Mass spectrometric detection of candidate protein biomarkers of cancer cachexia in human urine

RICHARD J.E. SKIPWORTH[1], GRANT D. STEWART[1], MISHAIM BHANA[1], JENNIFER CHRISTIE[2], CATHARINE M. STURGEON[3], DENIS C. GUTTRIDGE[4], ANDREW D. CRONSHAW[2], KENNETH C.H. FEARON[1] and JAMES A. ROSS[1]

[1]Tissue Injury and Repair Group, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB; [2]Institute of Structural and Molecular Biology (ISMB), Michael Swann Building, King's Buildings, University of Edinburgh, Mayfield Road, Edinburgh EH19 3JR; [3]Department of Clinical Biochemistry, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA, UK; [4]Human Cancer Genetics Program, Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, 554A Tzagournis Medical Research Facility, 420 W 12th Ave, Columbus, OH 43210, USA

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Abstract. Increased membrane permeability and myofibrillar protein breakdown are established features of cancer cachexia. Proteins released from cachectic muscle may be excreted in urine to act as biomarkers of the cachectic process. One-dimensional SDS polyacrylamide gel electrophoresis followed by matrix-assisted laser desorption/ionisation or liquid chromatography tandem mass spectrometry was used to compare the protein content of urine from cachectic (>10% weight loss) (n=8) and weight-stable (n=8) gastro-oesophageal cancer patients and healthy controls (n=8). Plasma creatine kinase concentration was used as a marker of gross muscle breakdown. The number of protein species identified in cachectic samples (median 42; range 28-61; total 199) was greater than that identified in weight-stable cancer (median 15; range 9-28; total 79) and control samples (median 12.5; range 5-18; total 49) ($P<0.001$). Many of the proteins identified have not been reported previously in the urine of cancer patients. Proteins identified specifically in cachectic samples included muscle (myosin species), cytoskeletal (α-spectrin; nischarin) and microtubule-associated proteins (microtubule-actin crosslinking factor; microtubule-associated protein-1B; bullous pemphigoid antigen 1), whereas immunoglobulin κ-light chain and zinc α-2 glycoprotein appeared to represent markers of cancer. The presence of myosin in urine (without an increase in plasma creatine kinase) is consistent with a specific loss of myosin as part of the cachectic process. Urinary proteomics using mass spectrometry can identify muscle-specific and non-muscle-specific candidate biomarkers of cancer cachexia.

Correspondence to: Professor James Ross, Tissue Injury and Repair Group, 1st Floor Chancellor's Building, University of Edinburgh, Royal Infirmary of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, UK
E-mail: j.a.ross@ed.ac.uk

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Introduction

Cancer cachexia is a condition of severe malnutrition and negative nitrogen balance that occurs in up to one half of all cancer patients and which results in an increase in patient morbidity and mortality (1). Once cachexia is clinically evident (e.g. by deterioration of nutritional status or muscle strength), the syndrome is more difficult to reverse (2). Thus, a systematic approach to the management of cachexia requires early identification of patients at risk and institution of prophylactic measures to attenuate its progression. However, there are currently no agreed early biomarkers (clinical or biochemical).

Systemic inflammation is considered to be one of the key mechanisms leading to cancer cachexia (3). Pro-inflammatory cytokines not only induce breakdown of skeletal muscle protein, but have more general effects, including an increase in capillary permeability and activation of the hepatic acute phase response (4-6). Thus, systemic inflammation is often evidenced by an increase in plasma concentrations of positive acute phase reactants [e.g. C-reactive protein (CRP) >10 mg/l]. However, despite the importance of pro-inflammatory cytokines in cachexia, plasma concentrations tend to fluctuate, thereby reducing their potential as effective biomarkers.

During the catabolic response in skeletal muscle, release of myofilaments from the sarcomere by calcium/calpain-dependent pathways is an early and perhaps rate-limiting, step (7). Activation of the ubiquitin-proteasome pathway (UPP) then degrades released myofibrillar proteins (3). In murine tumour models, cancer cachexia involves the selective reduction of myosin heavy chain (MyHC) protein expression within skeletal muscle (8). This reduction in MyHC levels is associated with increased activity of the UPP, suggesting that cachexia is the result of targeted depletion of specific

myofibrillar proteins. Studies in cytokine-dependent animal tumour models have also identified specific changes in skeletal muscle membrane structure and function during cachexia (9). In particular, the dystrophin glycoprotein complex appears dysregulated and the sarcolemma becomes permeable to vital dyes. However, the severity of membrane damage would appear, at first glance, to be insufficient to cause a free permeation of intracellular proteins, particularly as circulating levels of creatine kinase (CK), a marker of muscle damage, are not elevated (9,10).

