RESEARCH ARTICLE

Ginger phytochemicals mitigate the obesogenic effects of a high-fat diet in mice: A proteomic and biomarker network analysis

John H. Beattie[1], Fergus Nicol[1], Margaret-Jane Gordon[1], Martin D. Reid[1], Louise Cantlay[1], Graham W. Horgan[2], In-Sook Kwun[3], Ji-Yun Ahn[4] and Tae-Youl Ha[4]

[1] Rowett Institute of Nutrition and Health, University of Aberdeen, Bucksburn, Aberdeen, UK
[2] Biomathematics and Statistics Scotland, Aberdeen, UK
[3] Department of Food Science and Nutrition, Andong National University, Andong, Kyungpook, Republic of Korea
[4] Food Function Research Center, Korea Food Research Institute, Gyeonggi-Do, Republic of Korea

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Scope: Natural dietary anti-obesogenic phytochemicals may help combat the rising global incidence of obesity. We aimed to identify key hepatic pathways targeted by anti-obsogenic ginger phytochemicals fed to mice.

Methods and results: Weaning mice were fed a high-fat diet containing 6-gingerol (HFG), zerumbone (HFZ), a characterized rhizome extract of the ginger-related plant *Alpinia officinarum* Hance (high fat goryankang, HFGK) or no phytochemicals (high-fat control, HFC) for 6 wks and were compared with mice on a low-fat control diet (LFC). Increased adiposity in the HFC group, compared with the LFC group, was significantly ($p<0.05$) reduced in the HFG and HFGK groups without food intake being affected. Correlation network analysis, including a novel residuals analysis, was utilized to investigate relationships between liver proteomic data, lipid and cholesterol biomarkers and physiological indicators of adiposity. 6-Gingerol significantly increased plasma cholesterol but hepatic farnesyl diphosphate synthetase, which is involved in cholesterol biosynthesis was decreased, possibly by negative feedback. Acetyl-coenzyme A acyltransferase 1 and enoyl CoA hydratase, which participate in the β-oxidation of fatty acids were significantly ($p<0.05$) increased by consumption of phytochemical-supplemented diets.

Conclusion: Dietary ginger phytochemicals target cholesterol metabolism and fatty acid oxidation in mice, with anti-obesogenic but also hypercholesterolemic consequences.

Keywords:
6-Gingerol / Correlation network / Obesity / Proteomics / Zerumbone

1 Introduction

Over consumption of high-fat, Western-type diets and reduced exercise is leading to a sustained increase in obesity [1]. Concerted attempts to encourage the modification of dietary and lifestyle habits of individuals are necessary, but are having limited impact [2]. Bariatric surgery is an effective approach for some individuals with morbid obesity [3] but drug-based methods targeting energy metabolism have also been used [4, 5]. The potential of natural phytochemicals to influence energy metabolism has been investigated as a possibly effective way of influencing fat accretion

Correspondence: Professor John H. Beattie, Division of Lifelong Health, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, Scotland, UK
E-mail: J.Beattie@abdn.ac.uk
Fax: +44-1224-716629

Abbreviations: ACAA1, acetyl-coenzyme A acyltransferase 1; **ACSBG2,** long-chain fatty acid CoA ligase; **EF1δ,** elongation factor 1δ; **EFP,** epididymal fat pad; **FABP,** fatty acid-binding protein; **FPPS,** farnesyl diphosphate synthetase; **GST,** glutathione-*S*-transferase; **HFC,** high-fat control; **HFG,** high-fat gingerol; **HFGK,** high-fat goryangkang; **HFZ,** high-fat zerumbone; **LFC,** low-fat control; **LHPP,** phospholysine phosphohistidine inorganic pyrophosphate phosphatase; **PCA,** principal component analysis; **SREBP,** sterol regulatory element-binding protein; **TC,** total cholesterol; **TG,** triglyceride

without the need for major adjustments to dietary habits and without the risks of surgery or synthetic drug therapy [6]. In animal studies, ginger phytochemicals are reported to have anti-obesogenic properties [7, 8].

The underground rhizome of the plant *Zingiber officinale*, otherwise known as root ginger, and related plant species have been used for medicinal purposes in Asia for thousands of years. In a medicinal context, ginger consumption is used as a remedy for many purposes, including nausea, dyspepsia and gastroparesis as well as for heart disease and cancer [9, 10]. Active chemical constituents in ginger and rhizomes of related species include gingerols and shogaols. The bioactivity of 6-gingerol has been widely investigated in relation to its anti-cancer properties, and its chemical structure is shown in Fig. 1 [11].

Another ginger phytochemical, zerumbone, is an active oil-soluble biocompound derived from the wild ginger, *Zingiber zerumbet*, otherwise known as Asian ginger [12, 13]. This bioactive sesquiterpene (2,6,9,9-tetramethyl-(2*E*,6*E*,10*E*)-cycloundeca-2,6,10-trien-1-one) has a cross-conjugated ketone in an 11-membered ring (Fig. 1). It is reported to have anti-cancer properties [14] but information about its bioavailability and metabolism is limited.

Ginger phytochemical supplements are commercially available and may be of medicinal value. The rhizome extract of a plant in the ginger family (*Alpinia officinarum* Hance), otherwise known as Lesser Galangal, is produced in East Asian countries and is referred to as goryangkang in Korea ([15]; Korea Food and Drug Administration, http://www.kfda.go.kr/herbmed/), and contains 1,8-cineol, methyl cinnamate and α-cadinene as major constituent phytochemicals (0.5–1.0% by weight) [16].

There have been various attempts to understand the mechanisms by which ginger phytochemicals such as 6-gingerol affect lipid/energy metabolism, some studies using cell culture systems [7]. Interpretation of such studies may be limited because direct treatment of cells in culture with dietary phytochemicals may not account for absorption efficiency and/or metabolic transformation in vivo. For example, 6-gingerol is rapidly metabolized to the glucuronide form when absorbed [17], and this metabolite may have different or no biological activity. Other metabolic products with biological activity may be present in vivo and so need to be considered when using model systems in vitro. The aim of our study was to try and identify mechanisms of ginger phytochemical effects on lipid/energy metabolism using an in vivo model and a proteomic approach.

