum gall bladder volume change was the highest for the milk, indicating the fastest emptying response, while the high-fat emulsion meals yielded a lower slope value, indicating a slower (albeit deeper) gall bladder emptying.



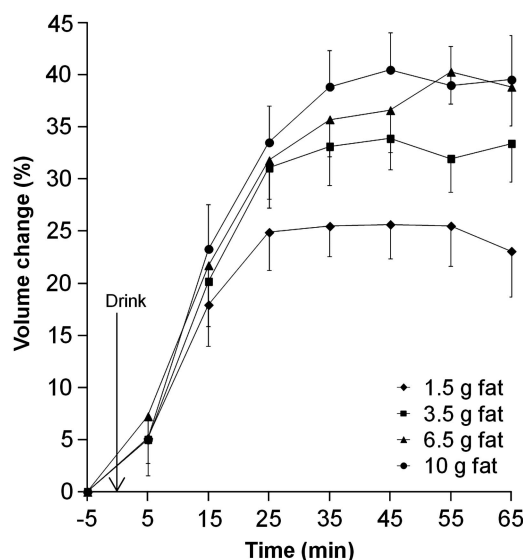
**Figure 1.** Representative example of a coronal, single-shot, turbo spin-echo magnetic resonance image taken across the fasting gall bladder (indicated by the closed arrows) of a healthy volunteer. In this heavily T2-weighted imaging sequence the liquid in the gall bladder appears very bright and the rest of the surrounding organs appear quite dark aiding data processing.

### Study 2

We confirmed previous work showing that, of all the ingredients tested, fat produced the strongest gall bladder response with a maximum % gall bladder volume change dose response to fat ( $P < 0.003$ ). The least amount of fat (1.5 g) achieved already a mean maximum percentage gall bladder volume change of 33%. The drinks containing 6.5 g and 10 g fat achieved significantly higher maximum % gall bladder volume change ( $P < 0.01$  for both). The mean maximum % gall bladder volume change data are plotted in Figure 3 and the other data are summarized in Supplementary Table 2.



**Figure 2.** Maximum % gall bladder volume change between  $t = -5$  and  $t = 65$  min for healthy volunteers after they consumed each of the 10 test ingredients in Study 1. Values are mean  $\pm$  s.e.m.,  $n = 8$ . A = 25 ml dairy emulsion; B = 25 ml dairy emulsion + 300 mg potato protease inhibitor; C = 67.5 g yogurt drink + 3 g protein; D = 67.5 g yogurt drink + 80 mg curcumin; E = 250 ml coffee; F = 250 ml tea; G = 250 ml semi-skimmed milk; H = 20 ml high-fat emulsion; I = 20 ml high-fat emulsion; and J = 4 ml high-fat emulsion. <sup>a</sup>Significant difference versus ingredient J, 4 ml high-fat emulsion ( $P < 0.05$ ); <sup>b</sup>Significant difference versus ingredient J, 4 ml high-fat emulsion ( $P < 0.01$ ).



**Figure 3.** Plot of the % gall bladder volume change with time for healthy volunteers after they consumed each of the four milk-based beverages of increasing fat content in Study 2. Values are mean  $\pm$  s.e.m.,  $n = 21$ .

Looking at the other outcomes, there was a significant effect of fat content on gall bladder volumes ( $P < 0.0001$ ).

There was a significant effect of meal fat on gall bladder emptying as measured by AUC of the volume curves ( $P < 0.01$ ) and also as % gall bladder volume change at  $t = 45$  min ( $P < 0.0008$ ).

There was a dose response of the effect of meal fat on maximum plasma CCK levels ( $P < 0.007$ ). There was a significant effect of meal fat on plasma CCK as measured by overall AUC (Figures 4,  $P < 0.004$ ) and on plasma CCK at  $t = 45$  min ( $P < 0.0008$ ).

The maximum % gall bladder volume change correlated significantly with the maximum plasma CCK (Spearman's  $r = 0.42$ ,  $P < 0.0001$ ).

#### Fasting gall bladder volumes normal ranges

The test-retest repeated Studies 1 and 2 yielded also a unique collection of fasting gall bladder volumes that enable assessment of size and inter- and intra-subject variability. The individual data are shown in Figure 5. The mean volume of the fasted gall bladder per subject showed wide variability between 12 ml and 52 ml with test-retest coefficients of variation ranging between 3% and 42%. The overall mean of the individuals' mean fasted gall bladder volumes was  $26 \pm 2$  ml. There was a strong correlation of mean gall bladder size with subjects' weight ( $r = 0.68$ ,  $P < 0.0001$ ), height ( $r = 0.50$ ,  $P < 0.006$ ) and BMI ( $r = 0.48$ ,  $P < 0.008$ ) but not with age ( $r = 0.12$ ,  $P < 0.5$ ).

