Ovarian cancer ascites induces skeletal muscle wasting in vitro and reflects sarcopenia in patients

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Abstract

Background Cachexia-associated skeletal muscle wasting or ‘sarcopenia’ is highly prevalent in ovarian cancer and contributes to poor outcome. Drivers of cachexia-associated sarcopenia in ovarian cancer remain elusive, underscoring the need for novel and better models to identify tumour factors inducing sarcopenia. We aimed to assess whether factors present in ascites of sarcopenic vs. non-sarcopenic ovarian cancer patients differentially affect protein metabolism in skeletal muscle cells and to determine if these effects are correlated to cachexia-related patient characteristics.

Methods Fifteen patients with an ovarian mass and ascites underwent extensive physical screening focusing on cachexia-related parameters. Based on computed tomography-based body composition imaging, six cancer patients were classified as sarcopenic and six were not; three patients with a benign condition served as an additional non-sarcopenic control group. Ascites was collected, and concentrations of cachexia-associated factors were assessed by enzyme-linked immunosorbent assay. Subsequently, ascites was used for in vitro exposure of C2C12 myotubes followed by measurements of protein synthesis and breakdown by radioactive isotope tracing, qPCR-based analysis of atrophy-related gene expression, and NF-κB activity reporter assays.

Results C2C12 protein synthesis was lower after exposure to ascites from sarcopenic patients (sarcopenia 3.1 ± 0.1 nmol/h/mg protein vs. non-sarcopenia 5.5 ± 0.2 nmol/h/mg protein, P < 0.01), and protein breakdown rates tended to be higher (sarcopenia 31.2 ± 5.2% vs. non-sarcopenia 20.9 ± 1.9%, P = 0.08). Ascites did not affect MuRF1, Atrogin-1, or REDD1 expression of C2C12 myotubes, but NF-κB activity was specifically increased in cells exposed to ascites from sarcopenic patients (sarcopenia 2.2 ± 0.4-fold compared with control vs. non-sarcopenia 1.2 ± 0.2-fold compared with control, P = 0.01). Protein synthesis and breakdown correlated with NF-κB activity (r_s = −0.60, P = 0.03 and r_s = 0.67, P = 0.01, respectively). The skeletal muscle index of the ascites donors was also correlated to both in vitro protein synthesis (r_s = 0.70, P = 0.005) and protein breakdown rates (r_s = −0.57, P = 0.04).

Conclusions Ascites of sarcopenic ovarian cancer patients induces pronounced skeletal muscle protein metabolism changes in C2C12 cells that correlate with clinical muscle measures of the patient and that are characteristic of cachexia. The use of ascites offers a new experimental tool to study the impact of both tumour-derived and systemic factors in various cachexia model systems, enabling identification of novel drivers of tissue wasting in ovarian cancer.

Keywords Protein synthesis; Protein breakdown; Atrogenes; NF-κB; Interleukin-6; C2C12 cells; Myotubes; Translational research

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Introduction

Cachexia and skeletal muscle wasting are highly prevalent in patients with ovarian cancer and are associated with poor disease outcome. The mechanisms underlying the development of cachexia in ovarian cancer remain elusive, although several lines of evidence have implicated tumour-derived compounds and their direct and indirect effects on skeletal muscle and adipose tissue. In particular, skeletal muscle protein metabolism alterations induced by changes in muscle RING-finger protein-1 (MuRF1), Atrogin-1, REDD1 (regulated in development and DNA damage responses) and/or NF-κB activity have been the focus of research.

About one-third of all newly diagnosed ovarian cancer patients and almost all patients with recurrent disease accumulate ascites. In the intraperitoneal cavity, small amounts of free fluids are produced by capillary membranes to allow for lubrication of serosal surfaces. Under physiological conditions, the vast majority of these fluids are reabsorbed by the lymphatic system. However, under influence of tumour-derived growth factors such as vascular endothelial growth factor, the peritumoural microvasculature becomes increasingly leaky. Furthermore, disseminated disease can cause obstruction of the lymphatic vessels. This combination of increased build-up and decreased reabsorption causes rapid accumulation of ascites fluid in the peritoneal cavity under malignant conditions. Importantly, excessive accumulation of ascites has been associated with detrimental nutritional status in ovarian cancer patients.

Ovarian cancer ascites is a complex reservoir of soluble factors and cell components, which collectively provide a pro-inflammatory and tumour promoting micro-environment. Interestingly, cytokine expression profiling of ascites from ovarian cancer patients revealed high levels of IL-6, IL-8, and Mcp-1, factors that have been suggested to promote tissue wasting in individuals with cachexia. Furthermore, the concentration of cachexia-related inflammatory cytokines in ovarian cancer ascites has been shown to be significantly higher in comparison with the serum concentrations of the same patient. Because ovarian cancer ascites is relatively easily accessible, generally present in large quantities, and contains a high concentration of tumour-derived compounds, it represents an attractive experimental tool to study the impact of ovarian cancer-derived factors on skeletal muscle physiology.

We hypothesized that factors present in ovarian cancer ascites from sarcopenic patients would induce protein metabolism disturbances characteristic of cachexia-associated sarcopenia in skeletal muscle cells. C2C12 skeletal muscle cells were exposed to ascites from well-phenotyped sarcopenic vs. non-sarcopenic patients with malignant or benign ovarian tumours, followed by analysis of protein synthesis and breakdown. We found that ascites from sarcopenic cancer patients decreased C2C12 protein synthesis in correspondence with their degree of sarcopenia.