Proteomics is a useful technique for identifying targeted metabolic or signalling pathways especially when changes in protein expression can be related to physiological and biochemical parameters that respond to the treatment of interest [18]. We have previously successfully used correlation networks to identify clusters of proteins with related functions responding to dietary treatments [19], and in the present study we have incorporated physiological and biochemical parameters to link with the novel protein responses on 2D gels. Appreciating that the strength of correlations between two variables can be influenced by treatment effects, we have also investigated the utility of correlation networks created from residual values (individual values minus the mean value). This removes treatment effects revealing underlying relationships between variables and a network analysis of residuals is a novel approach for proteomic data interpretation. In this study, we aimed to compare residual and treatment networks of hepatic proteins and other parameters to understand how 6-gingerol, zerumbone and goryangkang fed to mice challenged with a high-fat diet, influence adiposity.

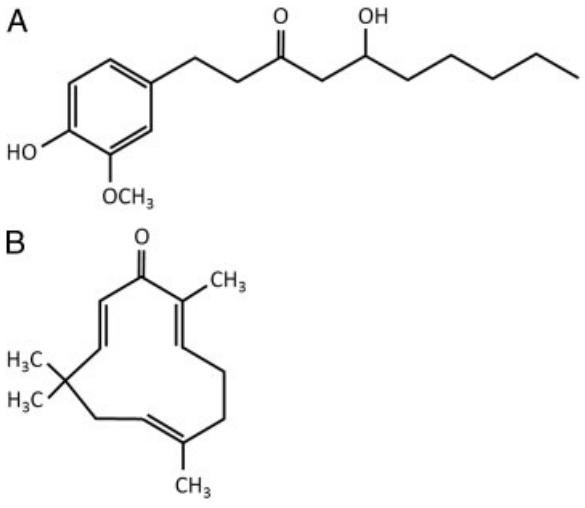

Figure 1. Chemical structure of 6-gingerol (A) and zerumbone (B).

2 Materials and methods

2.1 Ginger Phytochemicals

The 6-gingerol used in this study was >98% pure based on HPLC analysis and was obtained from Phytomarker (Tianjin, China). Zerumbone was obtained from Kingherbs (Hainan, China) and the purity was 99%. In order to prepare goryangkang, a powdered preparation of rhizome from *A. officinarum* Hance was purchased from Omni Herb (Seoul, Republic of Korea). The dried powder (1 kg) was extracted thrice with 4 L of 70% ethanol overnight at room temperature. The ethanol extracts were filtered through a filter paper (Whatman Grade No. 2, USA), and concentrated under a vacuum at 40°C. The concentrated extracts were then freeze-dried and the dry weight yield from the original powder was 4.45%. The total polyphenol content in the sample, which was determined using the Folin–Ciocalteu method [20], was 110 mg/g of extract. The total flavonoid content, which was determined using a spectroscopic method [21], was 100 mg/g of extract.

2.2 Animals, diets and tissues

The animals selected for use in this study were C57BL/6 mice, because this mouse strain has a propensity to become obese on a high-fat diet. The mice of Specific Pathogen-Free status were obtained from the Orient Bio (Sungnam, Republic of Korea) at 4 wks of age and were maintained in a barrier facility with microbiological control and HEPA filtered air. The animals were acclimatized for 1 wk on mouse chow diet and 32 were randomly allocated to four groups of six mice and one group of eight mice. Mice in three groups of six animals were given high saturated fat (20% total fat by weight, 38.9% of total energy intake as fat) semi-synthetic diets (see Table 1) containing 0.05% 6-gingerol (HFG diet), 0.025% zerumbone (HFZ diet) or 0.5% goryangkang extract (HFGK diet). The values for 6-gingerol and zerumbone are equivalent to daily consumption of about 25 g of fresh ginger rhizome per kg of diet, or the equivalent as an extract supplement. The group with eight mice was given the same high-fat diet but with no added ginger phytochemical and this is referred to as the high-fat control (HFC) group. The remaining group of mice was fed a low saturated fat diet (5% total fat by weight, Table 1), which is referred to as the low-fat control (LFC) group.

Diets were fed ad libitum for 8 wks and food intake was monitored. The animal facility environmental temperature was $22 \pm 2^{\circ}C$ and a 12-h light–dark cycle was used. The mice were grouped three to a cage and were given free access to deionized water and the study was approved by the Korea Food Research Institute – Institutional Animal Care and Use Ethics Committee. No permission or accreditation number was assigned for this study. At termination of the study, mice were killed and liver was perfused in situ with 10 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose, before being excised and snap-frozen in liquid nitrogen. Blood was collected to EDTA-treated tubes (BD Falcon, San Jose, USA) and was centrifuged at $2000 \times g$ for 15 min to obtain plasma, which was divided into aliquots and snap-frozen in liquid nitrogen.

Table 1. Composition of low- and high-fat semi-synthetic diets (g/kg)

	Low fat	High fat
Casein	200	200
Corn oil	50	50
Lard	–	50
Cocoa butter	–	70
Coconut oil	–	30
Cholesterol	–	5
Corn starch	350	195
Sucrose	300	300
Cellulose	50	50
Mineral mix[a)]	35	35
Vitamin mix[a)]	10	10
Methionine	3	3
Choline bitartrate	2	2

a) Vitamin and mineral mixes were prepared according to AIN 76 recommendations.

2.3 Plasma and liver analyses

Triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL) were enzymatically analysed with a commercial kit (Shinyang Chemical, Seoul, Republic of Korea). Hepatic lipids were extracted using the Folch method [22]. Hepatic TG and TC concentrations were determined using an enzyme assay kit (Shinyang Chemical). Plasma insulin and leptin levels were determined using enzyme-linked immunosorbent assay kits (ELISA) according to manufacturer's protocol (Shinayagi, Tokyo, Japan). Plasma glucose levels were enzymatically analysed using a commercial kit (Shinyang Chemical).