The multifactorial catabolic and inflammatory mechanisms at work in cancer cachexia result in an increase in circulating proteins, leading to significant proteinuria and urinary nitrogen loss (2). Such proteinuria has been shown to be associated with worsened patient prognosis (11). However, the exact identity of protein species within the urine has not been elucidated. We hypothesized that the identification of proteins within the systemic compartment of cachectic patients may identify proteins that have been degraded selectively. The identification of proteins within patient plasma (as a representative matrix of the systemic compartment) is highly complex due to the multiplicity of species involved in non-cachectic processes found therein (12). However, mass spectrometry (MS) has been successful at species identification within urine (13). Thus, in a preliminary attempt to identify urinary proteins that might be used in future biomarker studies to target patients for therapeutic intervention, we aimed to use MS to identify and compare the protein contents of urine from cachectic cancer patients, weight-stable cancer patients and healthy controls.

Materials and methods

Patients and controls. Patients provided written, informed consent and the study was approved by the Lothian Research Ethics Committee. Patients with newly diagnosed gastro-oesophageal (GO) adenocarcinoma or squamous cell carcinoma who were undergoing elective resection with curative intent were recruited for the study (n=16), prior to July 2008. GO cancer patients have a high incidence of weight loss (1) and were therefore chosen as a representative group of patients who develop cachexia. Cancer patients were separated into two groups: those who had lost >10% of pre-morbid body weight and were therefore considered to be cachectic (CC group) (n=8), and those who had lost minimal weight and were thus considered to be weight-stable (WS group) (n=8). Urinary samples were also collected from healthy, weight-stable volunteers who served as controls (HC group) (n=8). Individuals with known previous renal failure were excluded.

Anthropometry. Height was measured using a wall-mounted stadiometer with the patient standing erect without shoes. Patients were weighed on spring balance scales without shoes and wearing light clothing. Pre-illness stable weight was self-reported by patients. Mid-arm circumference (MAC) was measured at the mid-point between the acromion and olecranon processes. Triceps skinfold thickness (TSF) was measured with Harpenden skin callipers (Holtain, Crymych, UK). Mid-arm muscle circumference (MAMC) was calculated according to the formula: MAMC = MAC-[π x TSF]. Karnofsky performance score (KPS) was documented by the recruiting physician.

Plasma tests. Plasma creatinine concentration was measured using a kinetic Jaffe technique (Olympus AU2700 analyzer: Olympus UK Ltd, Watford, UK; normal defined as <120 μmol/l). Plasma CK concentration was measured using a CK-NAC (IFCC) technique (Olympus AU2700 analyzer; normal defined as <170 U/l in males and <135 U/l in females). Within and between-run CVs were <2.0% at all concentrations for both tests. Plasma CRP concentration was measured using quantitative immunoturbidimetry (Olympus AU2700 analyzer; CRP>10 mg/l considered evidence of a positive acute phase response) with CVs of 3.4% at concentrations <15 mg/l and 1.6% at 80 mg/l.

Urinary sampling and protein precipitation. Morning urine samples were taken and stored at -20˚C until analysis. First urine samples of the day were not used in order to avoid urine which may have remained in the bladder overnight. Following centrifugation to remove debris, proteins were precipitated from 20 ml urine by incubation at -20˚C for 2 h with 40 ml cold acetone containing 20 mM dithiothreitol (DTT). Samples were centrifuged and the protein pellet was re-dissolved in 3 ml PBS/3 ml rehydration buffer (8 M urea, 2% CHAPS, 20 mM DTT). A 200 μl aliquot was centrifuged and the supernatant assayed for protein concentration using the Bradford technique (Bio-Rad Protein Assay kit, USA) Absorbance was read at 630 nm (MRX II spectrophotometer, Dynex Technologies). A standard curve was created by diluting bovine-γ-globulin protein standard with PBS and re-hydration buffer at a 1:1:1 ratio.

1D SDS-PAGE gel electrophoresis. Preliminary unpublished study with 2D gels of urine demonstrated a low level of complexity compared to previous experience with plasma, serum and saliva; thus, 1D SDS-PAGE was used for initial separation (NuPAGE Novex 4-12% Bis-Tris gradient gels, Invitrogen). In order to avoid potential 'masking' of normal urinary proteins by novel species that may be highly prevalent in WS/CC urine, equal volumes (rather than equal amounts) of precipitated protein supernatant were loaded onto gels. Thirteen microliters of protein sample were prepared with 5 μl of 4X SDS sample buffer and 2 μl of 10X reducing agent. Gels were loaded with low and high molecular weight markers (GE Healthcare UK Ltd, Little Chalfont, UK) and stained with GelCode Blue (Pierce). Using a clean scalpel, bands were excised in an identical fashion from each gel lane in 7 separate blocks (Fig. 1).

