Original Article

A proteomic analysis of excreted and circulating proteins from obese patients following two different weight-loss strategies

Assim A Alfadda[1,2], Abdullah Arif Turjoman[1,3,4], Amr S Moustafa[1,3,5], Mohammed Y Al-Naami[6], Muhammad A Chishti[1,3], Reem M Sallam[1,3,5], David Gibson[4,7] and Mark W Duncan[1,4]

[1]Obesity Research Center, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia; [2]Department of Medicine, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia; [3]Clinical Chemistry Unit, Department of Pathology, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia; [4]Division of Endocrinology, Diabetes & Metabolism, Department of Medicine, School of Medicine, Anschutz Medical Campus, University of Colorado Denver, Aurora, CO 80045, USA; [5]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Ain Shams University, Cairo, Egypt; [6]Department of Surgery, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia; [7]Arthritis Research Group, Queen's University of Belfast, Centre for Infection and Immunity, Health Sciences Building, Belfast BT9 7BL, Northern Ireland
Corresponding author: Assim A Alfadda. Email: aalfadda@ksu.edu.sa

Abstract

Bariatric surgery is the most successful therapeutic approach to weight loss, but how it leads to weight loss, and how it resolves obesity-related complications, including type-2 diabetes, are poorly understood. This study, comprising two groups of individuals, one on a low-calorie diet ($n = 5$) and one undergoing bariatric surgery ($n = 7$), used both targeted and untargeted proteomic approaches to determine changes in protein levels pre- and post-intervention (i.e. 3–6 months later). Changes were observed in both circulating and excreted proteins following weight loss. Targeted multiplexed biochip arrays measured 12 plasma peptides/proteins involved in metabolism and inflammation: C-peptide, ferritin, interleukin-6, interleukin-1 alpha, resistin, insulin, tumor necrosis factor alpha, leptin, plasminogen-activator inhibitor-1, adiponectin, cystatin C, and C-reactive protein. Following a low-calorie diet, plasma insulin and C-reactive protein levels were significantly reduced ($P = 0.045$ and $P = 0.030$, respectively); adiponectin increased and leptin decreased following surgery ($P = 0.014$ and $P = 0.005$, respectively). Untargeted proteomic analysis employing 2D difference in-gel electrophoresis (DIGE) showed 28 protein spots with $\geq$1.5-fold changes in expression following weight loss by a low-calorie diet; comparison of pre- and post-intervention urine samples from the bariatric surgery group showed changes in excretion of 110 protein spots.

The combination of targeted protein analysis by multiplexed arrays and an exploratory (i.e. an unbiased or discovery) proteomic assessment of hundreds of proteins offers valuable insights into the mechanistic differences between alternative weight-loss strategies. This is a powerful hypothesis-generating approach to study complex, multifactorial syndromes such as obesity. The findings that arise from these studies can then be validated in targeted, hypothesis-directed investigations.

Keywords: Obesity, proteomics, multiplex biochip array, bariatric surgery, low-calorie diet

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Introduction

Obesity is a complex, multifactorial syndrome resulting from a combination of genetic, environmental, psychological, social, and cultural factors.[1] Obesity adds billions to health care costs, decreases the quality of life, and is associated with a variety of metabolic diseases and co-morbidities.[2]

There are currently three major approaches to treat obesity, but none is optimal. Lifestyle modification involves decreased calorie intake and/or increased energy expenditure. Although this is the preferred approach, it does not lead to a satisfactory long-term result in most individuals. As an alternative, considerable effort has been directed at developing pharmacologic approaches, but these currently

achieve only modest body weight reduction.[3] The third and most successful of these options is bariatric surgery, but this is a drastic and invasive procedure that is impractical on a wide scale and has significant risk. Nevertheless, on a positive note, bariatric surgery very successfully reduces both body weight and obesity-associated co-morbidities, including type-2 diabetes, but the underlying mechanisms driving these positive changes are poorly understood.[4,5]

Given the scope of the obesity problem and the ineffectiveness and/or impracticality of existing treatment options, elucidating the underlying molecular events associated with successful weight loss is important. In particular, it would be beneficial to understand any unique molecular changes associated with bariatric surgery.

In this study, we introduce and apply a novel hypothesis-generating proteomic strategy: i.e. the simultaneous application of both targeted and untargeted protein analysis. The approach is a powerful first-step for advancing our understanding of complex, multifactorial disorders such as obesity because it delivers a wealth of high-quality data, even from a relatively small sample set, that can then be verified through hypothesis-driven follow-up studies. This strategy does not aim to provide definitive answers – that would be prohibitively expensive and time-consuming because very large sample numbers would be required to offset chance findings. Nevertheless, the approach highlights associations/relationships worthy of further investigation, some of which have not been considered previously. Further, the data generated can be stored in a widely accessible database and used by others to develop or support their own hypotheses, theories, or models relating to the complex biological processes underlying obesity and weight loss.

The aim of this study was to use the approach outlined above to identify and compare differentially abundant proteins following two weight loss interventions: i.e. bariatric surgery and a low-calorie diet. Because obesity is a state of chronic low-grade inflammation, we targeted a panel of 12 circulating adipocytokines and inflammatory cytokines for precise quantification by biochip analysis. In addition, an untargeted proteomic approach was used to explore unanticipated changes in urinary protein excretion.

The overarching hypothesis guiding this work is that the molecular changes associated with both interventions – a low calorie diet vs. bariatric surgery – are the same in nature and are only exaggerated when the process of weight loss is accelerated by the surgical approach. We therefore propose that any differences in protein expression and/or clearance observed between these two groups post-intervention, whether qualitative or quantitative, can offer insights into the biochemical events associated with bariatric surgery.

Subjects and methods

Twelve subjects who were classified as obese (i.e. body mass index (BMI) of 30 kg/m^2 or above) were included in this study. Subjects were recruited at the Obesity Research Center, College of Medicine, King Saud University, Riyadh. Each subject gave their written informed consent and the project was approved by the local Ethics Committee. Recruited subjects were obese but otherwise healthy (Table 1). Five of the subjects (all men, average BMI = 35.6 kg/m^2, range 30–50 kg/m^2) elected to participate in a dietary control program as an approach to weight reduction; the other seven elected to undergo bariatric surgery (4 men and 3 women, average BMI = 45.4 kg/m^2, BMI range 39.5–55.5 kg/m^2).

Table 1 Clinical and demographic characteristics of the study subjects

Intervention	Diet	Surgery
Number	5	7
Age (years)	31 ± 12	36 ± 6
Men/women (n)	5/0	4/3
BMI (pre)	36 ± 8	45 ± 6
BMI (post)	31 ± 5	35 ± 5
Duration between pre and postsamples (months)	3 ± 0	4 ± 1
% Wt loss	11 ± 9	22 ± 7
% loss of excess body Wt following Intervention	34 ± 27	42 ± 13

Wt = weight; BMI = body mass index (kg/m^2). Values are mean ± standard deviation, or the number of subjects.