2.4 Proteomic analysis

2.4.1 Soluble hepatic protein preparation and 2D gel proteomics

Quantification and correlation network analysis of 2D gel protein spots relies on there being sufficient replication of proteomic analyses within a treatment group. For this reason, each liver from 6 to 8 replicate animals in a group was analysed individually yielding a total of 32 gel separations (6–8 gels per group). Tissue samples (approximately 100 mg) from the same location on the same liver lobe were homogenized in 400 μL extraction buffer (50 mM Tris-HCl, pH 7.1, containing 100 mM KCl, 20% glycerol and Roche complete protease inhibitor) using a Precelys 24 homogenizer (Bertin, Montigny-le-Bretonneux, France) and 1 mm zirconia beads (5.5 g/cm^3, BioSpec Products, Bartlesville, OK, USA) for 2×10 s. The resulting homogenate was centrifuged at $100\,000 \times g$ for 30 min to remove insoluble cell particles. The supernatant was withdrawn and the pellet re-homogenized in 200 μL of extraction buffer. After re-centrifugation the resulting supernatant was pooled with the first supernatant and the protein content was measured using the Bradford assay. The protein sample was further purified by TCA precipitation as described previously [23]. Precipitated proteins were resuspended in 0.5 mL of solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, 2% Biolyte ampholite pH 3–10. The proteins were separated by isoelectric focussing in the first dimension using immobilized pH gradient (IPG) strips (pH 3–10, Bio-Rad, Hemel Hempstead, UK) and SDS–PAGE in the second dimension on 8–16% gradient gels (18×18 cm), as described elsewhere [19]. The IPG strips were rehydrated with 300 μg of liver protein and gels were stained with Coomassie blue. Images were obtained from a high-resolution scanner and analysed using the SameSpots software (Nonlinear Dynamics,

Newcastle, UK). Spot data were normalized and exported for quantitative statistical analysis.

2.4.2 Mass spectrometry

Spots affected by ginger phytochemicals were cut robotically, proteolysed in a Mass Prep Station using trypsin and analysed by LC-MS/MS using an Ultimate Nano-LC capillary chromatography system (Dionex, Camberly, UK) hyphenated with an Applied Biosystems 2000 Q Trap. Samples processed by the Mass Prep Station (trypsinized and dissolved in 40% ACN and 1% formic acid in water) were dried down and dissolved in 0.1% formic acid prior to injection of 10 μL onto a 15 cm × 75 μm PepMap C-18 nano-column. Laddered peptide fragment mass spectra yielded the sequence of separated peptides and the peptide fingerprinting web resource program. "MASCOT" (version 1.6b9, Matrix Science, Boston, USA) was used to interrogate the MSDB database (Release 20060831) for matching proteins. Search parameters were as follows: fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); missed cleavages, up to 1; peptide tolerance, 1.5 Da.

2.4.3 Data analysis and statistics

Group differences for all physiological and protein data were examined using R (R Foundation for Statistical Computing, Vienna, Austria) by applying a one-way analysis of variance on log transformed data. Spots from 2D gels showing significant differences ($p<0.05$) between groups were then analysed by principal component analysis (PCA) and presented as 2D plots of the first and second component scores for observations (mice). A PCA of all spots was also examined.

All 2D gel protein spots, physiological and biochemical data from all treatment groups were then correlated in a matrix and statistically significant associations with a correlation coefficient of ≥ 0.3 or ≤ -0.3 were displayed as a correlation network using the Cytoscape software (http://www.cytoscape.org/). When correlating two variables (e.g. leptin and white adipose tissue weight) after pooling data from different treatment groups, the strength of the correlation (correlation coefficient) can be influenced by treatment effects on both variables. In order to remove these effects and look at the underlying relationship between the variables, the residuals (individual values minus the mean value) were calculated for data within each treatment group before pooling and correlating them.

3 Results

3.1 Physiological and biochemical parameters

Animals consuming the HFC diet with no added ginger phytochemicals were significantly heavier than those consuming the LFC diet (Table 2). This weight increase in HFC rats was at least in part due to increased adiposity, since the epididymal fat pad (EFP) to body weight ratio increased significantly. Addition of 6-gingerol and goryangkang but not zerumbone to the mouse diets significantly reduced body weight gain and adiposity. Food intake was not significantly affected by the fat content of the diet or any of the phytochemicals (Table 2). Compared with the HFC diet, the HFG, HFZ and HFGK diets significantly ($p<0.05$) decreased plasma TG levels as well as plasma insulin and leptin levels, which tend to reflect adiposity (Table 3). Circulating plasma TC levels were raised, which may be attributed to an increase in plasma HDL cholesterol level, especially in the case of the HFGK group (Table 3).

Table 2. Influence of the diets HFG, HFZ and HFGK on body and liver weight, food intake and adiposity in comparison with the HFC and LFC diets

	Food intake (g/d)	Body weight (g)	EFP/body (%)
LFC	2.85 ± 0.07^{a}	28.7 ± 0.6^{a}	3.86 ± 0.27^{a}
HFC	2.89 ± 0.08^{a}	36.5 ± 1.3^{b}	6.37 ± 0.21^{b}
HFG	2.95 ± 0.07^{a}	$32.8 \pm 1.1^{c,d}$	5.53 ± 0.25^{c}
HFZ	3.04 ± 0.07^{a}	$34.7 \pm 0.8^{b,d}$	6.31 ± 0.11^{b}
HFGK	3.07 ± 0.18^{a}	$31.5 \pm 1.1^{a,c}$	4.99 ± 0.27^{c}

Values are the mean ± SE of 6 (LFC, HFG, HFZ, HFGK) or 8 (HFC) mice and different superscripted letters in one column indicate significant differences ($p<0.05$). Adiposity is indicated by EFP ratio with body weight.

Table 3. Physiological and biochemical parameters increased (+) or decreased (−) by the diets HFG, HFZ and HFGK and also LFC, as compared with the HFC

Ref.	LFC	HFG	HFZ	HFGK	Identity
Bod	**−1.3**	**−1.1**	−1.1	**−1.2**	Body weight
Liv	**−1.5**	+1.1	+1.1	+1.1	Liver weight
EFP	**−2.1**	**−1.3**	−1.1	**−1.5**	Epididymal fat pad weight
RFP	**−1.8**	−1.1	1.0	**−1.4**	Retroperitoneal fat pad weight
BAT	**−1.6**	−1.3	−1.3	**−2.0**	Brown adipose tissue weight
Glu	−1.4	−1.1	1.0	−1.1	Plasma glucose
TG	+1.1	−1.1	**−1.2**	**−1.3**	Plasma triglycerides
TC	**−1.6**	**+1.1**	**+1.2**	**+1.2**	Plasma total cholesterol
HDL	**−1.4**	1.0	+1.2	**+1.2**	Plasma high density lipoprotein
Ins	**−1.8**	**−1.7**	**−1.9**	**−1.4**	Plasma insulin
Lep	**−3.6**	**−1.9**	**−1.6**	**−2.5**	Plasma leptin
LLip	**−2.1**	1.0	+1.2	1.0	Liver lipids
LTG	**−1.9**	1.0	+1.2	1.0	Liver triglycerides
LTC	**−1.4**	1.0	**+1.1**	1.0	Liver total cholesterol

Fold differences of parameter mean values compared with the means for the HFC group are shown and data in bold are significantly different ($p \leq 0.05$) from HFC.