In-gel tryptic digestion. Gel blocks were incubated three times in 200 mM NH_4HCO_3 in 50% acetonitrile (ACN) at 30˚C for 30 min. Each block was then incubated in 20 mM DTT/200 mM NH_4HCO_3 in 50% ACN at 30˚C for 1 h to reduce the protein, followed by three washes in 200 mM NH_4HCO_3 in 50% ACN. Cysteines were alkylated by incubation in 50 mM iodoacetamide/200 mM NH_4HCO_3 in 50% ACN at room temp for 20 min in the dark, followed by 3 further washes. Gel blocks were cut into pieces and

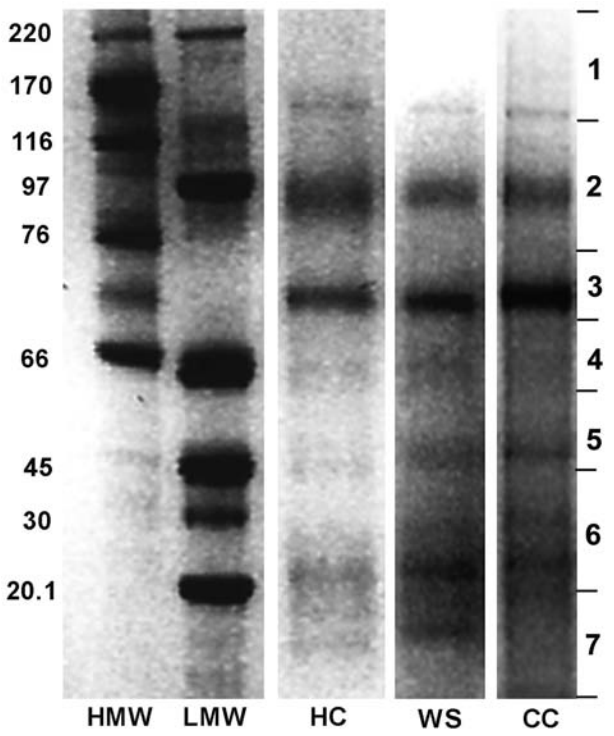

Figure 1. Representative 4-12% Bis-Tris gradient gel lanes from a healthy control (HC), weight-stable cancer patient (WS) and cachectic cancer patient (CC) with high (HMW) and low (LMW) molecular weight markers demonstrating the sites of gel block excision (blocks 1-7) for mass spectrometric analysis and locations of the abundant urinary proteins. Locations of the abundant urinary proteins: Block 1, c-myc intron binding protein 1 (MIBP1), JmjC domain-containing histone demethylation protein 2A, maltase glucoamylase, microtubule associated protein 1B (MAP1B), myosins, spectrins, talin-2; Block 2, chromosome 14 open reading frame 78; Block 3, α-fetoprotein, uromodulin; Block 4, albumin; Block 5, kininogen, zinc-α-2 glycoprotein; Block 6, α-1-microglobulin, microtubule actin crosslinking factor 1 (MACF1).

incubated with 100% ACN until white. Gel pieces were then air-dried before incubation with 0.5 μg trypsin (Promega, UK) in 50 mM NH_4HCO_3 for 16 h.

MS analysis. Tryptic digests were sonicated for 10 min. Samples were zip-tipped (C18 Zip-Tip, Millipore) to reduce salt contamination. For MALDI-TOF MS analysis, 0.5 μl aliquots of the tryptic digests were mixed with 0.5 μl of 10 mg/ml CHCA matrix, and analysed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). Processed spectra were searched against the Swissprot and National Center for Biotechnology Infomation (NCBI) non-redundant databases using Protein Prospector MSFit and Mascot PMF (Matrix Science). Search parameters included a maximum of 1 missed cleavage per peptide and a mass tolerance of 100 ppm. Proteins with a significant MOWSE score ($P<0.05$) are reported. For LC tandem MS analysis, tryptic digests from gel blocks were pooled into two groups by molecular weight: MW ≤30 kDa and MW >30 kDa. Pooled samples were spun through a 0.22 μm filter (Millipore). Each pooled sample (100 μl) was loaded onto a PepMap C18 column (3 μm particle size, 75 μm x 15 cm) on a Famos/Swichos/Ultimate high performance liquid chromatography (HPLC) system (Dionex, UK) at a flow rate of 200 nl/min. The column was equilibrated with solvent A (0.1% formic acid) and eluted with a linear gradient from 0 to 70% solvent B (0.1% formic acid in 100% acetonitrile) over 45 min. Flow was controlled by Chromeleon software and interfaced to the LCQ deca MS (Thermo Scientific) with a PicoTip (FS-360-20-10-N-20-C12 DOM, New Objective, UK). Output was monitored at 214 nm. Data-dependent acquisition was controlled by Xcalibur software and database searching was achieved using TurboSequest software and Mascot. Redundancy was avoided within the protein counts by identifying the gene of origin of hypothetical and KIAA proteins wherever possible.