Methods

Patients and cachexia screening

Between March 2017 and March 2018, 15 consecutive patients with a suspected malignancy of the ovary, as indicated by computed tomography scan analysis and the presence of abdominal ascites, were prospectively enrolled at the Maastricht University Medical Centre+. Patients were eligible for either primary cytoreductive surgery or neoadjuvant chemotherapy. Before start of the treatment, patients received a physical screening including assessment of handgrip strength, triceps skinfold assessment, upper arm circumference, and wrist circumference. The physical screening was complemented with the Patient-Generated Subjective Global Assessment, Mini Nutritional Assessment, and subjective assessment of fat, muscle, and fluid status. Patient-reported weight loss was assessed over the past 6 months. Venous blood was drawn, and concentrations of haemoglobin, leucocytes (and differentiation), kidney function markers, liver function indicators, lipids, insulin, glucose, and acute phase proteins were assessed to characterize cancer cachexia and to identify possible promoters of sarcopenia.

For body composition analysis, one single axial slice of the abdominal computed tomography scan at the third lumbar level was used. Standard Hounsfield unit ranges of −30 to +150 Hounsfield units (HU) for skeletal muscle, −190 to −30 HU for intramuscular adipose tissue and subcutaneous adipose tissue, and −150 to −50 HU for visceral adipose tissue were used to demarcate tissue using SliceOmatic software (v5.0, TomoVision, Montreal, Canada). Following demarcation, surface areas were standardized by height to compute the skeletal muscle index (SMI) in cm²/m². Skeletal muscle radiation attenuation was calculated using the mean HU values of skeletal muscle. Patients with a malignancy (n = 12) were divided into a sarcopenic and a non-sarcopenic group based on their L3-SMI. The cut-off for sarcopenia was determined by 1 SD below the mean SMI (SMI 39.1 cm²/m²). Three patients with a benign ovarian condition served as non-sarcopenic controls. This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre+ and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and all its revisions. All patients gave their informed consent prior to their inclusion in the study.

Collection and analysis of ascites

Ascites was collected either during an abdominal paracentesis or during primary cytoreductive surgery, but in all instances before any systemic treatment was started. Although more ascites was present, between 30 mL and 200 mL of ascites were collected for further analysis (see

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Table 1). After aspiration, the ascites was kept on ice before centrifugation for 10 min at 200× g. The supernatant was centrifuged again for 15 min at 350× g. Cell-free supernatant was aliquoted and stored at −80°C. The ascites was processed under sterile conditions in a flow cabinet.

The concentrations of IL-6, IL-8, GDF-8, GDF-15, tumour necrosis factor (TNF)-α, IL-1β, leukaemia inhibitory factor, and Mcp-1 in ascites were quantified with enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA) as per the manufacturer’s protocol.

C2C12 cell culture

C2C12 murine myoblasts (American Type Culture Collection No. CRL1772, Manassas, VA, USA) were cultured in growth medium (GM), composed of low-glucose (1 g/L) Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Dublin, Ireland) supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco). Cells were maintained at 37°C and 5% CO2 until 70–80% confluency was reached at which point the cells were passaged or used for experiments.

Depending on the experiment, cells were seeded at a density of 1.5 × 10^5 cells/cm² on BD Matrigel-coated (Matrigel® Matrix Basement Membrane—Growth Factor Reduced, Corning) (1:50 in low-glucose DMEM) cell culture plates (Eppendorf). Myoblasts were cultured for 24 h in GM. After 24 h, differentiation was initiated after washing the cells with Dulbecco’s phosphate-buffered saline (PBS) (Gibco) and switching the GM to differentiation medium (DM), which consisted of high-glucose (4.5 g/L) DMEM supplemented with 1% (v/v) heat-inactivated foetal bovine serum (30 min at 56°C), 1% (v/v) sodium pyruvate, and 0.5% (v/v) antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin). DM was refreshed every 48 h for 6 days at which point the cells were used for experiments (see Supporting Information, Video S1, for an example of a fully differentiated myotube culture used for experimentation). Myotubes were monitored during experiments using an IncuCyte® S3 Live-Cell Analysis System (Sartorius). Phase-contrast images were captured every 2 h using the 10× objective.

Analysis of protein synthesis

Fully differentiated C2C12 myotubes were treated with 25% v/v acetone. Controls consisted of Hank’s balanced salt solution (HBSS) in DM (25% v/v), 100 nM insulin (Eli Lilly, Indianapolis, IN, USA) in DM (positive control), or 10 µM dexamethasone (Sigma, St. Louis, MO, USA) solubilized in absolute ethanol and diluted in DM (negative control). All compounds and media were pre-warmed to 37°C before they were added to the cells. Before treatment, the C2C12 cells were washed twice with warm PBS. After treatment, cells were washed again with pre-warmed PBS and incubated in DM containing 0.3 mM L-phenylalanine (Sigma) and 0.1 µCi/mL 14C-L-phenylalanine (PerkinElmer, Waltham, MA, USA) for 8 h. After incubation, cells were washed with ice-cold PBS, and intracellular proteins were subsequently precipitated in 1 M of perchloric acid (PCA) for 1 h at 4°C. After washing, the cells were left overnight in 1 M of sodium hydroxide (NaOH) + 1% sodium dodecyl sulfate (SDS) solution. Subsequently, cells were scraped, and protein concentrations in the lysates were measured with the BCA assay (Thermo Scientific, Waltham, MA, USA). Protein lysates were suspended in Ultima Gold multi-purpose liquid scintillation cocktail (LSC) (Sigma), and 14C-L-phenylalanine incorporation was measured with a Tri-Carb 2910 low activity liquid scintillation analyser (PerkinElmer). Protein synthesis was expressed as nmol 14C-L-phenylalanine incorporated per hour of exposure per milligram of total protein (nmol/h/mg). (see Figure S1 for an overview of the experimental set-up).