3.2 Proteomics

Two-dimensional gel proteomics (Fig. 2) revealed 29 significantly affected protein spots out of a total of 585 verified spots. Of the 29 spots, 23 were considered to be credible candidates for identification, i.e. present as identifiable discreet spots with a sufficiently high staining density to allow sequencing. Significant changes in spot density due to different dietary treatments ranged from 1.2- to 3-fold, which is consistent with commonly observed protein level changes due to nutritional interventions [18, 19]. The identities of 19 of 23 proteins were successfully obtained by sequencing and are presented in Table 4. Compared with the HFC dietary group, the HFGK and HFZ diets significantly increased or decreased around half the identified hepatic proteins (10–12 spots), whereas 6-gingerol affected about one-third (7 spots). Ginger phytochemicals affected the hepatic levels of proteins relating to lipid and energy metabolism. 6-Gingerol decreased hepatic farnesyl diphosphate synthetase (FPPS), which is involved in cholesterol biosynthesis. Acetyl-coenzyme A acyltransferase 1 and enoyl CoA hydratase, which participate in the β-oxidation of fatty acids, were significantly ($p < 0.05$) increased by consumption of phytochemical-supplemented diets. Elongation factor 1-delta (EF1δ) and P-lysine P-histidine inorganic pyro-P phosphatase were consistently decreased by all the three ginger compounds.

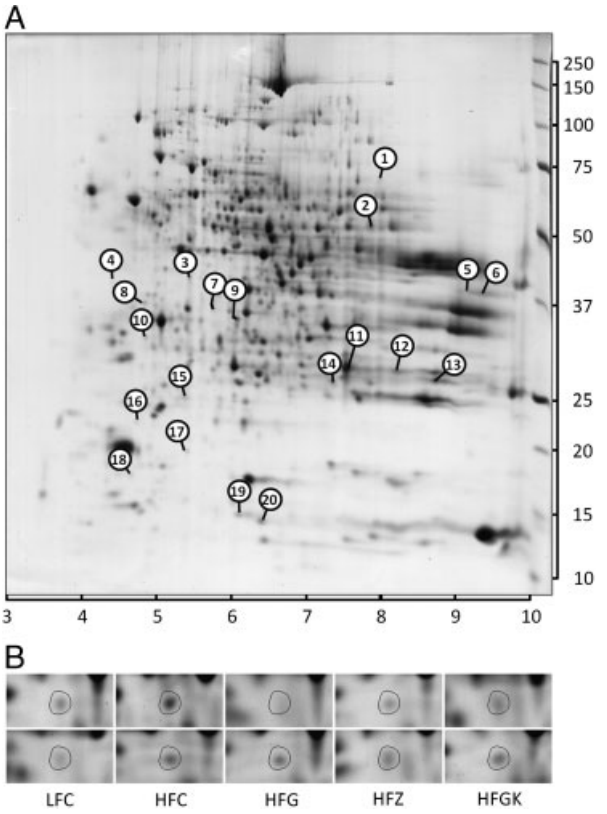

Figure 2. (A) Example 2D gel separation of soluble liver extract indicating the location of each identified protein that was significantly affected by ginger phytochemicals and/or dietary fat. The identities of each protein spot can be seen in Table 4. Molecular weight markers are shown with units in kDa on the vertical axis, and the pH gradient is shown on the horizontal axis. (B) Example spots for protein 10 (P-lysine P-histidine inorganic pyro-P phosphatase) from 2 of 6 or 8 replicate gels for each dietary treatment (LFC, low fat control; HFC, high fat control; HFG, high fat gingerol; HFZ, high fat zerumbone; HFGK, high fat goryangkang).

3.3 Principal component analysis

PCA of data for protein pots showing significant differences revealed separations relating to treatment (Fig. 3). There was a clear cluster separation between the high-fat dietary groups and the LFC group and there was also notable clustering of replicate animals for phytochemical treatments. There were marked similarities between the PCA plots for the spot data and the physiological parameters (Fig. 3) demonstrating that similar group differences were apparent for both the protein spot data and the physiological data. This indicates that diet-related physiological and biochemical differences, such a body fat content, which might be expected due to differences in dietary fat intake, are also reflected in the expression of hepatic proteins, giving further confidence in the validity of the proteomic data analysis.

In Fig. 3A (physiology), most variables contributed positively to the first component, with EFP weight having the highest loading, while the second component revealed a contrast between positive and negative contributions, the two most positive being insulin and brown adipose tissue weight and the two most negative being HDL cholesterol and TC.

In Fig. 3B (proteomics), several proteins contributed positively to the first component, including acetyl-coenzyme A acyltransferase 1 and fatty acid-binding protein (FABP), with FPPS contributing negatively with the highest loading. In the second component, contributors with the highest loadings included (positive) long-chain –fatty aci d CoA ligase (ACSBG2), NG,NG-dimethylarginine dimethylaminohydrolase 1, carbonic anhydrase 3, glutathione-*S*-transferase (GST) and (negative) gremlin 2 homolog, cytochrome *b*5 and EF1δ.