Table I. Tumour details of the recruited gastro-oesophageal cancer patients (n=16).

	Weight-stable cancer (n=8)	Cancer cachexia (n=8)	Overall (n=16)
Tumour site			
Oesophageal	3	5	8
Gastric	5	3	8
Histology			
Adenocarcinoma	7	5	12
Squamous	1	3	4
Disease stage			
I	4	3	7
II	1	0	1
III	1	4	5
IV	2	1	3

Statistical analysis. All statistical analyses were performed using Statistical Package for Social Services version 13.0 (SPSS 13.0; Chicago, IL, USA). Concentrations of the precipitated protein supernatants were used to derive protein concentrations of the initial urine samples in mg/l. Differences between distribution functions of data for the 3 patient groups was determined by Kruskal-Wallis (KW) test. Subsequent analysis to determine differences between any 2 groups was determined by Mann-Whitney U test (MW) and Fisher's exact test (FE). All quoted P-values are two-sided. Asymptotic P-values are quoted for KW test whereas two-sided exact P-values are quoted for MW test. Statistical significance was set at $P<0.05$ level.

Results

Patient demographics. Tumour site, histology and stage of disease did not differ between the WS and CC groups (Table I). The HC group was younger than both the WS group ($P=0.001$) and CC group ($P=0.002$) (Table II). However, there was no difference in age or gender ratio between the WS and CC groups. The CC group had lost a median of 17.9% of pre-morbid weight (range 12.7-25.6) compared with both the WS

Table II. Characteristics of healthy, weight-stable, non-cancer controls, the weight-stable cancer patients and the cancer cachexia patients (>10% weight loss).

Patient Group	n	Gender M:F	Age[g] yrs	Weight loss[g] %	Weight kg	BMI[g] kg/m^2[h]	MAC[h] cm	TSF mm	MAMC[i] cm	KPS	Creat μmol/l	CK U/l	CRP mg/l	UPC mg/l
Healthy controls	8	6:2	31.5 (21-59)	0 (0.0-0.0)	73.7 (62.8-82.9)	23.7 (23.0-28.6)	30.8 (29.9-34.5)	16.0 (8.4-26.6)	26.0 (21.5-31.6)	100 (100-100)	NA	NA	NA	174.6 (74.7-343.5)
Weight-stable cancer	8	7:1	72[a] (56-74)	0 (-1.67-1.00)	73.0 (66.8-111.0)	32.0[b] (25.3-33.5)	31.3 (29.0-35.2)	13 (6.2-25.8)	26.7 (24.4-32.1)	100 (90-100)	98 (76-193)	79 (22-128)	11 (<5-39)	105.3 (35.1-202.8)
Cancer cachexia	8	5:3	70[a] (48-82)	17.9[a,d] (12.7-25.6)	52.2[a,f] (39.6-90.0)	20.4[c,d] (15.5-25.2)	24.3[b,e] (18.0-31.0)	8.6 (3.0-17)	21.2[c,e] (15.9-26.9)	100 (80-100)	90 (66-108)	43 (24-68)	5 (<5-47)	157.2 (65.4-288.9)

Data are presented as medians with ranges in parentheses. BMI, body mass index; CK, plasma creatine kinase; Creat, plasma creatinine; KPS, Karnofsky performance score; MAC, mid-arm circumference; MAMC, mid-arm muscle circumference; NA, not assessed; TSF, triceps skinfold thickness; UPC, urinary protein concentration. [a,b,c]Difference from HC group on MW test [a]P≤0.001; [b]P≤0.01; [c]P<0.05. [d,e,f]Difference from WS group on MW test [d]P≤0.001; [e]P<0.01; [f]P<0.05. [g,h,i]Difference between the 3 groups on KW test [g]P≤0.001; [h]P<0.01; [i]P<0.05.

and HC groups which had not lost weight (P<0.001). Other nutritional parameters (BMI, mid-arm circumference, mid-arm muscle circumference) were also reduced in the CC group compared with the WS group, consistent with a specific loss of skeletal muscle mass in the CC group. The WS group exhibited an elevated BMI compared with the HC group, reflecting the role of obesity in the pathogenesis of GO adenocarcinoma.