## DISCUSSION

The emptying of the gall bladder has been studied using gamma scintigraphy or ultrasound techniques.<sup>3,4,23–29</sup> In recent years, MRI has been shown to measure serially the emptying of the gall bladder accurately, non-invasively and with high spatial resolution.<sup>30–34</sup> In these studies, serial MRI performed well and it was an effective, non invasive and accurate way of monitoring the effects of various food products on gall bladder emptying.

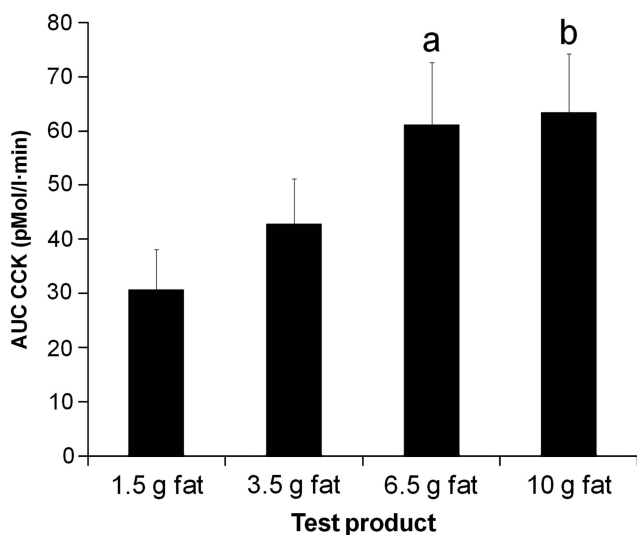
The main hypothesis of Study 1 that different food ingredients will have a different effect on stimulating gall bladder emptying was proven to be correct. The choice of ingredients varied from common (occasion driven) items like coffee, tea and milk to

actives like curcumin and potato protease inhibitor (food manufacturing driven).

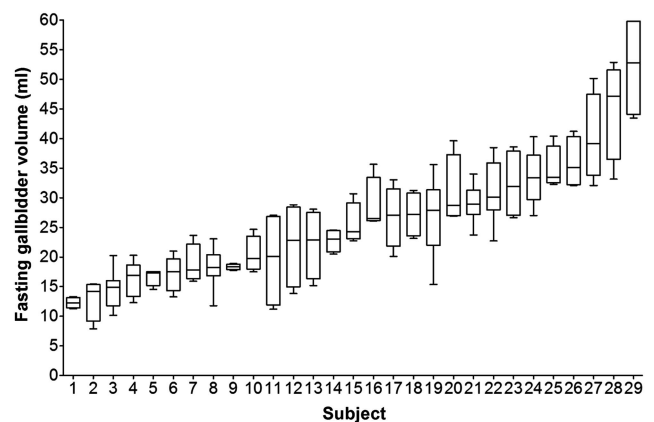
Semi-skimmed milk performed well but it is difficult at this stage to discriminate whether its composition of fat and protein would have an additive effect in contracting the gall bladder. The protein contribution would need to be the subject of future studies.

The largest gall bladder volume change was observed with the positive fat-containing control (20 ml of high-fat emulsion). However, gall bladder emptying induced by amounts of fat as low as 7 g (perfused in the jejunum)<sup>35</sup> had been observed previously. Our data show that a very low amount of fat (2 g fat in 4 ml of high-fat emulsion) nevertheless achieved a marked gall bladder volume change of 27%. The potato protease inhibitor achieved a modest gall bladder volume change. The same potato protease inhibitor did not significantly affect healthy volunteers' plasma CCK at twice the dose we used.<sup>36</sup> The 80 mg curcumin stimulus was the fourth best performer achieving a mean 23% maximum gall bladder volume change. This was, however, much lower than the 40% volume change reported at 1 h in a previous ultrasonography study.<sup>37</sup> Whether the reason for this difference might rest on the different imaging technologies used or in the delivery vehicle (a yogurt drink in our study versus plain curcumin ingested with a glass of water in that report) is unclear. When looking at the correlation with meal components, the maximum % gall bladder volume change correlated positively and significantly with the fat content of the samples (with or without the stanol included in the calculation, Pearson's  $P < 0.0052$  and  $P < 0.0012$ , respectively) but neither with the protein nor with the volume of the meals.

The main conclusion from this initial scouting study was that long-chain fat with a high degree of emulsification was the best performer in stimulating gall bladder emptying, likely to be explained with current knowledge<sup>2</sup> via the cholecystokinin (CCK) duodenal response and to be tested in our second study. Semi-skimmed milk performed well and this was one of the reasons driving the choice of a milk-based beverage as vehicle for increasing amounts of emulsified fat, while keeping protein and volume the same, in Study 2. Protein is also a potent stimulant of CCK, whereas carbohydrate is not.<sup>10</sup> Gastric emptying of stable fat emulsions is slow<sup>8</sup> that may be a factor contributing to the longest time to maximum gall bladder volume change shown by the 20 ml high-fat emulsion.