Analysis of protein breakdown

Fully differentiated C2C12 myotubes were pretreated with 0.2 µCi/mL 14C-L-phenylalanine and 0.3 mM unlabelled L-phenylalanine in DM for 24 h. The pretreatment was followed by a 2 h chase period in which the cells were exposed to 0.3 mM unlabelled L-phenylalanine in DM to prevent the reincorporation of labelled phenylalanine released from degraded protein during protein synthesis. The chase period was followed by a 24 h period of treatment with experimental conditions. The same experimental conditions and controls as for the synthesis experiment were used for analysis of protein breakdown. After treatment, the culture medium was collected, proteins in the medium were precipitated with 1 M PCA, and after centrifugation, the supernatant was added to LSC and counted (Reading A). The precipitates were resuspended and solubilized by adding 1 M NaOH + 1% SDS, and after incubation at 37°C for 2 h, they were added to LSC and counted (Reading B). The cells in the plate were washed with PBS, solubilized with 1 M NaOH + 1% SDS, followed by overnight precipitation in 1 M PCA at 4°C. Cells were scraped, and protein concentrations were measured with the BCA assay. Protein samples were suspended in LSC, and 14C-L-phenylalanine incorporation was measured with a low activity liquid scintillation analyser (Reading C). Incubated protein was expressed as nmol 14C-L-phenylalanine incorporated per hour of exposure per milligram of total intracellular protein (nmol/h/mg). Breakdown was calculated as Reading A/(Reading A + Reading B + Reading C) * 100 and expressed as % breakdown (see Figure S2 for an overview of the experimental set-up).
| Patient and tumour characteristics | Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Non-sarcopenic benign controls (n = 3) | P-value | Reference values |
|-----------------------------------|------|-----------------------|-------------------|----------------------|---------------------------------------|---------|------------------|
| **Age**                           | Years | Median (range)   | 65 (22–81) | 68 (65–75) | 59.5 (22–63)* | 67 (66–81) | 0.008 |
| **Body mass index**               | kg/m² | Mean ± SD           | 25.8 ± 3.7  | 22.4 ± 1.4* | 29.0 ± 2.3  | 26.0 ± 3.6 | 0.011 |
| **Weight loss**                   | % (range) | 1.6 (0–4.4)  | 3.3 (0–4.4) | 1.5 (0–3.6) | 0.8 (0–1.3) | 0.164 |
| **FIGO stage**                    | II n (%) | 2 (13.3) | 2 (33.3) | 0 (0) n.a. |
|                                  | III n (%) | 8 (53.3) | 3 (50) | 5 (83.3) n.a. |
|                                  | IV n (%) | 2 (13.3) | 1 (16.7) | 1 (16.7) n.a. |
| **Tumour grade**                  | Low n (%) | 2 (13.3) | 1 (16.7) | 1 (16.7) n.a. |
|                                  | High n (%) | 10 (66.7) | 5 (83.3) | 5 (83.3) n.a. |
| **Ascites volume**                | mL Median (range) | 1200 (200–8000) | 1200 (500–8000) | 2750 (500–7000) | 350 (250–500) | 0.264 |

| Measurements | Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|--------------|------|-----------------------|-------------------|----------------------|----------------|---------|------------------|
| Wrist circumference (WC) | cm Mean ± SD | 16.8 ± 1.3 | 15.8 ± 1.2 | 17.4 ± 0.9 | 17.5 ± 0.7 | 0.211 |
| Triceps skinfold assessment (TSA) | cm Mean ± SD | 1.7 ± 0.4 | 1.3 ± 0.2* | 2.0 ± 0.4 | 1.9 ± 0.1 | 0.005 |
| Upper arm circumference (UAC) | cm Mean ± SD | 27.7 ± 3.1 | 24.9 ± 2.7 | 30.0 ± 1.1 | 29.0 ± 1.4 | 0.069 |
| Handgrip strength | kg Mean ± SD | 24 ± 4.2 | 22.5 ± 4.9 | 26.2 ± 2.4 | 22 ± 5.7 | 0.069 |
| L3-SMI | cm²/m² Mean ± SD | 41.8 ± 4.8 | 36.6 ± 2.9* | 45.2 ± 2.4 | 41.7 ± 3.6 | 0.002 |
| L3-SAT | cm²/m² Mean ± SD | 95.5 ± 70.1 | 48.9 ± 40.1 | 105.2 ± 66.6 | 169.4 ± 65.4 | 0.06 |
| L3-IMAT | cm²/m² Mean ± SD | 14.6 ± 9.3 | 12.4 ± 10.1 | 17.9 ± 10.5 | 12.3 ± 4.0 | 0.448 |
| L3-MRA | HU Mean ± SD | 37.6 ± 10.7 | 40.1 ± 9.0 | 38.4 ± 13.8 | 30.9 ± 6.2 | 0.164 |

| Electrolytes and kidney function | Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|----------------------------------|------|-----------------------|-------------------|----------------------|----------------|---------|------------------|
| Sodium                           | mM Median (range) | 139 (135–144) | 138 (135–140) | 139.5 (137–141) | 142 (140–144) | 0.06 | 135–145 |
| Potassium                        | mM Median (range) | 4.6 (4.0–5.2) | 4.5 (4.0–5.2) | 4.7 (4.1–5.0) | 4.4 (4.4-4.6) | 0.875 | 3.6–5.0 |
| Urea                             | mM Median (range) | 4.4 (2.2–10.8) | 4.1 (2.2–10.8) | 4.6 (3.5–7.0) | 4.4 (3.8–5.4) | 0.875 | 3.0–8.0 |
| Creatinine                       | μM Median (range) | 68 (52–88) | 63.5 (52–74) | 73 (56–88) | 68 (65–73) | 0.448 | 50–100 |
| MDRD                             | mL/min Median (range) | 80 (60.5–90) | 86.8 (73.5–90) | 77.70 (60.5–90) | 76.8 (74.2–80.3) | 0.448 | >90 |