Plasma levels. Plasma creatinine was elevated in one patient in the WS group suggesting mild renal impairment, but there was no overall difference between the WS and CC groups (P=0.234). Six (37.5%) cancer patients exhibited evidence of an acute phase response (plasma CRP >10 mg/l), but there was no difference in CRP between the WS and CC groups (P=0.613). Plasma CK was not elevated in any of the cancer patients and did not differ between the WS and CC groups (P=0.083).

Cachectic cancer urine contains more protein species than weight stable cancer and control urine. There was no significant difference in the urinary protein concentration of the three patient groups (P=0.149) (Table II). Thus, the finite amounts of protein loaded onto gels were comparable statistically. Using both MS approaches, the number of proteins identified in each CC urine sample (median 42, range 26-61) was greater than that identified in each WS sample (median 15; range 9-28) and HC sample (median 12.5; range 5-18) (P<0.001) (Table III). Furthermore, the total number of different protein species identified in all samples of the CC group combined (n=199) was higher than that identified in the WS (n=79) and HC (n=49) groups (Table III). There was no difference between the total number of proteins identified in the WS group compared with the HC group (P=0.234). In all 3 groups, the number of proteins identified by MALDI-TOF MS was higher than that identified by LC-MS/MS.

Candidate biomarkers of cachexia. The distribution of gel bands did not differ observably between the 3 patient groups (Fig. 1). Thirty-five protein species were identified that appeared in at least 3 urine samples within any patient group of 8 (i.e. at least 37.5%). Of these 35 species, 21 were not specific to the CC group. These non-cachexia-specific protein species included common urinary proteins such as albumin, cytokeratins 1 and 10 and uromodulin, plus many proteins not previously reported in the urine of cancer patients (Table IV). Immunoglobulin (Ig) κ-light chain was identified in 6 (75%) CC samples and 4 (50%) WS samples compared with 1 (12.5%) HC sample (CC vs. HC P=0.041), whereas zinc α-2 glycoprotein (ZAG) (either precursor chains A-D or the whole molecule) was identified in 5 (62.5%) CC samples and 5 (62.5%) WS samples compared with 1 (12.5%) HC sample. These two proteins may therefore represent markers of cancer or ill-health.

All of the CC samples exhibited at least one species of myosin, including sarcomeric myosin heavy chain 2 and myosins 3a, 4, 5a, 5c, 7a, 8, 9a, 9b, 10, 13 and 18b. In comparison, none of the WS samples expressed myosin species whereas only one (12.5%) HC sample expressed myosin 18b. Furthermore, all of the CC samples expressed

Table III. Number of urinary proteins identified within each patient group by each mass spectrometric technique.

Patient group	n	Urinary proteins identified			
		Number	MALDI[e]	LC	Combined[e]
Healthy controls	8	Total	37	23	**49**
		Median	8.5	7	12.5
		Range	(5-16)	(3-11)	(5-18)
Weight-stable cancer	8	Total	64	21	**79**
		Median	11.5[a]	6	15
		Range	(6-23)	(4-10)	(9-28)
Cancer cachexia	8	Total	187	18	**199**
		Median	41.5[b,c,d]	6.5	42[c,d]
		Range	(26-59)	(4-9)	(26-61)

Total number of proteins identified within a patient group shown in bold. Median number of proteins identified within a single urine sample shown in normal type with range in parentheses. [a,b]Difference from LC technique within same patient group on MW test [a]P<0.05; [b]P<0.001. [c]Difference from HC group on MW test P<0.001. [d]Difference from WS group on MW test P<0.001. [e]Difference between the 3 patient groups on KW test P<0.001.

species of spectrin compared with none of the WS or HC samples. Fourteen proteins were found exclusively in at least 3 out of 8 (37.5%) CC samples and may therefore represent specific markers of cachexia. All 14 of these proteins were detected by MALDI-MS, and not LC-MS/MS. The identities and functions of these proteins are shown in Tables V and VI. Retrospective analysis of 1D gels suggests that some markers (e.g. myosins and α-spectrin) were excreted in complete or near-complete form, whereas other biomarkers (e.g. MACF1) appear to have been digested into smaller peptides prior to excretion.

Discussion

MS has been utilised successfully for the identification of urinary proteins (13), oligosaccharides (14) and nucleosides (15). Furthermore, MALDI-MS has been employed in the detection of biomarkers in kidney disease (16), genitourinary tumours, including bladder (17) and prostate cancer (18), and tumours outwith the genitourinary tract, including pancreatic (19) and colon cancer (20). In this preliminary study, MS was used to demonstrate that urine from cachectic GO cancer patients contained significantly more protein species than urine from weight-stable GO cancer patients and healthy controls, in the absence of an elevated CK level. Cancer patients were older than healthy controls, but ages of the WS and CC groups were comparable, and thus age alone is unlikely to explain the different numbers of protein species between groups.