3.4 Correlation networks

The correlation networks used in this study are a visual way of demonstrating inter-relationships between proteins identified by proteomics, biomarkers (such as cholesterol and leptin) measured by independent methods and physiological characteristics such as body weight and body white fat depot size. Some relationships, such as between body white fat and leptin, might be expected from existing

Table 4. 2D gel protein spots increased (+) or decreased (−) by the diets HFG, HFZ and HFGK, and also LFC compared with the HFC diet

Spot	LFC	HFG	HFZ	HFGK	Identity	NA	MS	SC	UniProt ID	Functional context
1	+**1.8**	1.0	+**2.0**	+1.3	Long-chain fatty acid CoA ligase	ACSBG2	62	1% (1)	Q2XU92	Lipid metabolism
2	−**1.4**	−1.1	+1.1	1.0	Retinal dehydrogenase 1	–	221	17% (7)	AL1A1	Retinal-binding protein
3	+1.4	−**2.1**	−1.7	−1.4	Farnesyl diphosphate synthetase	FPPS	246	17% (5)	Q5M8R9	Cholesterol synthesis
4	−1.4	+1.2	−**1.5**	+**1.4**	Tropomyosin 1	TPM1	261	19% (5)	TMRTF1	Cytoskeleton stabilisation
5	−1.8	+1.3	+1.1	+**1.6**	Acetyl-coenzyme A acyltransferase 1	ACAA1	468	45% (10)	Q3TUU3	Lipid metabolism
6	−**1.5**	+1.1	−1.1	+**1.6**	Acetyl-coenzyme A acyltransferase 1	ACAA1	334	17% (8)	Q3TUU3	Lipid metabolism
7	−1.1	+1.1	−1.1	+**1.4**	NG,NG-dimethylarginine dimethylaminohydrolase 1	DDAH	375	45% (11)	DDAH1	NO generation
8	−**1.5**	−**1.7**	−**1.8**	−**1.4**	Elongation factor 1-δ	EF1δ	130	8% (2)	EF1D	tRNA delivery
9	−**1.6**	1.0	−1.1	+1.1	Unknown	–	93	29% (6)	BAC40160	–
10	−**1.5**	−**1.5**	−**1.3**	−**1.4**	P-Lysine P-histidine inorganic pyro-P phosphatase	LHPP	50	9% (2)	Q6P070	Oxidative phosphorylation
11	−1.1	+**1.2**	+1.1	+1.1	Carbonic anhydrase 3	CA3	200	50% (10)	CAH3	Hydration of CO_2
12	1.0	1.0	+**1.3**	+**1.2**	Enoyl CoA hydratase	Echs1	195	23% (5)	AAH02178	Lipid metabolism
13	−1.3	1.0	+**1.5**	−1.2	Glutathione-*S*-transferase	GST	165	38% (8)	GSTM1	Glutathione metabolism
14	−**1.4**	−1.1	1.0	+1.1	Triose-phosphate isomerase	TPI	84	8% (2)	ISMST	Glucose metabolism
15	+1.4	+1.1	−1.1	+**1.8**	Long-chain fatty acid CoA ligase	ACSBG2	63	1%(1)	Q2XU92	Lipid metabolism
16	−1.3	−**1.7**	−**1.7**	−1.3	Peroxiredoxin-2	–	132	14% (3)	PRDX2	Peroxide quenching
17	−1.1	−**1.4**	−**1.7**	−1.2	Gremlin 2 homolog	Grem2	Ab	8%(1)	Q3TST1	BMP2/4/7 inhibitor
18	−**1.7**	−1.4	−**1.6**	+1.1	Cytochrome *b*5	–	58	41% (4)	CYB5	Oxidative phosphorylation
19	−**1.4**	+1.1	1.0	+1.2	Fatty acid-binding protein, intestinal	I-FABP	88	32% (3)	FABPI	Lipid metabolism
20	1.0	1.0	1.0	+**1.4**	D-Dopachromedecarboxylase	DDT	135	57% (5)	DOPD	D-Dopachrome metabolism
21	1.0	−1.2	+1.1	−**2.3**	Unidentified	–	–	–	–	–
22	1.0	1.0	−1.2	−**2.3**	Unidentified	–	–	–	–	–
23	−**2.2**	−**2.4**	−**3.0**	−**1.8**	Unidentified	–	–	–	–	–

Fold -differences are shown along with protein spot identities, common nomenclature abbreviations (NA), probability-based Mowse scores (MS) and sequence coverage (SC), with number of non-overlapping peptides shown in parentheses. Data in bold are significantly different ($p \leq 0.05$) from HFC.

knowledge, but we were particularly interested in less well defined or unknown associations between proteins identified from proteomic data and factors which are related to body fat, cholesterol and energy metabolism. We have therefore correlated the pooled replicate data from all treatment and control groups for proteins and biomarkers that were significantly affected by the ginger phytochemical treatments, and also some physiological factors. Examples of the data used to calculate these correlations are shown in Fig. 4, and although the replicate values for each treatment or control are given different symbols, correlation coefficients were obtained from the pooled data sets.

Given the large number of correlations generated, we have presented them in a network (Fig. 5B) for better interpretation of all the inter-relationships together. The coloured circles represent the different proteins, biomarkers and physiological factors, and the lines joining them represent the correlations, with line thickness indicating the strength of the correlation and colour indicating whether it is positive (green) or negative (red). As is evident in Fig. 4A–D, the correlations were highly dependent on changes in the dietary fat or phytochemical content. These ''treatment effects'' appeared to be much stronger than any within-group correlation of replicate values. We therefore calculated residual values (replicate value minus the group mean) for all the replicates in order to remove treatment effects and look at the underlying correlation between the variables. The resulting residuals network is shown in Fig. 5A. Comparing Fig. 5A with B, confirms that treatment effects had a much stronger influence on correlations than replicate effects, because many more and stronger correlations can be seen in Fig. 5B as compared with Fig. 5A.

The treatments most influencing the strength of particular correlations when comparing Figs. 5A and 5B can be determined by reference to Tables 3 and 4. For example, a residuals plot of spot 3 (FPPS) and TC showed no strong correlation (Fig. 5A) but the same plot of the untransformed data (Fig. 5B) indicated a relatively strong treatment-related