Subjects in the dietary control group were given a folder listing different combinations of meals and snacks that collectively would provide 1500 kcal/day. They then used their food lists to self-select three meals and two snacks a day during the three-month duration of the study. Follow-up telephone calls were made once every two weeks to ensure compliance. Subjects were also advised at the first visit to maintain a healthy lifestyle in general, but they did not participate in any organized physical activity programs. Of the bariatric surgery group, four subjects received a laparoscopic sleeve gastrectomy and three subjects received a laparoscopic gastric bypass (Roux-en-Y). All subjects achieved significant weight loss (defined as a reduction of more than 4% of total body mass over the 3–6 months duration of the study). In addition to the obese subjects, seven healthy, lean, and age- and gender-matched subjects were included as a control group for the targeted biochip array analysis.

Sample preparation and protein extraction

For every patient, a midstream spot urine sample (50–100 mL) and a fasting blood (10 mL into K_3EDTA-vacutainer tubes) were collected on enrollment and at follow-up, 3–6 months later. Blood samples were immediately centrifuged (3300 *g*, 15 min, 4°C), the plasma separated, aliquoted, and stored at –80°C. Proteins were isolated from the urine samples by precipitation with methanol and chloroform (i.e. 4 parts of methanol and 1 part of chloroform to 1 part sample) as previously described.[6] Total protein determinations were performed by the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Targeted biochip array analysis of plasma analytes

An Investigator® metabolic biochip array (Randox Laboratories Ltd., Crumlin, UK) was used to simultaneously quantify multiple analytes from a single patient plasma sample.[7] Levels of C-peptide (CPEP), ferritin (FERR), interleukin-6 (IL-6), interleukin-1 alpha (IL-1α), resistin (RETN), insulin (INS), tumor necrosis factor alpha (TNFα), leptin (LEPT), plasminogen-activator inhibitor-1 (PAI-1), adiponectin (ADIP), cystatin C (CYSTC), and C-reactive protein (CRP) were measured by two biochips: i.e. solid substrates with multiple specific antibodies attached at predefined sites on the surface. With this approach, each protein is captured by a specific antibody, paired enzyme-labeled secondary antibodies are added, and the target proteins are quantified by chemiluminescence using a charge-coupled device camera and imaging system.

Plasma was thawed immediately before analysis and each sample (100 µL) was applied directly to individual reaction wells in the biochip. The concentration of each analyte was calculated by reference to its calibration curve. Quality control procedures-required predefined acceptance criteria were met for each assay to guarantee accuracy, sensitivity, and precision. For Randox metabolic arrays, inter-assay and intra-assay precision CVs are typically 5–10%. Each biochip contains internal control sites with set target levels used to determine if a particular assay fails to perform within the normal detection range. Calibrators and multi-analyte controls were also used per assay. Biochip array data were analyzed using GraphPad Prism v5.03 (GraphPad Software Inc., La Jolla, CA, USA). Independent *t* tests were used to ascertain the statistically significant inter-group differences in protein levels between the enrollment and follow-up time points; paired *t* tests were used to compare intra-group changes in protein levels between time points.

DIGE of the urine proteome

First-dimension isoelectric focusing (IEF) and second-dimension SDS-PAGE of urine proteins were carried out as previously described.[8] Briefly, 50 µg of urine protein was 'minimally labeled' with 200 pmol of CyDye (Cy2, Cy3 or Cy5; GE Healthcare, Piscataway, NJ, USA) for analytical gels. For preparative gels, an additional 750 µg of unlabeled protein was added to give a total of 800 µg. The entire sample was diluted with IEF rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, pH 8.3), and the sample was applied to a 24-cm IPG strip (pH 3–10, nonlinear, Bio-Rad) for passive rehydration (24 h). A stepwise protocol was used for the first-dimension IEF. Second-dimension SDS-PAGE was performed at 24°C and at 20 Watts/gel (Ettan DALT 12 vertical system, GE Healthcare, Piscataway, NJ, USA) on precast polyacrylamide gels prepared on low-fluorescence glass (5–20% Sigma-Aldrich, St. Louis, MO, USA). Voltage and current were continuously monitored throughout all runs for quality control purposes. Fluorescent images were acquired on a Typhoon 9400 Variable Mode Laser Imager (GE Healthcare, Piscataway, NJ, USA).

Protein identification by mass spectrometry

For the purposes of protein identification, preparative 2D gels were visualized with Lava Purple (Fluorotechnics, Collingswood, NJ, USA), major protein spots were excised, and the proteins identified by MALDI-TOF mass spectrometry (MS) as previously described.[8,9] Briefly, an Ettan Spot Handling Workstation (GE Healthcare) was used for spot excision and in-gel trypsin enzymatic digestion. The peptide solution (0.5 µL) derived from each digested protein spot was spotted on a MALDI plate together with the matrix solution (0.5 µL). Dried spots were then analyzed by MALDI-TOF MS (Voyager DE-STR, Applied Biosystems). Mass calibration with Bruker peptide mass calibration standards was performed prior to every batch of MALDI-TOF analyses. Spectra were collected over the range *m/z* 500–5000. Peptide mass fingerprints were internally calibrated to monoisotopic trypsin peaks. Spectra were processed using ProTS Data (Efeckta Technologies, Steamboat Springs, CO, USA) to generate a peak list which was then submitted to Mascot (Matrix Science Ltd., London, UK) for database searching. Database searches were conducted using the mammal subset of the non-redundant SwissProt protein database.

Not all proteins of interest could be identified. This is because some spots could not be excised from the gels successfully, some proteins were of low abundance and did not yield sufficiently intense mass fingerprints, and some spots were mixtures of multiple proteins.

Image, cluster, and pathway analysis

Gel image analysis was performed with Progenesis SameSpots software version 3.3 build 3420.25059 (Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK). The software incorporated gel warping, DIGE normalization, and comparison modules. All gel images were aligned to a reference gel and the SameSpots outlines were overlaid onto them to ensure no data were omitted. The software calculated the normalized volume (NV) of each spot on each gel from the Cy3 (or Cy5) to Cy2 spot volume ratio. The software performs log transformation of the spot volumes to generate normally distributed data. Log normalized volume (LNV) was used to quantify differential expression.

Urine samples were collected from all subjects pre- and post-intervention and were analyzed by DIGE gels. One gel was used for each patient. Each gel included total protein from the pre-intervention sample (stained with either Cy3 or Cy5), total protein from the pre-intervention sample (stained with the alternative dye, either Cy3 or Cy5), and an aliquot of a pool of all urine samples that served as the internal standard (stained with Cy2). To facilitate comparison, four average (i.e. master) gels were created with the software: one for all pre-diet samples ($n = 5$), one for all post-diet samples ($n = 5$), one for all pre-surgery samples ($n = 7$), and one for all post-surgery samples ($n = 7$).