MALDI-MS was capable of identifying more protein species than LC-MS/MS. Furthermore, all cachexia-specific protein species were detected exclusively by MALDI-MS. These results probably reflect the increased sensitivity of MALDI-MS, and the fact that, during LC-MS/MS analysis, highly abundant proteins will mask less abundant species (21). Masking may have taken place during LC-MS/MS analysis within the present study as, for example, uromodulin was detected by LC-MS/MS in all members of the HC group, but was found in only 2 of the WS group and 1 of the CC group.

The protein species identified specifically in the cachectic urine samples could be considered to share a common structural function, and thus their urinary excretion might point to a *prima facie* deterioration in muscle structure during cachexia. The myofibrillar proteins detected in cachectic urine were predominantly myosin species. Titin and cytoplasmic actin 1 were found in individual cachectic patients but none of the other core myofibrillar proteins, such as troponin T and tropomyosin, were detected. Myosins can be subdivided into 18 different classes based on homologous myosin head domain sequences (22). Conventional myosins, such as those found in muscle tissue, are composed of two myosin heavy-chain (MyHC) subunits, each associated with two light-chain subunits that bind at the neck region and play a regulatory role. Unconventional myosins, believed to function in intracellular motion and signal transduction (23), may contain one or two heavy chains, and associated light chains. The seven MyHC isoforms that predominate in mammalian skeletal muscles include two developmental isoforms (MyHC-embryonic and MyHC-perinatal); three adult skeletal muscle isoforms (MyHC-IIa, MyHC-IIb, MyHC-IIx/d); and MyHC-ß/slow, which represents the main MyHC component in slow, oxidative, type I skeletal muscle fibres and cardiac muscle (24). This last isoform (specifically MyHC 7/cardiac muscle/ß variant) was identified specifically in cachectic urine within the present study. At first glance, this result might appear contradictory to previous studies, which have predominantly found a reduction in type II fast fibres in cachectic muscle (9). However, studies have also found slight reductions in type I MyHC within cachectic muscle (9) and switching of fibre-type from I to IIa in animal tumour models (25).

The remaining four myosins (5c, 7a, 9a and 10) detected exclusively in cachectic patients were all unconventional myosins. Myosin 18b was detected in 3 cachectic patients,

Table IV. Protein species that were not cachexia-specific and were identified in at least 3 individual samples of any patient group of 8 (i.e. 37.5%).

Protein	Accession no.	MW (kDa)	MALDI peptides	MALDI % coverage	LC MS/MS peptides	No. of individuals excreting protein (out of 8)		
						Healthy controls	Weight-stable cancer	Cancer cachexia
Acetyl-CoA carboxylase 2	O00763	276555	21	12	NA	2	3	0
α-1-Acid glycoprotein 1 precursor	P02763	23512	NA	NA	6	2	3	5
Albumin	P02768	69367	34	61	34	8	8	8
Centriolin	Q7Z7A1	268886	23	15	NA	0	1	3
Collagen type VI, α3	P12111	343552	23	15	NA	0	1	3
Cytokeratin 1	P04264	66018	18	38	10	5	5	4
Cytokeratin 10	P13645	59511	17	35	7	5	2	2
α-Fetoprotein	P02771	68678	33	56	3	6	5	7
Immunoglobulin κ light chain	Q6GMX8	25707	8	50	7	1	4	6[a]
Leucine-rich repeat-containing protein KIAA1731	Q9C0D2	295146	20	14	NA	3	1	0
Kininogen-1	P01042	71957	12	37	3	3	2	4
α-1-Microglobulin/bikunin	P02760	38999	17	58	12	7	7	7
Myosin 18B	Q8IUG5	285185	25	16	NA	1	0	3
Neuroblastoma-amplified protein	Q8NFY8	268585	22	18	NA	0	1	3
Probable G-protein coupled receptor 135	Q8IZ08	51736	NA	NA	1	5	2	0[a]
Prostaglandin D2 synthase	Q5SQ09	22836	NA	20	4	1	6[a]	5
ß-spectrin I (non-erythrocytic)	Q59ER3	274609	26	17	NA	1	0	4
ß-spectrin IV (non-erythrocytic)	Q71S07	289061	28	19	NA	0	1	5[a]
Talin-2	Q9Y4G6	271555	26	16	NA	1	1	4
Uromodulin	P07911	69761	27	34	15	8	5	6
Zinc-α-2-glycoprotein	P25311	33872	21	61	11	1	5	5

Results reported as maximum number of peptides detected by MALDI-MS and LC-MS/MS within a single sample and maximum peptide percentage coverage detected by MALDI-MS within a single sample. [a]Difference from HC group on FE test $P<0.05$.