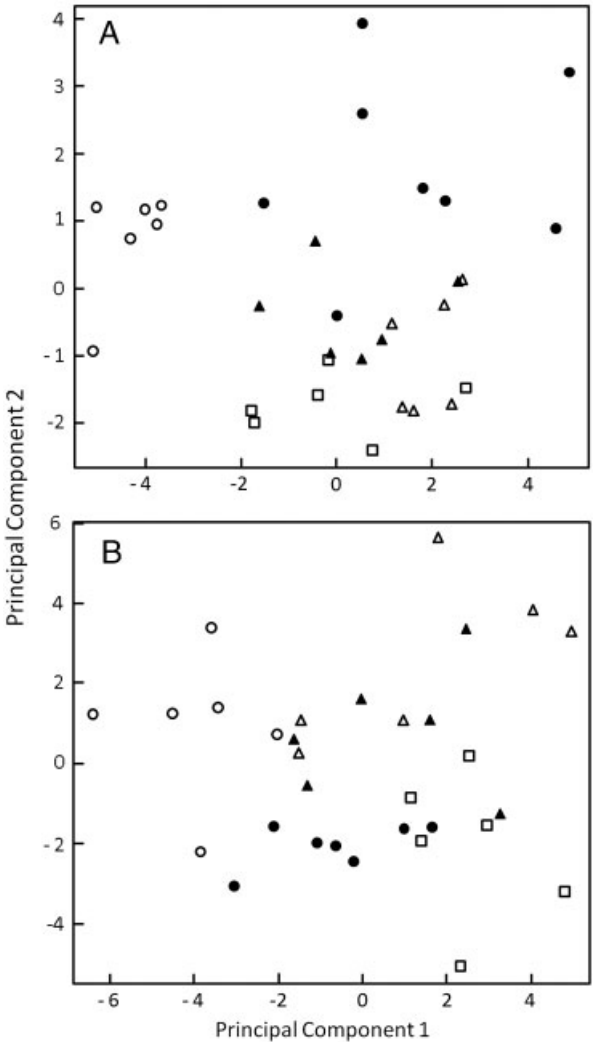

Figure 3. Scores plots for principal components analysis of physiological parameters (A) and spot densities (B) from mice consuming the low fat diet (open circles), the high fat diet (closed circles), the high fat diet with 6-gingerol (closed triangles), the high fat diet with zerumbone (open triangles) and the high fat diet with goryangkang extract (open square).

negative correlation, with the influencing treatment (mainly 6-gingerol) being identified from Table 3 (TC) and Table 4 (spot 3/FPPS, an enzyme involved in cholesterol biosynthesis).

4 Discussion

We have shown that phytochemicals from the rhizomes of ginger family plants significantly diminished obesogenesis in C57BL/6 mice on a high-fat, Western-type diet. Since food intake was unaffected by consumption of ginger extract or phytochemicals, energy expenditure may have been affected, although this would need to be confirmed by direct measurement. The thermogenic activity of gingerols and shogaols have been evaluated during perfusion of these phytochemicals into the hindlimbs of rats [24]. Oxygen consumption and lactate production both increased significantly and thermogenesis was found to be partly associated with vasoconstriction. However, rats given ginger-containing chow for 5 days showed no changes in body temperature or physical activity [25]. Likewise, ginger added to a meal of human volunteers did not increase the post-prandial metabolic rate of subjects as compared with the meal alone [26]. Intraperitoneal injection of 6-gingerol in rats caused a marked decrease in metabolic rate which resulted in reduced body temperature [25]. Gingerols have been shown to be agonists of the vanilloid receptors which are found in capsaicin-sensitive neurones [27] and the role of transient receptor potential vanilloid-1 channels in thermoregulation have been reviewed [28]. Another possible explanation for the anti-obesogenic effects of ginger phytochemicals is that they affect the efficiency of fat absorption. There is some evidence to support this idea since mice fed a high-fat diet containing an extract of *Z. officinale* Roscoe for 8 wks showed reduced pancreatic lipase activity and were also found to accrete significantly less parametrial adipose tissue [8]. It is possible that ginger phytochemicals influence both absorption and energy metabolism and we have focused on the latter in this study.

Our study design aimed to investigate the modifying effect of a longer term nutritional intervention with ginger phytochemicals on the obesogenic impact of a high-fat diet. We aimed to identify changes in protein expression and also protein associations using proteomics in order to identifying the key proteins affected by these phytochemicals. Liver was targeted because it is a primary site of lipid and steroid metabolism and the hepatic levels of ten proteins were significantly affected by dietary fat intake (Table 4). These included several proteins known to be influenced by elevated dietary fat intake, such as acetyl-coenzyme A acyltransferase and FABP. The proteomic data also showed that compared to the HFC, 16 proteins, of which 8 were related to cholesterol, lipid and energy metabolism, were significantly increased or decreased by consumption of one or more of the ginger compounds. In order to search for biologically meaningful treatment effects, we prepared correlation networks of physiological/biochemical parameter values and protein spot density data. The residuals plot of Fig. 5A shows treatment-independent correlations between parameters and proteins. Some expected correlations were observed, such as between EFP weight and plasma leptin level, which has been well documented in the literature [29]. The correlation network of the untransformed data, which included ginger phytochemical treatment effects (Fig. 5B), clearly shows a large increase in the number and strength of protein and parameter correlations compared with the residuals network. This indicates a strong influence of treatment on the relationship between proteins and parameters, which was confirmed by reference

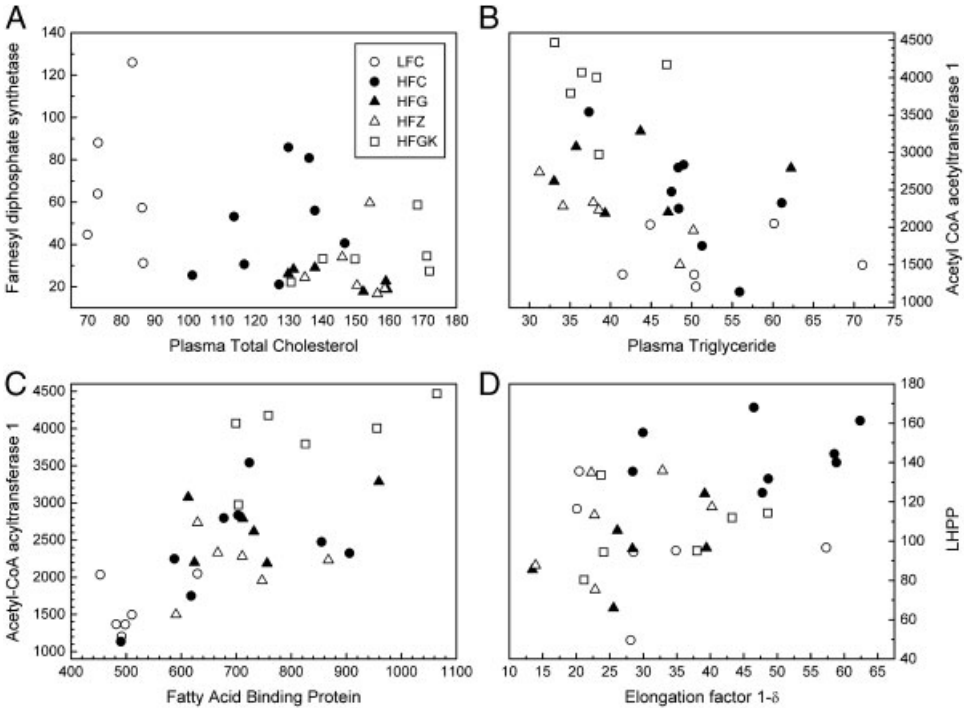

Figure 4. Example scatter plots of hepatic protein and other physiological parameters showing changes relating to addition of ginger phytochemicals to a high fat diet, as compared to a high fat control (HFC) diet alone. The phytochemicals used were 6-gingerol (HFG), zerumbone (HFZ) and goryangkang (HFGK), and a low fat dietary control (LFC) was also included. Spot densities are in arbitrary units and cholesterol and triglyceride values are in mg/dL. In order to calculate correlation coefficients for each of the scatter plots A–D and all of the correlations in Fig. 5A and B, replicate values for all treatments were pooled in a single dataset.