| Liver function and parameters    | Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|----------------------------------|------|-----------------------|-------------------|----------------------|----------------|---------|------------------|
| Alkaline phosphatase             | U/L Median (range) | 93.5 (47–751) | 92 (61–751) | 100 (47–403) | 89 (83–95) | 0.819 | <98 |
| γ-GT                            | U/L Median (range) | 25 (8–657) | 25 (11–407) | 88 (8–657) | 25 (25–25) | 0.517 | <38 |
| ASAT                            | U/L Median (range) | 27 (14–197) | 23 (14–122) | 28 (17–197) | 18 (20–38) | 0.627 | <31 |
| ALAT                            | U/L Median (range) | 17 (5–22) | 11 (5–9) | 51 (16–221) | 24 (14–34) | 0.11 | <34 |
| Lactate dehydrogenase           | U/L Median (range) | 185 (125–375) | 171 (126–375) | 186 (125–218) | 185 (185–185) | 0.517 | 47–247 |
| Total bilirubin                 | μM Median (range) | 4.6 (2.1–6.5) | 5.0 (4.0–6.5) | 2.2 (2.1–5.2) | 5.4 (4.6–6.1) | 0.557 | <20 |
| Direct bilirubin                | μM Median (range) | 2.3 (2.0–4.4) | 2.7 (2.3–4.2) | 2 (2.0–4.4) | 2.0 (2.0–2.0) | 0.136 | <5 |
Table 1 (continued)

| Lipids         | Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|----------------|------|-----------------------|--------------------|-----------------------|----------------|---------|------------------|
| Cholesterol    | mM   | Median (range)        | 3.9 (2.4–8.8)      | 3.1 (2.4–4.7)         | 4.2 (5.5–8.8)  | 5.5 (5.1–5.8) | 0.264 | 5.0–6.4          |
| HDL            | mM   | Median (range)        | 1.0 (0.6–1.7)      | 1.0 (0.6–1.7)         | 1.1 (0.8–1.6)  | 1.2 (0.9–1.4) | 0.842 | >0.9             |
| LDL            | mM   | Median (range)        | 1.8 (1.0–6.1)      | 1.5 (1.0–3.0)         | 1.7 (1.4–6.1)  | 3.6 (3.1–4.0) | 0.264 | 3.5–4.4          |
| Triglycerides  | mM   | Median (range)        | 1.4 (0.8–3.1)      | 1.2 (0.8–1.5)         | 2.8 (2.7–3.1)  | 1.7 (1.3–2.1) | 0.036 | 0.8–1.94         |
| Free fatty acids | mM   | Median (range)        | 0.7 (0.2–2.0)      | 0.6 (0.5–0.8)         | 1.4 (0.2–1.9)  | 0.4 (0.2–0.7) | 0.325 | 0.1–0.6          |

| Proteins and inflammation Unit | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|-------------------------------|-----------------------|--------------------|-----------------------|----------------|---------|------------------|
| CRP mg/L                      | Median (range)        | 37 (1–212)         | 77 (2–212)            | 67 (16–87)     | 2 (1–3) | 0.145 | <10              |
| Total protein g/L             | Median (range)        | 61.1 (57.0–78.6)   | 60.7 (57.3–66.1)      | 61 (57.0–78.6) | 65 (62.5–66.9)| 0.246 | 60–80            |
| Albumin g/L                   | Median (range)        | 30.3 (15.9–36.9)   | 24.6 (15.9–32.1)      | 31 (20.6–36.9) | 32.7 (30.3–36.2)| 0.226 | 32–47            |
| Leucocytes 10^9/L             | Median (range)        | 7.6 (6.2–12.9)     | 10.9 (7.0–12.9)       | 8.1 (6.7–9.7)  | 6.4 (6.2–6.5) | 0.345 | 3.5–11           |
| Haemoglobin m/M               | Median (range)        | 7.7 (5.2–9.1)      | 6.8 (5.2–7.7)*        | 8.2 (6.0–9.1)  | 8.2 (8.1–8.4) | 0.008 | 7.3–9.7          |
| Glucose mM                    | Median (range)        | 5.8 (4.8–8.0)      | 5.4 (4.8–6.8)         | 5.9 (5.6–6.7)  | 7.2 (5.4–9.0) | 0.842 | 3.1–7.8          |
| Insulin p/M                   | Median (range)        | 46.3               | 28.7                  | 150.4          | 145.7   | 0.155 | 12–150           |

| Questionnaires                | All patients (n = 15) | Sarcopenia (n = 6) | No sarcopenia (n = 6) | Control (n = 3) | P-value | Reference values |
|-------------------------------|-----------------------|--------------------|-----------------------|----------------|---------|------------------|
| PG-SGA                        | Median (range)        | 12 (2–23)          | 11 (2–23)             | 13 (3–17)      | 8 (7–12) | 0.325 |                  |
| MNA                           | Median (range)        | 12 (6–14)          | 11 (6–14)             | 12 (12–12)     | 12 (12–13) | 0.773 |                  |

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; FIGO, International Federation of Gynaecology and Obstetrics; HDL, high-density lipoprotein; IMAT, intramuscular adipose tissue; LDL, low-density lipoprotein; MDRD, modification of diet in renal disease; MNA, Mini Nutritional Assessment; MRA, muscle radiation attenuation; PG-SGA, Patient-Generated Subjective Global Assessment; SAT, subcutaneous adipose tissue; SD, standard deviation; SMI, skeletal muscle index; VAT, visceral adipose tissue; γ-GT, γ-glutamyltransferase.