Table V. Protein species that were specifically identified in at least 3 of the 8 (37.5%) cancer cachexia urine samples and which therefore represent candidate biomarkers of cachexia.

Candidate biomarker Of cancer cachexia	Accession no.	MW (kDa)	MALDI peptides	MALDI % coverage	Number of individuals excreting protein (out of 8)		
					Healthy controls	Weight-stable cancer	Cancer cachexia
Bullous pemphigoid antigen 1 (isoforms 1-10)	Q03001	372210	22	18	0	0	3
Chromosome 14 open reading frame 78	Q8IVF2	616629	26	14	0	0	3
C-myc intron-binding protein 1 (MIBP1)	Q38G99	268803	20	16	0	0	3
Maltase-glucoamylase (intestinal)	O43451	209853	18	16	0	0	3
Microtubule-actin crosslinking factor (MACF1)	Q9UPN3	620418	49	16	0	0	3
Microtubule-associated protein 1B (MAP1B)	P46821	270620	21	15	0	0	5[a]
Myosin 5C	Q9NQX4	202794	22	19	0	0	5[a]
Myosin 7A	Q13402	254406	35	23	0	0	4
Myosin, heavy polypeptide 7, cardiac muscle, ß variant	Q2M1Y6	223097	22	18	0	0	4
Myosin 9A	Q9UNJ2	292707	39	27	0	0	3
Myosin 10	P35580	228939	24	19	0	0	3
Nischarin	Q6PGP3	166653	18	20	0	0	5[a]
α-spectrin 1 (non-erythrocytic)	Q13813	284539	39	24	0	0	5[a]
Zinc finger protein 106 homolog	Q9H2Y7	208883	27	20	0	0	6[b]

Results reported as maximum number of peptides and maximum percentage coverage found by MALDI within a single sample. [a]Difference from both HC and WS group on FE test $P<0.05$. [b]Difference from both HC and WS group on FE test $P<0.01$.

but was not cachexia-specific as it was also found in one healthy control. Myosin 18b is detected predominantly in all types of striated muscle but at much lower levels compared to class II sarcomeric myosins. It moves into the myonuclei on differentiation to possibly regulate muscle-specific genes (26).

The cytoskeletal proteins detected in cachectic urine included α-spectrin 1 (non-erythrocytic) and nischarin. The ß spectrins I and IV were also found in several cachectic samples but were not specific. The spectrins are a family of widely-distributed filamentous cytoskeletal protein heterodimers that consist of a constant α-chain and variable, tissue-specific ß-chains, which associate with short actin filaments to form a hexagonal mesh. They play an important role in the maintenance of plasma membrane integrity (27). In pathological processes such as diffuse axonal injury, cleavage of α-spectrin by calpain/caspase activity (an enzyme system hypothesized to be involved in the early stages of myofibrillar degradation) leads to membrane disruption and, ultimately, cellular death (27), suggesting that the cachectic process may involve membrane disruption. α-spectrin has been found to be over-expressed in intestinal-type gastric tumours (28), but in our cohort was found primarily in samples from patients with oesophageal cancer. Nischarin binds to the cytoplasmic domain of the integrin α5 subunit, thus inhibiting cell motility, and altering actin filament organization (29).

Another high-molecular weight cytoskeletal protein involved in integrin function and found in a high proportion of the cachectic samples, albeit non-specifically, was talin-2. Talin is concentrated at regions of cell-substratum contact and is capable of activating integrins enabling linkage to the actin cytoskeleton (30). It is also a substrate for the calpain system (31).

Table VI. Functions of candidate biomarkers of cachexia.