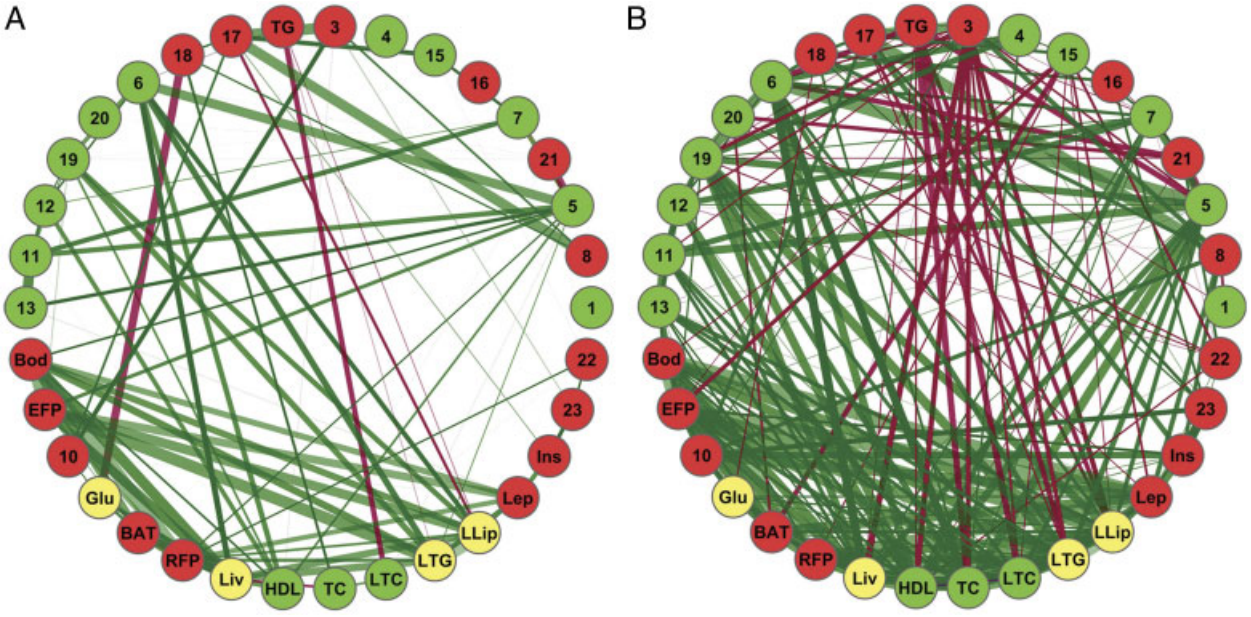

Figure 5. Correlation network of residuals (A) and the untransformed data (B) for 2D gel hepatic cytosol protein spots (numbered circles) and other biomarker/physiological factors (lettered circles) in C57BL/6 mice given low fat diets or high fat diets with or without addition of ginger phytochemicals. Green lines indicate positive correlations whereas red lines are negative correlations. Only correlations with an R value of $>=0.3$ or $<=-0.3$ are illustrated and the wider the lines, the stronger the correlation. Red circles and green circles are proteins or factors showing a significant decrease or increase ($p<0.05$), respectively, for one or more phytochemical treatment groups (HFG, HFZ and HFGK) compared to the high fat control group (HFC). Yellow circles are factors showing no significant phytochemical-related effect. Factor and protein identities are shown in Tables 3 and 4. The residuals network (A) excludes effects of treatment and shows only underlying correlations between the various proteins and factors (e.g. leptin (Lep) and epididymal fat pad weight (EFP) are positively correlated). The untransformed data network (B) includes the effect of dietary treatments, which in this study strongly promote existing and new correlations.

to particular correlations, examples of which are shown in Fig. 4. Of special interest were the multiple associations between particular proteins, notably FPPS (Protein 3), enoyl CoA hydratase, FABP (Protein 19) and acetyl-coenzyme A acyltransferase 1 (ACAA1, Proteins 5 and 6), and lipid and steroid parameters, including HDL, plasma TC, liver TC, liver TGs and liver lipids.

FPPS is involved in the formation of farnesyl diphosphate from isopentyl diphosphate [30] and is an upstream enzyme in the biosynthesis of cholesterol [31]. Gene expression of FPPS and other cholesterogenesis genes are regulated by sterol regulatory element-binding proteins (SREBPs) [32] and the activation of SREBP is inhibited by the presence of sterols, such as cholesterol. In conditions of high cholesterol therefore, it would be expected that FPPS expression will be suppressed, and negative correlations (red lines) between FPPS and plasma TC, LDL cholesterol and liver TC are apparent in Fig. 5B. Comparing Fig. 5A and B, it is clear that this correlation was greatly strengthened by one or more of the ginger phytochemical treatments, and reference to the correlation plot between FPPS and plasma TC in Fig. 4A shows that 6-gingerol had the greatest impact. However, as can also be seen in Fig. 4A, both zerumbone and goryangkang influenced the correlation since replicate animals tended to show higher plasma cholesterol and lower hepatic FPPS than HFC mice.