*For age-adjusted handgrip strength, see Table S1. For age-adjusted and BMI-adjusted SMI, see Table S2.

Statistical significance with post hoc Kruskal-Wallis testing, P < 0.05.
Analysis of signalling pathways

C2C12 cells stably transfected with a 6xB-TK luciferase plasmid (NF-κB reporter) were used for the assessment of NF-κB transcriptional activity, as previously described. After full differentiation, the cells were treated with ascites in DM (25% v/v, experimental condition) or with 1 nmol TNF-α in DM (positive control) or HBSS (25% v/v, control condition) for 8 h. NaHCO₃ and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma) were added to HBSS for buffering capacities (final pH 7.41). All compounds and media were pre-warmed to 37°C before they were added to the cells. Cells were lysed with 1× lysis buffer (Promega, Madison, WI, USA) (20% v/v in 1× DPBS), and protein lysates were scraped and stored at −80°C. Protein concentrations were measured with a BCA assay, and luciferase activity was determined according to the manufacturer’s instructions (Promega). Luciferase activity was normalized for protein concentration and expressed as a fold change of the benign control.

For analysis of MuRF1, Atrogin-1, REDD1, MYH1, MYH2, MYH4, and MYH7 gene expression, fully differentiated myotubes were treated with ascites in DM (25% v/v, experimental condition) or with HBSS (positive control) or with 100% HBSS as positive control for 24 h. RNA was isolated with TRizol Reagent (Thermo Fisher, Waltham, MA, USA), and RNA concentration was determined with a DS-11 microvolume spectrophotometer (DeNovix, Wilmington, DE, USA). RNA was reverse transcribed using the SensiFAST cDNA Synthesis Kit according to the manufacturer’s instructions (Bioline GmbH, Germany). cDNA was amplified with the SensiMix SYBR Hi-Rox Kit (Bioline) on a LightCycler 480 PCR platform (Roche, Almere, the Netherlands). LinRegPCR 11.0 software (Amsterdam, the Netherlands) was used to assess PCR efficiency. Expression of the genes of interest was normalized with a correction factor derived from several reference genes (cyclophilin A and β₂-microglobulin) using geNorm (qBase+, Belgium). Results are expressed as a fold change of the benign control (see Table S1 for primer sequences).

Statistical analysis

Baseline data between all groups were compared with one-way analysis of variance or Kruskal–Wallis tests where applicable. Relationships between continuous variables were tested with Spearman correlation coefficients. Logistic regression was performed for age and body mass index (BMI)-adjusted SMI. γ² testing was used for age-adjusted handgrip dynamometer strength. SPSS v23.0 (IBM Corp., Chicago, IL, USA) was used for all statistical analyses. A correlation matrix was used to visualize these correlations using R 3.6.1 for Microsoft Windows. GraphPad Prism v. 5.03 was used to make the box plots. Correction for multiple testing with the Kruskal–Wallis test was only applied to the experimental conditions for in vitro outcomes. P values < 0.05 were considered statistically significant.

Results

Cachexia-related patient phenotyping

The mean SMI of the sarcopenic group was 36.6 ± 2.9 cm²/m², which was significantly lower than the SMI of the non-sarcopenic (45.2 ± 2.4; P = 0.002) and benign control (41.7 ± 3.6; P = 0.05) groups. Although relative weight loss was higher in the sarcopenic vs. the non-sarcopenic vs. the benign control group, the differences were not significant (P = 0.16). The median age of the sarcopenic cancer patients was higher than the age of the non-sarcopenic cancer patients (68.0 vs. 59.5 years, P = 0.008). Conversely, mean BMI was lower in the sarcopenic group than in the non-sarcopenic group (22.4 vs. 29.0 kg/m², P = 0.011). However, when SMI was adjusted for BMI and age based on figures reported by van der Werf et al., all patients from the present study were above the 10th percentile for low skeletal muscle index in a healthy female population (Tables S2 and S3).

Mean triceps skinfold (1.30 cm) was lower in the sarcopenic group than in the non-sarcopenic group (1.98 cm, P = 0.005). C-reactive protein (CRP) levels tended to be higher in the group with a malignancy (P = 0.15), and serum albumin was markedly (but not significantly) lower in the sarcopenic group. These and other patient characteristics are listed in Table 1.

Figure 1 shows a correlation matrix that illustrates the correlation between body composition parameters and anthropometric measurements across all patients. As expected, SMI was significantly correlated with BMI (rₛ = 0.68, P = 0.005). Furthermore, triceps skinfold (rₛ = 0.83, P = 0.003), upper arm circumference (rₛ = 0.87, P = 0.001), serum albumin (rₛ = 0.64, P = 0.01), and subcutaneous adipose tissue (rₛ = 0.62, P = 0.01) were also all positively correlated with SMI. CRP levels were inversely correlated with SMI (rₛ = −0.61, P = 0.02). Patient-reported body weight loss was not associated with any of the other clinical cachexia-related parameters.