Quantitative differential spot analysis was performed on the 'master' gels for each intervention. Pairwise comparisons were made to establish patient-to-patient and Cy3-to-Cy5 dye variations. Within the SameSpots review module, each comparison was filtered to find the spots: (a) with a difference between the Cy3-to-Cy5 signals

with a $P \leq 0.05$ (paired t test) and (b) having greater than a 1.5-fold change in average LNV expression between the two groups. Spot filtering on the 'master' gels was used to remove dust particles and missed spots.

Expression data were analyzed using Epclust, a generic data clustering, visualization, and analysis tool (http://www.bioinf.ebc.ee/EP/EP/EPCLUST/). Hierarchical analysis reordered protein expression patterns in an agglomerative fashion, using the unweighted pair group method with an arithmetic mean (UPGMA) clustering procedure. Pearson's ranked correlation was used as the similarity measure to group or separate the expression data. A heat map was produced together with a dendrogram illustrating the extent of similarity between the different proteins within the samples.

The quantitative data on the identified proteins was imported into Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA). This software identifies biological relationships between proteins as networks and allows the user to determine which functional pathways are involved. By overlaying the experimental expression data on networks constructed from published interactions it is possible to generate a more complete view of the pathways affected by the study interventions.

Results

Weight differences and changes

The average BMI of the bariatric surgery subjects was higher than that of the diet group at enrollment, but this difference was not significant (Table 1). All subjects achieved a weight loss of 4% or more over the duration of the study (Table 1). As expected, bariatric surgery resulted in pronounced weight loss in all subjects ($n = 7$, average 22% weight loss); weight loss in the low-calorie diet group over 3 months was less ($n = 5$, average 11% weight loss).

Plasma levels of targeted proteins and their changes

Biochip array analysis revealed no significant differences in cytokine levels between the diet and surgery groups at enrollment. Statistically significant differences in pre- and post-intervention levels are shown in Figure 1 and Supplementary Table 1.

Changes 3 months after a low-calorie diet. Following a low-calorie diet, both plasma INS and CRP levels were reduced and these differences reached statistical significance (i.e. $P = 0.045$ and $P = 0.030$, respectively).

Changes 4 ± 1 months after bariatric surgery. Adiponectin increased following surgery ($P = 0.014$) and there was also a statistically significant decrease in LEPT ($P = 0.005$).

Changes in urinary protein excretion across groups

Changes 3 months after a low-calorie diet. For the five (male) obese subjects who elected to participate in the program of weight reduction through a low-calorie diet,

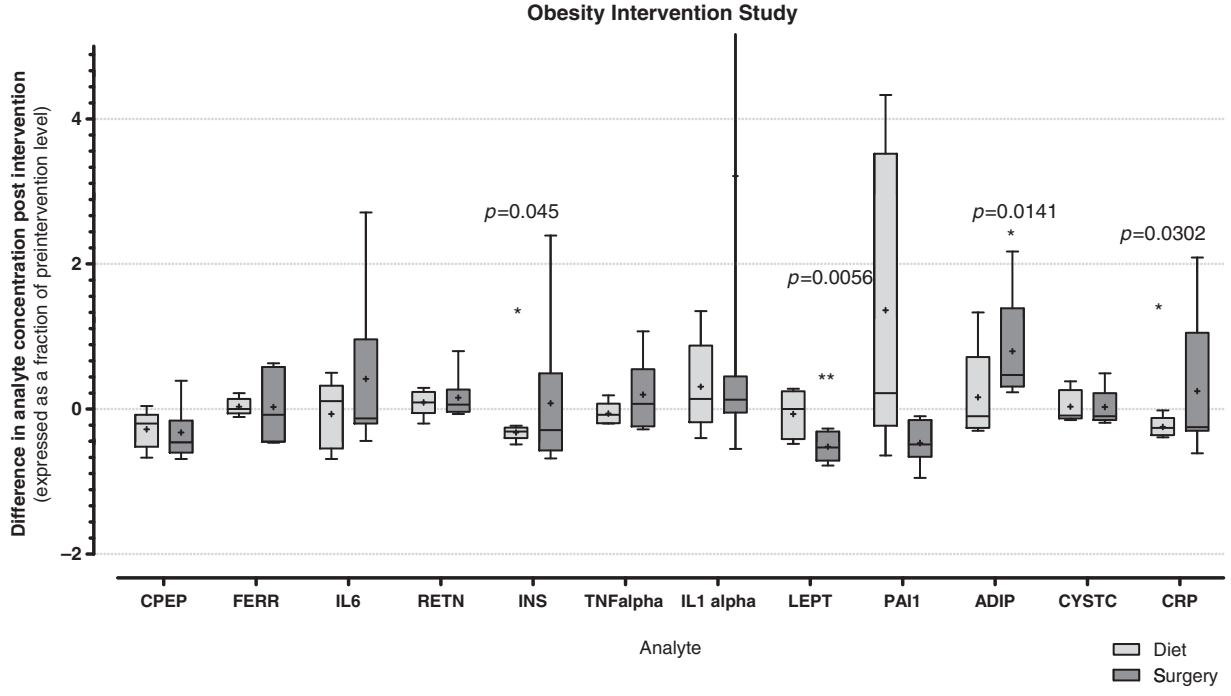

Figure 1 Differences in plasma levels of obesity-related proteins determined by biochip array. Plasma levels of C-peptide (CPEP), ferritin (FERR), interleukin-6 (IL-6), interleukin-1 alpha (IL-1α), resistin (RETN), insulin (INS), tumor necrosis factor alpha (TNFα), leptin (LEPT), plasminogen-activator inhibitor-1 (PAI-1), adiponectin (ADIP), cystatin C (CYSTC), and C-reactive protein (CRP) were measured by biochip. Fold difference values were calculated by dividing the difference between pre- and post-intervention levels by the pre-intervention level, on a patient by patient basis. Positive values therefore represent an increase after intervention, whereas negative values represent a decrease. Dietary intervention patient values are represented in light grey, surgery intervention patients in dark grey. The middle bar represents the median value, the outer box limits are the 25th and 75th percentile; mean is marked with '+'. Significant differences in plasma protein levels after intervention are highlighted with an asterisk: $^{*}P < 0.05$; $^{**}P < 0.005$

912 protein spots were consistently evident in their urine. After spot filtering, 86 protein spots (circled in Figure 2a) showed a 1.5-fold difference in NV between the pre- and post-intervention samples. Twenty-eight of these spots were identified following excision, digestion, MALDI-TOF analysis, and mass fingerprinting.

Changes 4 ± 1 months after bariatric surgery. For the seven obese subjects who elected to have bariatric surgery as an approach to weight reduction, 972 protein spots were consistently expressed in their urine. After spot filtering, 294 proteins (circled in Figure 2B) showed a 1.5-fold difference between the pre- and post-intervention samples and 110 of these were identified.