**Figure 4.** Area under the curve (AUC) for plasma cholecystokinin (CCK) for healthy volunteers after they consumed each of the four milk-based beverages of increasing fat content in Study 2. Values are mean  $\pm$  s.e.m.,  $n = 17$ . <sup>a</sup>Significant difference versus 1.5g fat drink ( $P < 0.05$ ); <sup>b</sup>Significant difference versus 1.5g fat drink ( $P < 0.01$ ).



**Figure 5.** Box and whiskers plot of the individual fasting gall bladder volumes for the eight healthy volunteers in Study 1 and the 21 volunteers in Study 2. For each participant, the box represents the 25 and 75 percentiles, the horizontal line in the box represents the median value and the whiskers represent the minimum and maximum gall bladder volume.

The main hypothesis of Study 2 that the best performer of Study 1 (emulsified fat in this case) will have a dose response effect on maximum % gall bladder volume change was correct. This correlated well with increased plasma CCK levels in keeping with the well-established role of fat in releasing CCK, which depends on fatty acid chain length,<sup>38,39</sup> and the role of CCK in regulating gall bladder emptying.<sup>40</sup> Interestingly the milk-based beverage with the lowest amount of fat (1.5 g) achieved already a marked mean maximum percentage gall bladder volume change of 33% over the length of time investigated. Our meals were all low calorie (for example, 90 kcal for the 20 ml high-fat emulsion). Much larger volume changes have been observed with higher calorie meals. A 70% gall bladder volume change was reported 45 min after a 465 kcal mixed solid-liquid meal<sup>41</sup> and 70% gall bladder volume change 70 min after a 675 kcal fat emulsion test meal<sup>8</sup> in healthy subjects.

From the test-retest studies, we have also observed that the fasted gall bladder volume varied remarkably between individuals and that individual day-to-day variability can have wide coefficients of variation of up to 42%. An influence of gender, age and body size on gall bladder volume has been reported from large surveys.<sup>42–44</sup> Our mean fasted value of 26 ml is higher than previously reported values in the 18-ml range,<sup>37</sup> and this could be because of imaging technology and/or subjects' cohort differences. One possible explanation for the large intra-individual variation in fasting gall bladder volumes is the cephalic phase response to food. The subjects may have been exposed to food-related sensory stimulation at home or on their way to the study site. Cephalic, pre-ingestive neural signals at the thought, sight or smell of food can trigger a number of physiological and secretory responses,<sup>45</sup> while sham feeding clearly induces gall bladder emptying.<sup>46</sup> Although in this study it was not possible to control the subjects' environment overnight before the morning measurements, future studies could consider this option in the study design. Gall bladder volume may also fluctuate with the cyclic variations of the migrating motor complex and motilin levels.<sup>47</sup> We observed a correlation between gall bladder fasting volume size and individuals' body frame but this was not surprising.<sup>43</sup>

Because of the different volume, taste/aroma, color and texture of the samples, we could not exclude the presence of a possible ingestive cephalic component of gall bladder emptying in these data. One thought for future studies would be to manufacture the new test samples as equal in volume/texture/flavor/color as possible. Cephalic effects could also help to explain the wide variation in time to maximum gall bladder volume change, which varied not just inter-subjects but also between study days. It is worth noting that in some of the experiments carried out the time to maximum gall bladder volume change was 65 min, indicating that the volume change could possibly have been greater at later times than the period investigated. The time period investigated is a limitation of this study, and it would be desirable in future studies to extend the monitoring time window to at least 120 min to fully capture the time course of the emptying of the gall bladder. Intravenous infusion of CCK<sup>48</sup> could be used as a gall bladder emptying standard for comparison.

In conclusion, various food ingredients are capable of inducing varying degrees of gall bladder emptying. Among the various food ingredients and actives tested here, fat and semi-skimmed milk induced the most profound volume changes. Fat showed the strongest effect, which correlated with plasma CCK levels. Improved knowledge of how to stimulate bile release using food ingredients will be useful to improve *in vitro/in vivo* correlation of bioavailability testing of hydrophobic drugs. It could improve performance of cholesterol-lowering plant stanol and sterol products and possibly aid understanding of some cholesterol gallstone disease.

## CONFLICT OF INTEREST

The Nottingham team was the recipient of this research project grant from Johnson & Johnson Consumer Services EAME Ltd. MR and RI are employees of Johnson & Johnson Consumer Services EAME Ltd. The remaining authors declare no conflict of interest.

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