Ascites of sarcopenic ovarian cancer patients reduces protein synthesis in C2C12 myotubes

We first studied the direct effects of ascites on protein synthesis and protein breakdown using a radioactive isotope tracer method. Interestingly, 14C-L-phenylalanine incorporation in C2C12 myotubes treated with ascites from sarcopenic patients was markedly and significantly lower in comparison
with cells treated with non-sarcopenic or benign ascites (3.1 ± 0.1 vs. 5.5 ± 0.2 vs. 5.8 ± 0.7 nmol/h/mg protein, \( P < 0.01 \) and \( P < 0.01 \), respectively; Figure 2A). In contrast, no significant differences in intracellular protein breakdown rates could be detected among the groups, although a trend to higher protein breakdown was observed after exposure of C2C12 myotubes to ascites from sarcopenic patients (Figure 2B, \( P = 0.14 \)). In line with these protein metabolism measurements, we detected consistently lower expression of all isoforms of myosin heavy chain, the main contractile protein of skeletal muscle cells, after exposure to ascites, although the differences were not statistically significant (Figure 2C).
Protein synthesis and breakdown rates as well as expression of myosin heavy chain isoforms in C2C12 myotubes exposed to ascites from ovarian cancer patients. Correction for multiple testing was performed within experimental conditions (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). The control/non-treated condition was 25% Hank’s balanced salt solution (HBSS) in differentiation medium. (A) Protein synthesis rates. Amino acid incorporation in cells treated with ascites from sarcopenic patients was significantly lower when compared with that in cells treated with 25% ascites in differentiation medium from non-sarcopenic cancer patients ($P < 0.01$) or patients with a benign ovarian condition ($P < 0.01$). The positive control was treated with 100 nM insulin. ***$P < 0.001$. (B) Protein breakdown. No significant differences in proteolytic rates were detected between the groups. The positive control was treated with 10 μM dexamethasone. (C) Expression of myosin heavy chain isoforms. Although mRNA expression of MYH isoforms was consistently lower in cells treated with ascites from sarcopenic patients compared with control/non-treated conditions, no significant differences were observed between the groups.

Figure 2
Figure 3  Concentrations of cachexia-inducing factors in ascites of sarcopenic and non-sarcopenic ovarian cancer patients and non-sarcopenic controls with a benign ovarian condition. The box and whiskers graphs display medians with min to max ranges. Testing for statistical significance by Kruskal–Wallis test. Also see Table S4. (A) Interleukin-6 (IL-6) concentration in ascites. (B) Interleukin-8 (IL-8) concentration in ascites. (C) Myostatin [growth differentiation factor-8 (GDF-8)] concentration in ascites. (D) Growth differentiation factor-15 (GDF-15) concentration in ascites. (E) Monocyte chemotactic protein-1 (MCP-1) concentration in ascites.
No overt alterations in myotube morphology were observed after exposure to ascites (Figure S3).

**Concentrations of cachexia-associated factors in ascites of sarcopenic and non-sarcopenic ovarian cancer patients**

We next analysed ascites for the concentrations of key factors previously implicated in the regulation of protein imbalances in cachexia-associated sarcopenia (Figure 3A–3E and Table S4). There was a trend towards higher median concentrations of IL-6 and IL-8 in ascites from sarcopenic patients vs. non-sarcopenic patients [IL-6: 6.7 vs. 2.6 ng/mL (P = 0.06), respectively; IL-8: 2.0 vs. 1.4 ng/mL (P = 0.08), respectively]. Interestingly, IL-6 levels in ascites were inversely correlated to protein synthetic rates in vitro (r₂ = −0.66, P = 0.02). IL-8 concentrations in ascites were also strongly correlated with serum CRP levels (r₂ = 0.70, P = 0.01) and with body weight loss (r₂ = 0.69, P = 0.01; Figure 1). Furthermore, serum IL-6 concentrations tended to correlate to ascites IL-6 concentrations (r₂ = 0.64, P = 0.09), even though the levels of IL-6 in ascites were much higher (see Table S4). Median concentrations of growth differentiation factor-15 (GDF-15), GDF-8/myostatin, and monocyte chemoattractant protein 1 (Mcp-1) did not differ between the groups. Concentrations of TNF-α, IL-1β, and leukaemia inhibitory factor were only detectable in four different samples; hence, no statistical analysis was performed. The total volume of ascites was not significantly different between sarcopenic patients vs. non-sarcopenic patients vs. benign controls.

**Ascites of sarcopenic ovarian cancer patients induces NF-κB activity in C2C12 myotubes**

To test if factors present in ovarian cancer ascites directly induced disturbances associated with cachexia in skeletal muscle cells, we exposed differentiated C2C12 myotubes to ascites from sarcopenic and non-sarcopenic patients with malignant or benign ovarian tumours for 24 h and analysed several regulatory factors implicated in skeletal muscle protein metabolism. Whereas treatment with 100% HBSS as a positive control markedly increased expression of MuRF1 and Atrogin-1, ascites of neither sarcopenic nor non-sarcopenic patients affected mRNA levels of Atrogin-1 (P = 0.39) or MuRF1 (P = 0.80) (Figure 4A and 4B). Likewise, expression of REDD1 was not significantly affected by ascites from sarcopenic and non-sarcopenic ovarian cancer patients or from benign controls (P = 0.07, Figure 4C).