The protein identity (ID), predicted isoelectric point (pI), predicted MW, Mascot score, sequence coverage, and matched peptides for each of the differentially abundant proteins we identified are included in Supplementary Table 2. The inter-individual variations among the differentially abundant proteins pre- and post-weight loss intervention are illustrated in the heat map (Figure 3). The data were reordered by hierarchical cluster analysis (HCA) using Pearson's centered correlation and UPGMA as a linkage measure. This approach reveals distinguishing expression patterns. A list of the proteins with the most statistically significant/interesting changes was compiled (Table 2) and was then used to construct molecular pathways (Figure 4a–c). Gene ontology analysis was used to illustrate the impact of each intervention on canonical pathways including those associated with inflammation and metabolism of lipids, proteins, and carbohydrates (Figure 5).

Discussion

Proteins are more closely linked to physiological events than the genes themselves and changes in protein levels reflect dynamic events that can pinpoint underlying mechanisms. However, examining a specific pathway or a few proteins can only reveal a fraction of the complex picture, and such a study is biased from its inception towards what is examined and what the investigator believes to be important. Unexpected changes/associations remain undiscovered, even if they are biologically relevant and highly statistically significant.

Proteomic methods can offer an attractive solution to this dilemma. Because approaches such as DIGE do not employ antibodies or aptamers directed towards specific target analytes, it is possible to 'discover' qualitative and quantitative information on hundreds or perhaps thousands of (untargeted) proteins. These methods therefore have the potential to offer unanticipated insights in complex biological processes. Despite their widespread adoption, there are, however, several limitations of multivariate proteomic strategies. First, detection limits are typically suboptimal, at least by the standards of immunoassays. Consequently, (very) low abundance proteins are frequently not detected. Second, shortcuts in the analytical process compromise analytical precision and therefore subtle differences in protein levels between samples cannot be detected. Third, and perhaps most importantly, the application of these strategies rarely, if ever, provides definitive information because of the high dimensionality of the data-set, the influence of chance, biological heterogeneity, and the practical constraints on study size and sample numbers.

To circumvent some of these problems we have combined a discovery (untargeted) proteomics strategy – in this case DIGE – with targeted assays for a set of proteins that might otherwise be missed but are anticipated to be central to the processes under investigation. We have applied this approach to an investigation of the biochemical changes associated with weight loss. Although this strategy overcomes some of the limitations of a conventional proteomic study, it can only *direct* the researcher to potentially fruitful areas for follow-up by hypothesis-driven study; it does not provide definitive answers in a single step.

In the sections that follow we discuss how we have applied this strategy and we highlight some of the key findings and future directions that follow from this work.

Measured changes in plasma levels of the targeted proteins

As anticipated, several of the targeted proteins were significantly altered following weight loss, both in the low-calorie diet and bariatric surgery groups. Numerous studies report changes in circulating adipocytokines and inflammatory cytokines levels following weight loss, either by diet or surgical intervention. Notably, Rao[10] recently collated and reviewed reports on changes associated with some of the same markers we measured following bariatric surgery. He concluded that changes are not consistent across studies but that bariatric surgery typically produces about 66% and 27% reductions in CRP and IL-6 levels, respectively; changes in TNFα do not approach statistical significance. Typically, reports indicate that weight loss improves obesity-associated inflammation as measured by both inflammatory markers (e.g. CRP, TNFα, IL-6, and leptin) and anti-inflammatory markers (e.g. adiponectin).[11] For example, Dalmas and colleagues[12] examined a range of adipocytokines and inflammatory cytokines at various time intervals following Roux-en-Y gastric bypass. They report a significant increase in adiponectin and a decrease in leptin, IL-6 and CRP 3 months following surgery.

In our study, we saw a significant reduction in CRP levels following a low-calorie diet ($P = 0.030$), and although there was a reduction in CRP of a similar magnitude post-bariatric surgery, here the change did not reach statistical significance, in large part because of the scatter in individual CRP levels within the group. In contrast to much of the published work, we saw no reduction in IL-6 levels in either the low-calorie diet or bariatric surgery groups following intervention. Consistent with the reports of Dalmas *et al.*[12] and Miller *et al.*,[13] we observed a significant increase in adiponectin ($P = 0.014$) following surgery; no increase was evident following weight loss associated with a low-calorie diet. This is consistent with other reports that indicate that there is no change in levels following moderate weight loss.[14] We also observed a highly significant reduction in leptin following bariatric surgery ($P = 0.005$), consistent with other