Fibrates are activators of the key transcription factor PPARα, which regulates fatty acid oxidation, increase HDL cholesterol and reduce plasma TGs [33]. Fibrate influences on cholesterol metabolism are thought to be mediated by PPARα-stimulated expression of FPPS through modulation of SREBP activation [31]. Expression of the ACAA1 gene (alternatively known as peroxisomal 3-ketoacyl CoA thiolase 1), is also directly regulated by PPARα and performs the fourth and final step in the peroxisomal β-oxidation of fatty acids [34]. The expression of ACAA1 is consequently raised when animals are fed a high-fat diet as part of a co-ordinated homeostatic response to help regulate adiposity through hepatic fatty acid β-oxidation. Another participant enzyme in the β-oxidation of fatty acids is enoyl CoA hydratase, whose expression is regulated by PPARα and which was also significantly raised in the present study in response to zerumbone and goryangkang consumption. Activation of PPARα induces β-oxidation of fatty acids, raises plasma HDL cholesterol and lowers plasma TGs [35], and a positive and negative relationship, respectively, have been noted with ACAA1 in the present study (Fig. 5B). PPARα agonists increase liver weight and decrease liver lipid in mice [36] but this was not observed in response to feeding any ginger phytochemical. However, 6-gingerol was found to significantly increase both hepatic PPARα gene and protein expression in mice on a high-fat diet (T. Y. Ha and J. Y. Ahn, Personal Communication) suggesting that the target for this phytochemical is upstream of PPARα activation. FABP transports fatty acids to their PPARα activation site and a positive correlation between FABP and ACAA1 is expected and has been found in the present study (Fig. 4C). However, the form of FABP that we have identified is the intestinal form, and is not known to be expressed in liver [37]. The identification of this protein originating from perfused liver was dependent on the sequence determination of three peptides covering 32% of the protein and the sequencing was repeated twice from two different gels. MASCOT analysis of the MSDB database and NCBI BLAST analysis of the NCBInr database yielded no likely alternative proteins and the liver form of FABP has a quite distinct sequence. There is little chance of misidentification and we therefore conclude that intestinal FABP has a hepatic role in lipid metabolism.

Consistent with the working hypothesis that ginger phytochemicals are promoting the β-oxidation of fatty acids, the ATP-dependent ACSBG2, which is another enzyme involved in fatty acid β-oxidation, was identified as two separate spots on 2D gels, and the levels of both proteins were significantly increased by consumption of HFZ and HFGK diets. ACSBG2 is reportedly expressed mainly in testis and brain stem [38], but we have found detectable levels in liver. The identification of the protein was dependent on a single peptide (FLCMLLTLK) and the more widely tissue distributed ACSBG1 protein has a single amino acid discrepancy in this sequence compared with ACSBG2 (ACSBG1 peptide sequence: FLSMLLTLK). Nevertheless, peptide sequencing of tryptic digests from both spots gave the sequence for ACSBG2, and NCBI BLAST or MASCOT analyses of the NCBInr and MSDB databases, respectively, revealed no likely alternative proteins other than ACSBG1.

The peroxisomal β-oxidation of fatty acids generates hydrogen peroxide and so raised levels of catalase and other antioxidants might be expected. Peroxiredoxin 2 was indeed significantly affected by the HFG and HFZ diets but was decreased compared with the HFC diet group. Hepatic GST was however raised in response to the HFZ diet. GSTs play an important role in the regulation of the intracellular concentrations of lipid peroxidation products [39] and raised GST may be a reflection of the cellular response to increased peroxisomal β-oxidation of lipids. Further investigation of the hepatic antioxidant response profile and redox indicators may give further insight into redox regulation following ginger phytochemical consumption.

Of the significant proteins identified, there were only two that responded significantly to all the three ginger phytochemicals, namely elongation factor 1δ (EF1δ or EF1Bα in revised nomenclature) and phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP), which showed a significant positive correlation when related to each other (Fig. 4D). In both cases, the high-fat diet alone (HFC) significantly increased the levels of these proteins in comparison to the LFC group. Incorporation of the ginger phytochemicals into the high-fat diet reduced the levels of both proteins back to those in the LFC rat liver. This was also found for plasma insulin and plasma leptin (not shown), and all these four proteins were significantly positively correlated to each other (Fig. 5B). EF1 is responsible

for the enzymatic delivery of aminoacyl tRNAs to ribosomes and the δ (Bα) subunit functions as a guanine nucleotide exchange factor [40]. LHPP catalyses the highly exergonic hydrolysis of the phosphoanhydride bond in pyrophosphate into two phosphates, and provides a thermodynamic driving force for biosynthetic reactions. It controls the level of inorganic pyrophosphate produced by biosynthesis of protein and is an essential housekeeping enzyme for cellular energy metabolism [41]. Insulin stimulates protein synthesis and so a correspondence between plasma insulin and hepatic EF1δ is likely. Insulin also regulates leptin gene expression in adipose tissue with leptin protein subsequently being secreted into circulation. Phytochemical-induced changes in the levels of these proteins tended to reflect body adiposity, and so may represent an effect rather than a cause of changes in energy metabolism.

As regards proteomic data analysis, we conclude that construction of residuals correlation networks in comparison with untransformed data networks is a very useful way of investigating treatment effects on protein expression. The addition of residuals analysis using a correlation network approach yields a wealth of information about biomarker and protein interdependence and the influence of a treatment on these relationships. Treatment-driven correlations between proteins from 2D gels and physiological/biochemical parameters, such as FPPS and plasma TC, give a clear indication of how certain identifiable treatments, in this case mainly 6-gingerol, are targeting particular pathways. While we have highlighted the major targets of relevance to ginger phytochemicals, this technique has much wider potential application for probing complex responses to dietary or other treatments.

We conclude that of the three phytochemical treatments, goryangkang and 6-gingerol had the greatest anti-obesogenic effect and goryangkang most influenced liver protein expression. Proteins targeted were predominantly related to cholesterol biosynthesis, fatty acid oxidation and energy metabolism with some, such as EF1δ and P-lysine P-histidine inorganic pyro-P phosphatase appearing to reflect changes in adiposity rather than promoting them. The finding that several proteins were related to the β-oxidation of fatty acids and that 6-gingerol consumption raises PPARα gene and protein expression suggests that at least this ginger phytochemical my reduce adiposity by promoting the catabolism of lipids. Lipoprotein metabolism in mice is distinctly different from that in humans and so further work is required to determine whether ginger phytochemicals target cholesterol and fatty acid metabolism in human subjects. This study identifies some key protein targets and biomarkers to make that assessment. Assuming a similar anti-obesogenic effect of fresh ginger consumption in humans as observed in this mouse study, the daily human dose of fresh ginger would need to be around 25 g/kg diet, which is high, and might be better consumed as a supplement for individuals not accustomed to eating ginger.

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