In contrast, NF-κB activity was higher in cells treated with ascites from sarcopenic patients (fold change 2.2 ± 0.4) than in cells treated with ascites from non-sarcopenic patients (1.2 ± 0.2, P = 0.01) or from benign controls (1.0 ± 0.1, P = 0.04) (Figure 4D). Relative expression of IL-8, an important target gene of NF-κB, showed the same pattern [2.46 ± 0.55 vs. 1.51 ± 0.37 (P = 0.02) or 0.99 ± 0.27 (P = 0.07) for cells treated with ascites from sarcopenic vs. non-sarcopenic patients or vs. benign controls, respectively]. Interestingly, in vitro protein synthesis and breakdown rates across the groups were strongly correlated to NF-κB transcriptional activity (r₂ = −0.60, P = 0.03 and r₂ = 0.67, P = 0.01, respectively). NF-κB activity across all groups was further strongly correlated to Atrogin-1 (r₂ = 0.63, P = 0.02) and REDD1 expression (r₂ = 0.82, P = 0.0003) as well as to IL-8 concentrations in ascites (r₂ = 0.62, P = 0.04).

**In vitro protein metabolism data correlate with cachexia-related patient phenotypes**

The simultaneous collection of ascites of patients and thorough phenotyping of their cachexia-related parameters allowed us to assess potential associations between the in vitro effects of ascites on skeletal myotubes and individual patient data. Importantly, a substantial number of sarcopenia-related patient measures were correlated to the in vitro protein synthesis and protein breakdown data (Figure 1). For example, C2C12 protein synthesis after ascites exposure was strongly correlated to triceps skinfold (r₂ = 0.88, P = 0.02) and upper arm circumference (r₂ = 0.80, P = 0.001). Conversely, in vitro protein breakdown was strongly inversely correlated to triceps skinfold (r₂ = −0.60, P = 0.04) and upper arm circumference (r₂ = −0.73, P = 0.007). Moreover, in vitro protein synthesis rates of ascites treated C2C12 myotubes strongly correlated with the SMI of the patients (r₂ = 0.70, P = 0.005). In line, in vitro protein breakdown rates of ascites-stimulated C2C12 myotubes (r₂ = −0.57, P = 0.04) were inversely correlated with their corresponding patient SMI. Thus, ascites from ovarian cancer patients alters protein turnover of skeletal myotubes in correspondence with the degree of sarcopenia of the patient providing the ascites.

**Discussion**

This study was performed to assess whether factors present in ascites of sarcopenic vs. non-sarcopenic ovarian cancer patients differentially affect protein metabolism in skeletal muscle cells and to determine if these effects are correlated to cachexia-related patient characteristics. Fifteen patients with a suspected malignancy of the ovary were included and underwent extensive physical screening; their ascites was used for in vitro exposure experiments. Ascites from sarcopenic cancer patients induced NF-κB activity and decreased protein synthesis in skeletal muscle cells. This was not observed with ascites collected from non-sarcopenic can-
Interestingly, several strong correlations between the in vitro effects of ascites factors and patient’s body composition as well as anthropometric measurements were observed, indicating that ascites represents a novel tool to investigate pathophysiologically relevant aspects of cachexia in vitro.

It is well established that sarcopenia is associated with adverse outcomes in ovarian cancer patients. As such, it is of major importance to identify the drivers behind muscle wasting in these patients. The application of ascites to skeletal muscle cells followed by monitoring of its effects on protein metabolism represents a first step towards the identification of the drivers behind muscle wasting in these patients.

**Figure 4** Expression of atrophy-related genes and NF-κB activity in C2C12 myotubes exposed to ovarian cancer ascites. Correction for multiple testing was performed within experimental conditions (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). (A) Relative mRNA expression levels of Atrogin-1 expressed as fold change of the non-sarcopenic benign control group. No differences in Atrogin-1 expression were detected between the groups. (B) Relative mRNA expression levels of MuRF1 expressed as fold change of the non-sarcopenic benign control group. No differences in MuRF1 expression were detected between the groups. (C) Relative mRNA expression levels of REDD1 expressed as fold change of the non-sarcopenic benign control group. No differences in REDD1 mRNA expression were detected between the groups. (D) Transcriptional activity of NF-κB expressed as fold change of the non-sarcopenic benign control group. NF-κB transcriptional activity was significantly increased in cells treated with ascites from sarcopenic patients when compared with cells treated with non-sarcopenic (P = 0.01) or benign ascites (P = 0.04). The control/non-treated condition was 25% Hank’s balanced salt solution (HBSS) in differentiation medium. *P < 0.05.
of factors inducing skeletal muscle protein metabolism aberrations in patients with ovarian cancer. Of note, tumour cell-derived conditioned medium has previously been used in similar \textit{in vitro} models of muscle wasting.\textsuperscript{24,25} In some of these studies, catabolic responses like myotube atrophy, up-regulation of Atrogin-1, NF-κB phosphorylation, and p38MAPK activation were observed after exposure of C2C12 cells to tumour-derived conditioned medium.\textsuperscript{24,26–28} Several advantages of using ascites over tumour cell-conditioned medium can be identified. First of all, ascites is a unique reservoir of highly concentrated tumour-derived compounds as well as systemic factors produced by metabolic tissues in response to tumour factors. Thus, combining ascites and tumour cell or organoid-conditioned medium approaches provides unique opportunities to distinguish between direct tumour-derived effects and indirect effects of metabolic tissue alterations caused by tumour factors from the same patient. Second, ascites is relatively easily accessible and available in large quantities. This enables sequential sampling during the course of cancer progression and treatment to study potential progression and treatment-related changes in factors that affect muscle protein metabolism. Third, acquired data can be correlated to clinical parameters when patients are thoroughly phenotyped before collection of ascites. Indeed, we observed several intriguing correlations between patient’s body composition and \textit{in vitro} ascites effects (low SMI and upper arm circumference correlated to low protein synthetic rates, NF-κB activation, and REDD1 expression) in the current study, suggesting that they are functionally relevant \textit{in vivo}. Nevertheless, it should be noted that there is no direct exposure of ascites to skeletal muscle in vivo, and concentrations of cytokines implicated in skeletal muscle wasting are much higher in ascites than in plasma or interstitial fluid.