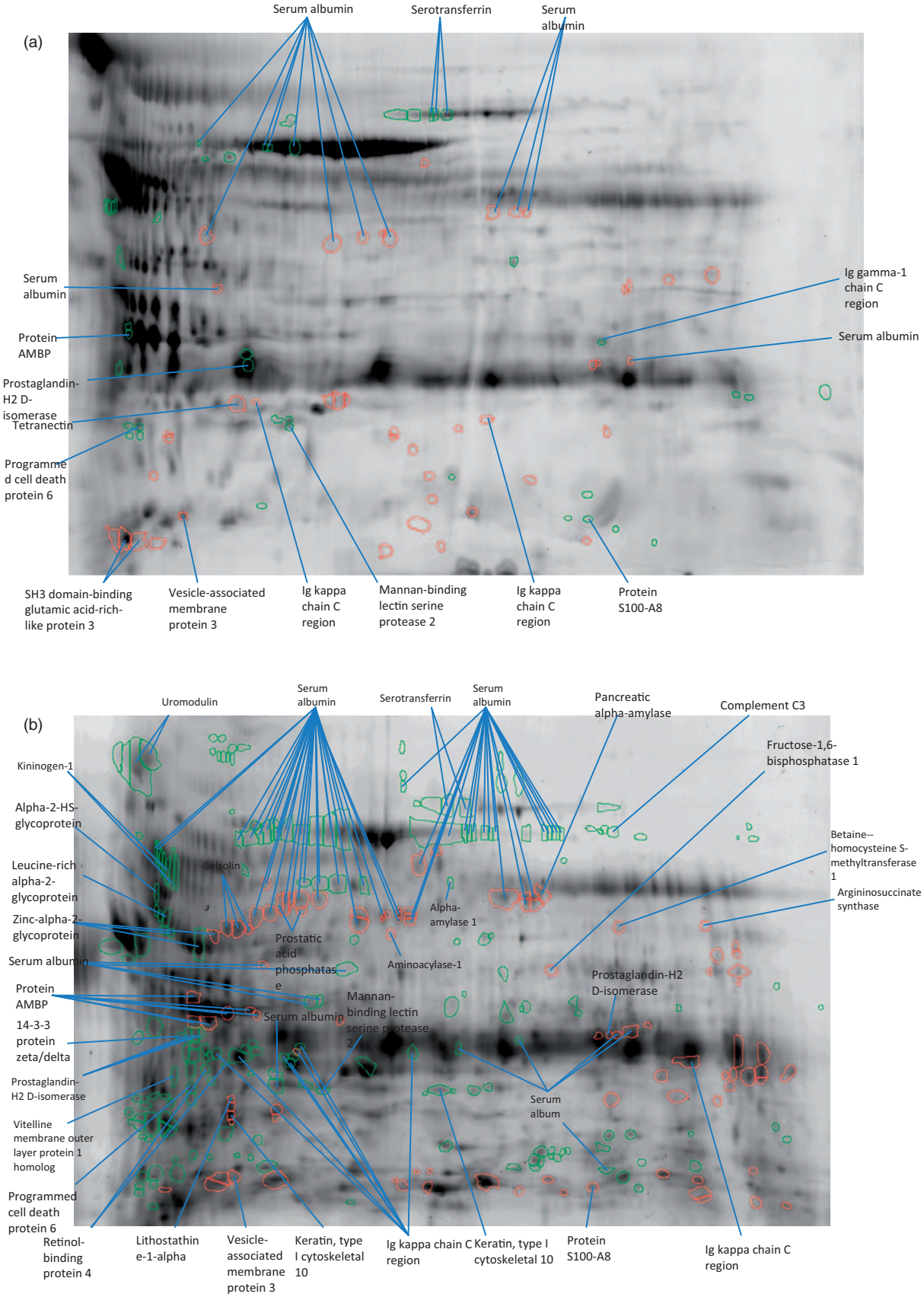

Figure 2 Identification of differentially-expressed proteins from pre- and post-intervention urine samples by DIGE. (a) Diet gel; (b) Surgery gel. Protein spots are highlighted according to a 1.5-fold difference pre- and post-intervention. Spots that were identified and either increased (red) or decreased (green) relative to the 'master' gel normalized volume are shown. (A color version of this figure is available in the online journal.)

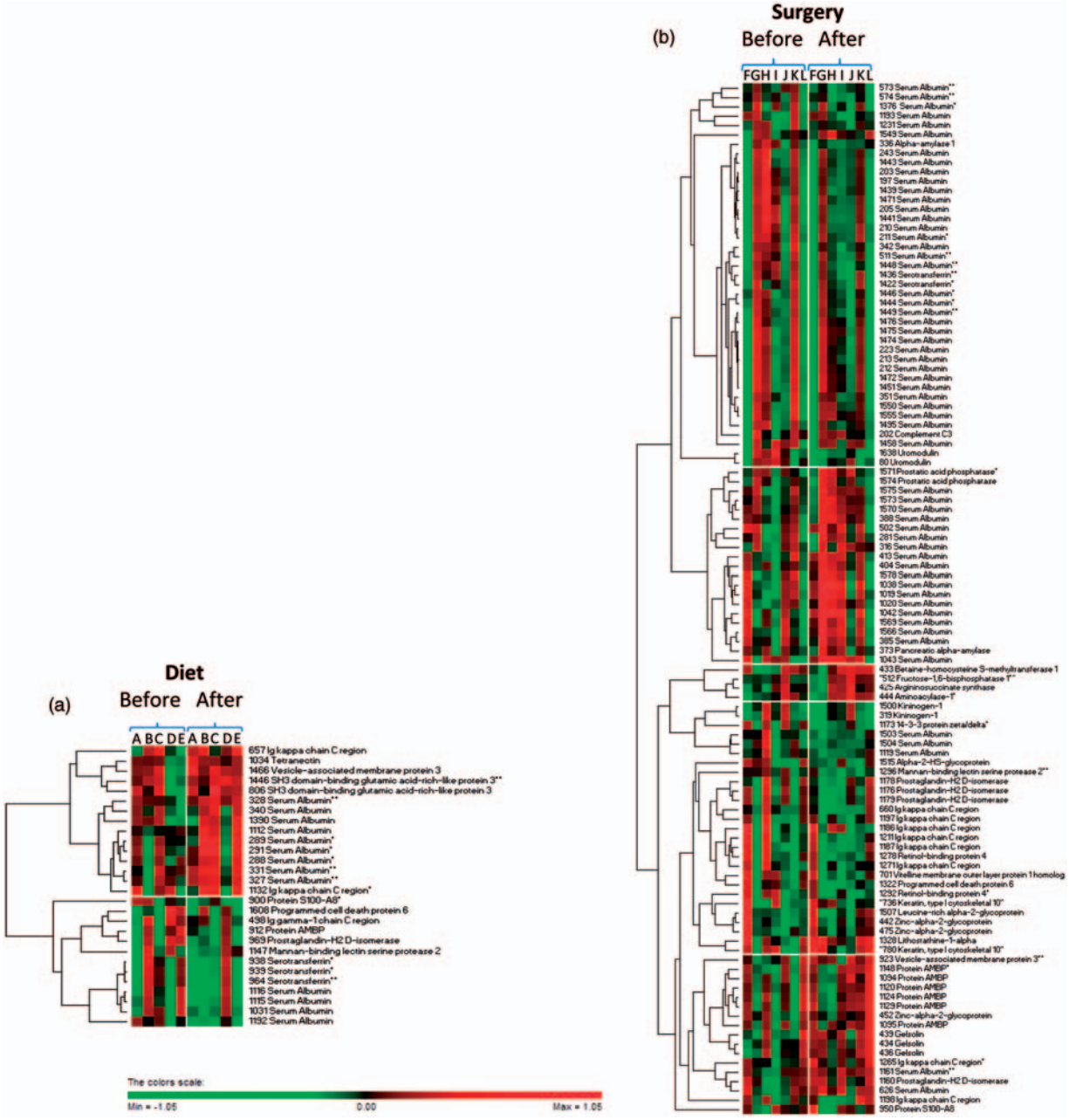

Figure 3 Hierarchical cluster analysis of differentially-expressed proteins with a 1.5-fold or greater from urine samples pre- and post-weight loss intervention. (a) The intra-individual variations among the identified proteins with a 1.5-fold or more change pre- and post-diet and (b) pre- and post-surgery. Data are represented in heat map form and are reordered by hierarchical cluster analysis (HCA) using Pearson's centered correlation by the unweighted pair group method with arithmetic mean (UPGMA). The figures reveal distinguishing expression patterns. Note that the vertical white line divides the heat map into the pre- (left) and post-intervention (right) data. Two (a) and five (b) main clusters of proteins are distinguishable. Each subject is represented by a separate labeled column. Proteins with two asterisks had significant differences ($P < 0.05$) pre- and post-intervention based on a paired *t*-test; those with single asterisk had a *P* value between 0.05 and 0.1. The locations of these spots on the comparative heat map are shown in Figure 2(a and b)

reports.[15,16] Of note, we saw no reduction in plasma leptin following diet-induced weight loss and this observation is worthy of follow-up because it is a clear difference in the results observed for the two weight-loss strategies. (See Figure 1 that indicates no shift in mean leptin values post–diet and acceptable scatter within the group results.) Finally, consistent with other reports,[17] we saw a significant reduction in insulin following weight loss associated with a low-calorie diet ($P = 0.045$). A reduction of similar magnitude was associated with weight loss following bariatric surgery, but this was not statistically significant (see Figure 1 and the very large scatter in individual values). Most other studies report a 50% reduction in plasma insulin levels following bariatric surgery,[5] but Whitson *et al.* observed a modest, but not-significant, *increase* in plasma insulin at 6 months following bariatric surgery in both diabetic and non-diabetic subjects.[18]