Candidate biomarker of cancer cachexia	Protein function
Bullous pemphigoid antigen 1 (isoforms 1-10)	Autoantigen of bullous pemphigoid (autoimmune subepithelial skin blistering disease). Member of spectraplakin family of cytoskeletal linker proteins. Highly expressed in skeletal muscle (34). Anchors intermediate filaments to inner plaque of hemidesmosomes.
Chromosome 14 open reading frame 78	Unknown.
C-myc intron-binding protein 1	Transcription factor. Belongs to the MHC binding protein family. May repress c-myc transcription from major promoter, P2 (45). Knockout mouse demonstrates defects in T-cell maturation (46).
Maltase-glucoamylase (intestinal)	Determines small intestinal starch digestion into glucose. Alternate pathway for when luminal α-amylase activity is reduced due to immaturity or malnutrition (47). May play role in digestion of malted dietary oligosaccharides used in food manufacturing.
Microtubule-actin crosslinking factor (MACF1)	Member of plakin family of cytoskeletal linker proteins (33). Functions in microtubule dynamics to facilitate actin-microtubule interactions at cell periphery and to couple microtubule network to cellular junctions.
Microtubule-associated protein 1B (MAP1B)	Binds to tubulin to regulate microtubule function. Commonly found in dendrites and axons. Promotes axon formation through regulation of microtubule dynamics and cytoskeletal organization. Muscle expression is increased in lean men by fat overfeeding (48).
Myosin 5C	Powers actin-based membrane trafficking, particularly of transferrin (49). Expressed in many secretory and glandular tissues.
Myosin 7A	Expressed in testis, kidney, lung, inner ear, retina and the ciliated epithelium of nasal mucosa. Gene mutations are responsible for Usher syndrome type 1B (50), an autosomal recessive condition characterized by deafness and gradual vision loss.
Myosin, heavy polypeptide 7, cardiac muscle, ß variant	Gene defects cause familial hypertrophic cardiomyopathy type 1 (51), myosin storage myopathy (involves type I fiber predominance and increased interstitial fat and connective tissue) (52), and Laing early-onset distal myopathy (53).
Myosin 9A	Expressed in all mammalian tissues. Involved in control of actin cytoskeleton by negatively regulating the small G-protein Rho (54). Gene mutations implicated in Bardet-Biedl syndrome (autosomal recessive disorder characterized by cognitive impairment, obesity, retinitis pigmentosa, syndactyly/polydactyly, short stature, and hypogenitalism) (55).
Myosin 10	Involved in mitotic spindle function and filopodia formation (56). Tail contains multiple pleckstrin homology domains (57).
Nischarin	Binds to cytoplasmic domain of integrin α5 subunit, inhibiting cell motility and altering actin filament organization (29). Inhibits Rac-induced migration and invasion of epithelial cells by affecting PAK signaling cascades (58).
α-spectrin 1 (non-erythrocytic)	Cleavage of α-spectrin by calpain/caspase leads to membrane disruption and cellular death (27). Overexpressed in some gastric tumours (28).
Zinc finger protein 106 homolog	Unknown.

The microtubule-associated proteins found specifically in the cachectic samples included microtubule associated protein 1B (MAP1B) and microtubule actin crosslinking factor 1 (MACF1). Microtubule-associated proteins (MAPs), including MAP1B, are thought to regulate the formation and stability of microtubules via their interaction with tubulin (32). MACF1 is a member of the plakin family of cytoskeletal linker proteins and is a structural hybrid of dystrophin (at the C-terminal) and bullous pemphigoid antigen 1 (BPAG1) (at the N-terminal) (33). BPAG-1 is another member of the plakin family which is highly expressed in skeletal muscle (34) and which was also identified specifically in the CC group.

Two-thirds of the cancer samples expressed Ig κ-light chain and/or ZAG. Ig light chains have been detected in the urine of ill patients since 1848 (so-called Bence-Jones protein). More recently, we have demonstrated Ig κ-light chains in the urine of patients with chronic heart failure and various malignancies (35). ZAG has been found previously in increased quantities in the urine of patients with bladder cancer (36). Furthermore, ZAG has been postulated as the lipid mobilizing factor in cachexia (37). However, the tumour-secreted proteolysis inducing factor (38) was not detected in any of the urine samples.

Some MS studies have suggested that the urinary proteome may contain ~1,500 species (39). Only a third of proteins identified are described as classical circulatory plasma proteins, which are known to be relatively abundant in urine despite their retention to a large extent during the glomerular filtration process. However, other studies have demonstrated that sequential preparation of urine samples by gel filtration and protein precipitation results in low amounts of detectable protein on 2D gels (40). These latter results are consistent with our own findings. Improvements in methodology, such as the use of storage agents (41); alternative strategies of protein precipitation (42); a return to 2D PAGE (43); or bioinformatics (43) may all increase the yield of potential biomarkers.

In the present study, weight loss alone was used as the definition of cachexia. We have shown previously that a 3-factor profile of cancer cachexia, incorporating weight loss, dietary intake and systemic inflammation, better defines groups with worsened prognostic outcome (44). Future biomarker studies which incorporate more detailed definitions of cachexia will help to elucidate changes caused independently by systemic inflammation and hyponutrition.

In summary, urinary proteomics using MS techniques can identify candidate molecules for potential targeting as novel biomarkers of cachexia in GO cancer patients. Biomarker strategies targeting myofibrillar, cytoskeletal and micro-tubule-associated proteins within the context of future longitudinal studies of large patient numbers will be required to assess fully the changing prevalence of biomarkers within different cachectic populations.

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