Importantly, factors specifically present in the ascites of sarcopenic patients inhibited protein synthesis and tended to increase protein breakdown in mature myotubes, contributing to a negative protein balance that has been previously shown to be characteristic of muscle wasting in cancer cachexia.\textsuperscript{29} Previous mechanistic studies have suggested that the ubiquitin-dependent proteasome pathway underlies protein breakdown in skeletal muscle wasting.\textsuperscript{5} Two muscle-specific ubiquitin ligases, MuRF1 and Atrogin-1, have been shown to be increased in skeletal muscle under atrophic conditions in several disease models and to contribute to protein degradation.\textsuperscript{30,31} However, MuRF1 and Atrogin-1 expression were not affected by ascites in our experiments, despite a trend to increased protein breakdown induced by ascites from sarcopenic patients. This is in line with a recent report on a cohort of lung cancer patients where MuRF1 and Atrogin-1 did not appear to be involved in skeletal muscle wasting, and with our own recent data using pancreatic tumour organoids.\textsuperscript{32,33} Although gene expression profiles for atrophy-related genes have been established in experimental models of cancer cachexia, they are not a direct or functional measure of skeletal muscle protein turnover. We therefore focused on assessing actual skeletal muscle protein synthesis and breakdown using radioactive isotope tracing with \textsuperscript{14}C-L-phenylalanine, thereby extending the relevance of our findings. Our data show that ascites from patients with muscle loss has more impact on skeletal muscle protein synthesis than on protein breakdown, suggesting that future studies in the field should not only focus on proteolysis-inducing pathways but also investigate protein synthesis pathways.

In this context, the mammalian target of rapamycin complex 1 (mTORC1) is known to promote protein synthesis.\textsuperscript{34} In response to environmental factors like nutrient availability, hypoxia, and energy stress, mTORC1 function is altered. REDD1 expression is considered to be an inhibitor of skeletal muscle protein synthesis by affecting mTORC1.\textsuperscript{35} In our experiments, REDD1 mRNA expression was not affected by ascites. However, strong correlations between REDD1 mRNA levels and \textit{in vitro} protein breakdown, NF-κB transcriptional activity, and inverse correlations to handgrip strength, serum albumin, and upper arm circumference were detected.

NF-κB is involved in the transcription of numerous genes encoding cytokines, chemokines, as well as growth regulatory and survival genes. In the context of skeletal muscle atrophy, it has been shown that TNF-α-induced NF-κB activation prevents myogenic differentiation.\textsuperscript{9} Consequently, it would be interesting to explore the effect of ascites from sarcopenic patients on myoblast differentiation. In the current study of differentiated myotubes, ascites derived from sarcopenic patients increased NF-κB transcriptional activity. Moreover, several interesting correlations between NF-κB transcriptional activity and other protein metabolism-related factors including REDD1 expression, Atrogin-1 expression, and protein breakdown as well as protein synthesis were observed, in line with a prominent role for NF-κB in ascites-induced skeletal muscle metabolism aberrations.

Conclusions

Collectively, our data show that factors present in ovarian cancer ascites negatively affect protein balance in skeletal muscle cells \textit{in vitro}, closely reflecting sarcopenia-related and cachexia-related characteristics of the patient providing the ascites. This indicates that the \textit{in vitro} use of ovarian cancer ascites has high potential to dissect direct and indirect tumour-induced skeletal muscle wasting. Consequently, efforts should be made to identify the compounds responsible for skeletal muscle protein metabolism aberrations induced by ascites, for example, using proteomics and genomics approaches. This will ultimately help to identify novel drivers of cachexia in ovarian cancer patients and aid in the development of treatment strategies for cancer-induced skeletal muscle wasting.
Ethics statement

All authors certify that they comply with the ethical guidelines for authorship and publishing as laid down by the Journal of Sarcopenia Cachexia and Muscle.36

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Video S1. Supporting Information.
Figure S1. Overview of the protein synthesis experiment.
Figure S2. Overview of the protein breakdown experiment.
Table S1. Primer sequences for the genes of interest.
Table S2. Age adjusted dynamometer handgrip strength. Differences between groups were not significantly different, $p = 0.5$. *, one missing value. Based on: Dodds RM, Syddall HE, Cooper R, Benzeval M, Deary IJ, Dennison EM, Der G, Gale p = 0.5. *, one missing value. Based on: Dodds RM, Syddall HE, Cooper R, Benzeval M, Deary IJ, Dennison EM, Der G, Gale

Conflict of interest

None declared.

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Table S3. Reference values for age and BMI adjusted values of SMI based on a healthy kidney donor population: van der Werf, A., et al., Percentiles for skeletal muscle index, area and radiation attenuation based on computed tomography imaging in a healthy Caucasian population. Eur J Clin Nutr, 2018. 72(2): p. 288–296. Since BMI was significantly lower in the sarcopenic group whilst age was significantly higher, SMI values were compared against reference percentiles of a healthy cohort of female kidney donors. When adjusted for BMI and age, SMI values from the current cohort were all above the p5-p10 of the reference cohort. Logistic regression analysis revealed no significant association between BMI, age, and SMI.

Figure S3. Representative images of differentiated myotubes after 24 hours of exposure to the indicated experimental conditions.

Table S4. Concentrations of cachexia-associated factors in ascites and plasma. GDF 8; growth differentiation factor-8, GDF-15; growth differentiation factor-15, MCP-1; monocyte chemoattractant protein 1. Medians with ranges are presented. The Kruskall-Wallis test was used to assess statistical significance between all groups.
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