Table 2 A list of the most statistically significant/interesting differentially-expressed proteins from urine samples following surgery or a low-calorie diet*

			Diet		Surgery	
Spot ID	Symbol	Entrez gene name	Fold change	*P* value	Fold change	*P* value
327;1448	ALB	albumin	**1.841**	**0.01**	**−1.981**	**0.02**
912;1148	AMBP	alpha-1-microglobulin/bikunin precursor	−1.516	0.23	1.685	0.09
1147;1571	IGKC	immunoglobulin kappa constant	1.822	0.08	1.818	0.09
1608;1296	MASP2	mannan-binding lectin serine peptidase 2	−1.536	0.26	**−1.635**	**0.03**
969;512	PDCD6	programmed cell death 6	−1.863	0.27	−2.085	0.10
900;1160	PTGDS	prostaglandin D2 synthase 21kDa (brain)	−1.698	0.18	1.533	0.14
1446;950	S100A8	S100 calcium binding protein A8	−1.909	0.08	1.613	0.48
964;1436	TF	transferrin	**–1.535**	**0.04**	**–1.621**	**0.03**
1466;923	VAMP3	vesicle-associated membrane protein 3 (cellubrevin)	1.854	0.21	**1.672**	**0.02**
498	CLEC3B	C-type lectin domain family 3, member B	1.573	0.176	–	–
1132	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	−1.524	0.24	–	–
1108	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	**1.625**	**0.0323**	–	–
1265	ACPP	acid phosphatase, prostate	–	–	2.088	0.0912
444	ACY1	aminoacylase 1	–	–	1.69	0.0667
1515	AHSG	alpha-2-HS-glycoprotein	–	–	−1.561	0.204
1638	AMY1A	i-amylase, alpha 1A (salivary)	–	–	−1.911	0.162
373	AMY2A	amylase, alpha2 (pancreatic)	–	–	1.531	0.437
425	ASS1	argininosuccinate synthase 1	–	–	1.654	0.139
1507	ATXN2L	ataxin 2-like	–	–	−1.625	0.459
452	AZGP1	alpha-2-glycoprotein1, zinc-binding	–	–	1.52	0.38
433	BHMT	betaine—homocysteine S-methyltransferase	–	–	1.61	0.134
1322	FBP1	fructose-1,6-bisphosphatase 1	–	–	1.511	0.099
434	GSN	gelsolin	–	–	1.76	0.103
1500	KNG1	kininogen 1	–	–	−1.599	0.147
780	KRT10	keratin 10	–	–	2.24	0.211
1278	RBP4	retinol binding protein 4, plasma	–	–	−2.121	0.219
1328	REG1A	regenerating islet-derived 1 alpha	–	–	1.754	0.268
336	UMOD	uromodulin	–	–	−4.415	0.162
701	VMO1	vitelline membrane outer layer 1 homolog (chicken)	–	–	−1.82	0.189
1173	YWHAZ	tyrosine 3/5-monooxygen activation protein, zeta polypeptide	–	–	−2.318	0.0579

*Protein Entrez Gene symbols, identity, and expression data are used to construct molecular pathways to illustrate the impact of study interventions. In the instance of isoforms of a protein identified from multiple gel spots (e.g. albumin, ALB), the DIGE expression data for the protein isoform with the most statistically significant spot ID was used. The upper part of the table details proteins differentially expressed after both interventions; the middle part of the table refers to diet intervention spots; the lower part refers to surgery intervention spots. Bold values indicate statistically significant results with $p < 0.05$

These results demonstrate that even a small-scale exploratory study of this nature can be used to generate testable hypotheses regarding the role of specific circulating proteins in complex process. Of special significance in our study are the statistically significant changes seen in the post-bariatric surgery group but not the low-calorie group. These proteins indicate the role of both inflammatory (e.g. leptin) and anti-inflammatory (e.g. adiponectin) proteins in weight control mediated by bariatric surgery. Our study also highlights the need for additional studies to clarify some of the less definitive associations.

Changes in urinary proteins between pre- and post-intervention

We used urine rather than plasma for the discovery studies because it offers some unique advantages. Urine is formed in the kidney by ultrafiltration of the plasma and although total protein excretion in normal urine is low – less than 150 mg/day – the sample can be collected non-invasively and is relatively free from the high molecular mass proteins that complicate proteomic studies of blood. Therefore, there is no need to use complex, costly, and potentially confounding depletion strategies to remove the abundant high mass plasma proteins.[19]

The DIGE study provides substantial fodder for hypothesis-driven follow-up. A few of our findings on differentially abundant urinary proteins are discussed below to illustrate the potential of this strategy.

1. *Albumin and its variant forms in urine*: Multiple variant forms of albumin were observed on the 2D gels, some of which change significantly following weight reduction (Figure 2a and b). Albumin isoforms – i.e. multiple variant forms differing both in size and isoelectric point – have also been reported following

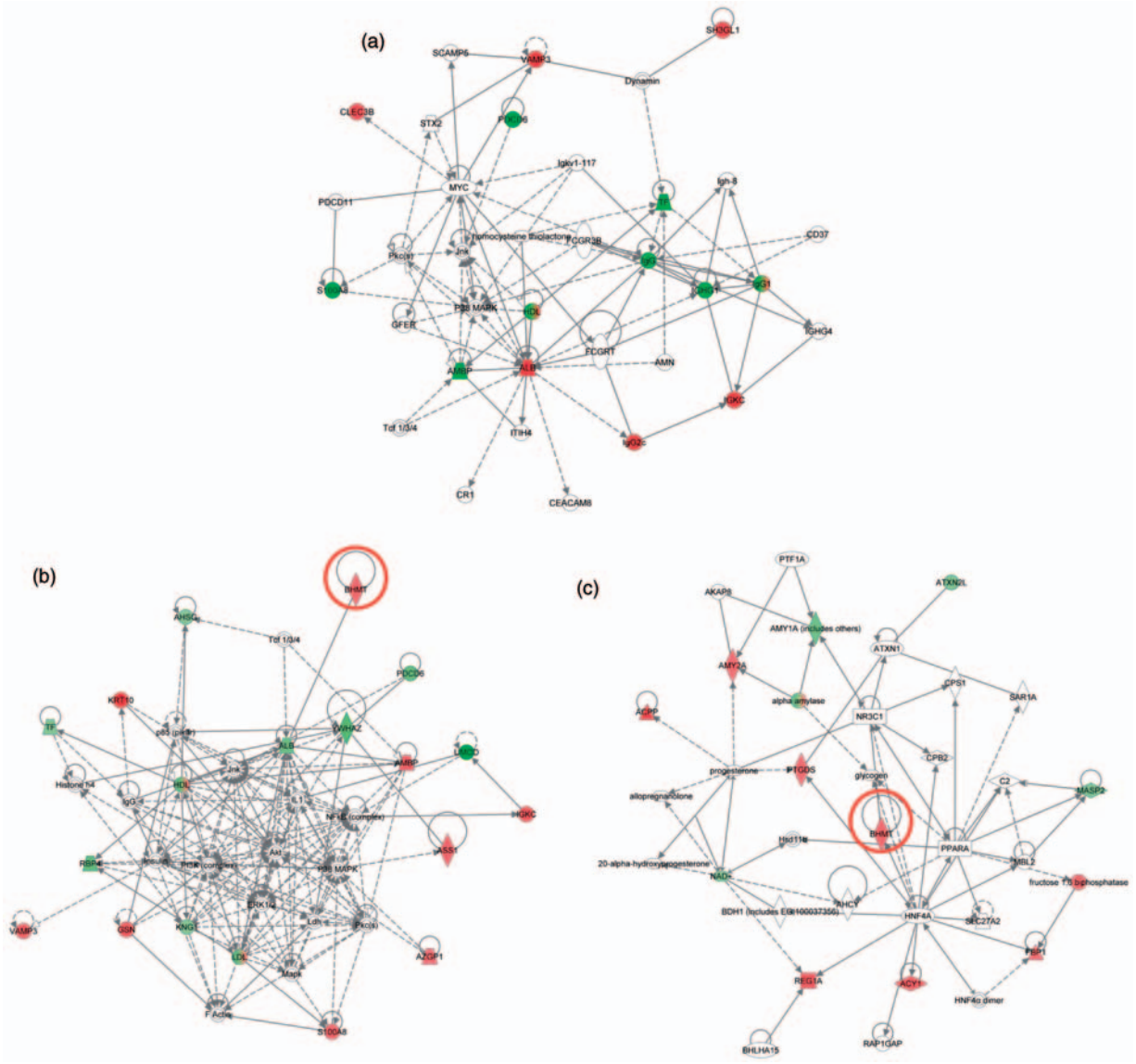

Figure 4 Ingenuity pathway analysis of protein expression after (a) diet and (b, c) surgical interventions (from data in Table 2). Proteins identified and quantified from post-intervention urine samples are shaded with respect to the difference in expression post-intervention; green is reduced expression and red is increased; grey molecules were added during the process of pathway construction. Molecules are identified by the Entrez Gene symbol (also listed in Table 2). Direct molecular interactions are signified by solid lines; indirect relationships involving a series of intermediary molecules are represented by dashed lines. (b, c) Betaine-homocysteine S-methyltransferase (BHMT), encircled in red, links two overlapping networks identified and differentially expressed post–surgery. (A color version of this figure is available in the online journal.)

bariatric surgery[20,21] and positively correlate with improved insulin sensitivity.[22] In addition, an association between albuminuria and urine adiponectin has been reported and is consistent with our findings.[22] The increased diversity in albumin isoforms evident following bariatric surgery is an intriguing observation that warrants further study.

Multiple forms of albumin have been identified in serum by various proteomic approaches and these have been critically reviewed by Hortin and Sviridov[23] and more recently, by Bruschi *et al*.[24] A partial list of albumin multiforms includes glycated forms, fatty acid-bound forms, oxidative sulfinic or sulfonic forms, and proteolytic fragments ranging in size from 10 to 50 kDa. These various forms can arise because of non-covalent binding of different ligands that can influence albumin's molecular mass, isoelectric properties, hydrophobicity, and/or conformation. Some degradation during sample storage and work-up could also contribute to the complexity of the urinary albumin profile.

2. *Transferrin*: Transferrin is a negative acute phase protein/reactant that might be expected to increase following weight loss. We found, however, a significant decrease in transferrin after both a low-calorie diet and bariatric surgery (Table 2). Consistent with our findings, Santos *et al*.[25] recently reported a statistically significant reduction in transferrin associated with

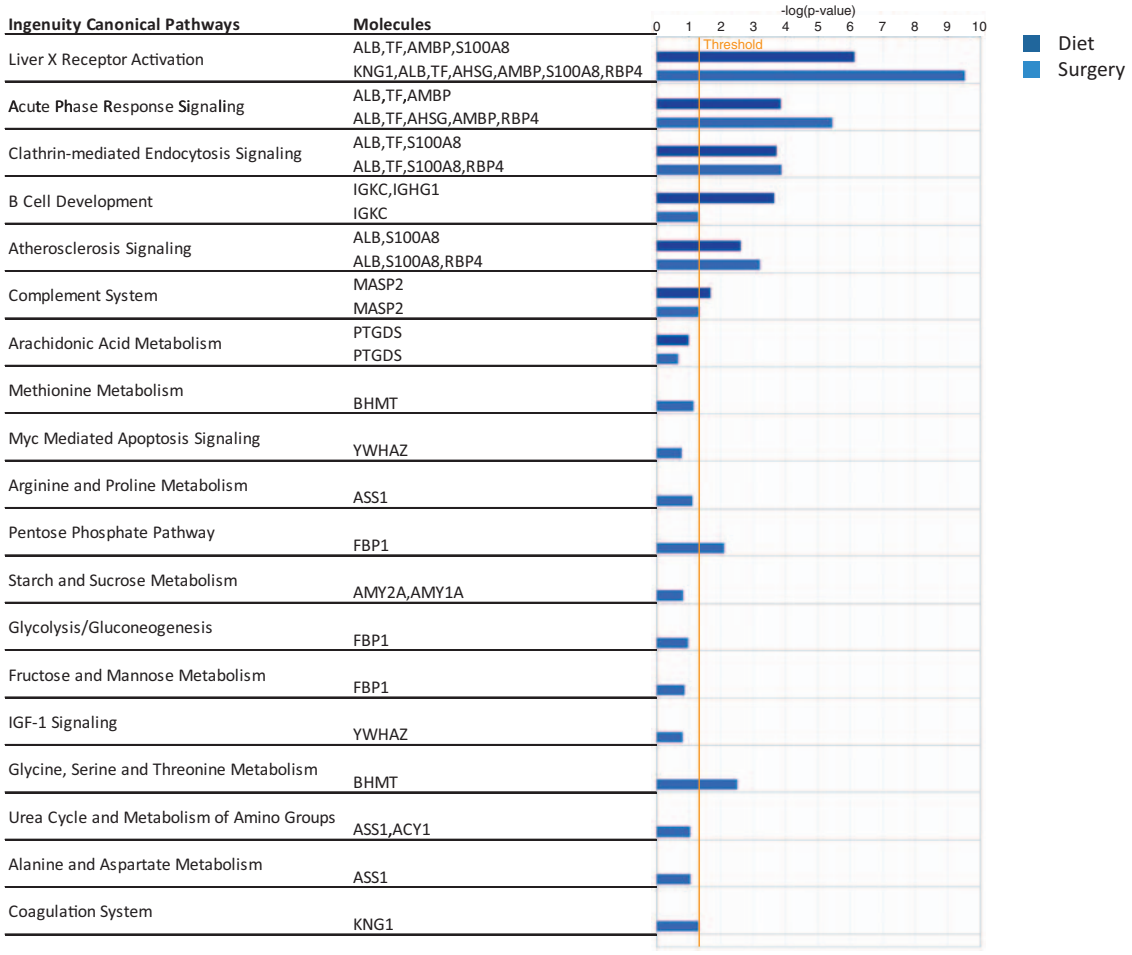

Figure 5 A summary of the most significant canonical pathways impacted by the detected differences in protein expression of post-intervention urine samples. The x-axis represents the –log of *P* values calculated by Fischer's exact test (right tailed); a threshold of $P = 0.05$ is represented by the orange vertical line. This gives a measure of the possible impact that each intervention has on each of the listed pathways. The light blue bars represent surgery data whereas the dark blue bars represent diet data. The molecules implicated from each intervention are also listed as Entrez Gene symbols (as used in Figures 4(a–c) and Table 2). (A color version of this figure is available in the online journal.)

weight loss following gastric banding surgery. They attribute this decline to changes in iron status and systemic hepcidin, although the complex inter-relationships between components of the iron transport system remain unclear.[26] Damms-Machado and colleagues also report a reduction in transferrin with weight loss following laparoscopic sleeve gastrectomy, but in this instance the change did not reach statistical significance.[27]

Belza *et al*.[28] reported a decrease in serum transferrin after a low-energy diet and they observed a tendency for levels to return to baseline after a subsequent maintenance period.[28] Additionally, a recent 12-months follow-up report on the effect of gastric banding on plasma proteins, including transferrin, showed a slight decrease in levels after surgery.[27]

3. *Mannan-binding lectin associated serine protease-2 (MASP-2)*: Our finding of a significant decrease in the excreted MASP-2 levels in patients following surgical intervention ($P < 0.05$) is also of interest. Obesity is considered a state of chronic inflammation that is ameliorated, at least in part, by the loss of excess body weight. In accordance with our finding, Frauenknecht and his coworkers[29] found that MASP-2 was associated with cardiovascular risk factors including obesity, dyslipidemia, and hypertension. Human MASP-2 is an essential component of the mannan-binding lectin (MBL)-dependent complement pathway[30,31] and the observed changes may represent a link between inflammation and thrombosis – two pathologic events that are closely associated with obesity and its complications. A more detailed investigation of MASP-2 and its role in obesity and weight loss is certainly warranted.
4. *SH3 domain-binding glutamic acid-rich-like protein 3 and weight loss*: SH3 domain-binding glutamic acid-rich-like protein 3 is a low-molecular-weight plasma protein (1.44 kDa) recently discovered by Greening and Simpson.[32] We observed a significant increase in excretion of SH3 domain-binding glutamic acid-rich-like protein 3 following a low-calorie diet ($P < 0.05$), but there was no change after surgical intervention

(<1.5-fold). This difference may have special significance because this is one of the few instances where a significant change was observed in the relatively small diet group, but not the surgical group. This observation may provide important insights into mechanistic differences between these two weight loss approaches.

5. *Vesicle-associated membrane protein 3 (VAMP-3) or cellubrevin*: We found a significant increase in excreted VAMP-3 levels following bariatric surgery ($P < 0.05$) and a non-significant decrease following a low-calorie diet. VAMP-3 is one of several proteins involved in GLUT4 translocation.[33] Recently, Schwenk *et al*.[34] speculated that over-expression of VAMP-3 – the scenario we observed in the bariatric surgery group – could help to protect insulin-stimulated GLUT4 translocation under conditions of insulin resistance (e.g. after acute exercise). They showed that VAMP-3, but not VAMP-2, is able to protect insulin-stimulated GLUT4 translocation during the early stages of diet-induced insulin resistance and that it preserves normal CD36 distribution. Others have shown that the simultaneous disruption of VAMPs 2, 3, and 8 completely inhibits insulin-stimulated GLUT4 insertion into the plasma membrane. These finding have been used to support the argument that VAMPs are essential for GLUT4 trafficking.[35]

Pathway analysis

Pathway analysis adds yet another perspective to these studies. Here, a pattern of differential protein expression influencing lipid metabolism and inflammation, including complements and immunoglobulins, was evident following both interventions; however, the most pronounced changes were within the postsurgery group (Table 2; Figure 5). Surgery induced profound changes in molecules influencing both carbohydrate and protein metabolism. Recent reports indicate that the metabolic alterations after gastric bypass occur by mechanisms other than those associated with weight loss by calorie control, and this may account for the improved glucose homeostasis observed following surgical intervention.[36,37]

Our findings identify specific proteins that likely play a key role in the mechanisms underlying weight loss following bariatric surgery. Changes in proteins involved with carbohydrate and protein metabolism are a clear focus for further studies.

Summary and limitations

We have used multiplex array assays, discovery approaches based on 2-D DIGE and pathway analysis to probe the molecular events associated with weight loss by two different strategies. We kept sample numbers to a practical minimum and did not attempt to generate definitive answers in a single study. In this way we were able to carry out a cost- and time-efficient study that provided a wealth of information for follow-up by hypothesis-driven investigation. These follow-up studies should incorporate an increase in the sample number and apply selective and precise analytical approaches capable of detecting small changes.

There was no control or oversight of the low-calorie diet group and this closely simulates the real-life situation. However, it does introduce uncertainties that can influence the results. In addition, our studies were of limited duration and longer follow-up, including serial samplings from each patient, would be beneficial.

Conclusions

We combined an untargeted hypothesis-generating approach and targeted analysis of several key proteins to identify changes following two different interventions. Studies of this type can be a practical and cost-effective approach to: (1) understanding the unique mechanistic events that underpin a complex biological process such as weight loss, (2) identifying individuals at risk of complications, and/or (3) developing novel approaches to manage weight loss.

Authors' contributions AAA designed the study and supervised the data collection and analysis, and co-wrote the manuscript. ASM, RMS, and MAC participated in experimental design, data collection, and analysis and manuscript preparation. AAT and DG performed experiments and participated in data analysis and manuscript preparation. MYA facilitated bariatric patient recruitment. MWD supervised experiments and participated in data analysis and manuscript preparation.

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GLOSSARY

MASCOT SCORE

The probabilistic scoring algorithm used to rank Mascot peptide mass fingerprint search results is based on both mass accuracy and sequence coverage. These data enable the algorithm to be used to judge whether a result is significant or not. The score is the probability that the observed match is, or is not, a random event. Scores greater than 67 are significant ($P < 0.05$).

SEQUENCE COVERAGE

The sequence coverage is the percentage of the database protein sequence covered by matching peptides. The larger the

sequence coverage, the greater the confidence in a protein identification based on the experimental data.

MATCHED PEPTIDES

The masses of tryptic peptides derived from a single spot and measured experimentally by MALDI-TOF are compared to the theoretical masses of peptides generated by *in silico digestion of* proteins stored in a database(s). Mascot compares the masses of the peptides (or peak lists) of the unknown protein to the theoretical peptide masses and the results are statistically analyzed to find the best match.

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