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Collagen fragment biomarkers as serological biomarkers of lean body mass – a biomarker pilot study from the DAHANCA25B cohort and matched controls

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Abstract

Background Loss of muscle mass and function is an important complication to ageing and a range of pathologies, including, but not restricted to, cancer, organ failures, and sepsis. A number of interventions have been proposed ranging from exercise to anabolic pharmacological therapy, with varying success. Easily applicable serological biomarkers of lean and/or muscle mass and change therein would benefit monitoring of muscle mass during muscle atrophy as well as during recovery. We set out to validate if novel peptide biomarkers derived from Collagen III and VI were markers of lean body mass (LBM) or change therein in head and neck cancer patients in the Danish Head and Neck Cancer Group (DAHANCA) 25B cohort subjected to resistance training as well as in an age-matched and gender-matched control group.

Methods Blood samples and dual X-ray absorptiometry data were measured at baseline, after 12 and 24 weeks in 41 HNSCC subjects of the DAHANCA 25B cohort of subjects recovering from neck and head cancer (stages provided in *Table 1*), and at baseline only in 21 healthy age-matched and gender-matched controls. Serum from blood was analyzed for the ProC3, IC6, and C6M peptide biomarkers and LBM were derived from the dual X-ray absorptiometry scans.

Results We were not able to show any correlation between biomarkers and LBM or C6M and anabolic response to exercise in recovering head and neck cancer patients. However, we did find that the biomarkers IC6, IC6/C6M, and ProC3 are biomarkers of LBM in the control group subjects (R^2/P of 0.249/0.035, 0.416/0.007 and 0.178 and $P=0.057$, respectively),

Conclusion In conclusion, the IC6, ProC3, and IC6/C6M biomarkers are indeed biomarkers of LBM in healthy individuals of both genders, but not in HNSCC patients.

Keywords Muscle; Biomarker; Head and neck cancer; Resistance training

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Introduction

Identifying pathological loss of lean mass and particularly muscle mass is an important task that may help target at-risk individuals for proper therapeutic interventions. This calls for development of novel early-response biomarkers of response to treatment, as these could help establish efficacy in clinical

trials of both pharmacological as well as physiotherapeutical interventions.

Rapid muscle loss, cachexia (literally, ‘poverty of flesh’), is a serious complication to most cancer diseases¹ and is a significant contributor to mortality and disability associated with cancers.^{2,3} Cachexia-inducing pathologies that impact the muscle mass do so through a number of endoparacrine and

paracrine mechanisms. These combine to induce anorexia, direct loss of muscle tissue, and resistance to anabolic stimuli like feeding and exercise, resulting in a net loss of lean body mass (LBM), predominantly muscle mass and strength, ultimately and negatively impacting daily life and quality of life of the patients.^{1,3} Loss of muscle mass may also manifest as a consequence of treatment, for example, radiotherapy or chemotherapy. This is particularly true for head and neck cancers, where the muscle loss is predominantly iatrogenic, caused by anorexia and dysphagia secondary to mucositis of the mouth and throat, caused by radiation therapy.^{2–5} This may be compounded by catabolic effects of most cytotoxic drugs used in combination with radiation therapy.⁶

Previous studies have shown that the serum levels of the Collagen type III propeptide (ProC3) levels in serum correlate well with LBM in young healthy men.⁷ As do the circulating levels of Collagen type VI peptides containing the IC6 epitope.⁸ In previous studies, we have shown that the anabolic response to reloading following immobilization was inversely related to the levels of the matrix-metalloproteinase (MMP)-generated Collagen type VI fragment C6M.⁸ Both Collagen types III and VI are known to be important constituents of the extracellular matrix of skeletal muscle.^{9–11} Therefore, fragments produced during muscle tissue turnover may quantitatively reflect the gross turnover and thus correlate with LBM. They could also be related to qualitative changes in metabolism associated with distinct physiological or clinical phenotypes, for example, fragments produced by a protease upregulated by cancer chemokines.⁸

Head and neck squamous cell carcinoma (HNSCC) patients have been shown to lose muscle mass as a consequence of their pathology and treatment, and therefore, they represent a physiological model in which muscle mass is already perturbed, making the model a candidate for muscle biomarker validation. Thus, in the present study, we tested whether the previous findings pertaining to the IC6 and ProC3 biomarkers could be reproduced or expanded in a group of HNSCC patients as well as a group of matched, healthy controls, both from the Danish Head And Neck Cancer Group (DAHANCA)25B study.^{6,12} This is a trial in which 41 HNSCC patients just after radiotherapy and chemotherapy treatment course were subjected to resistance training in order to restore muscle loss secondary to pathology or treatment. They were assigned to do exercise either immediately following their last treatment or 3 months later.

Thus, the purpose of the present study was to validate or expand our previous findings, i.e. (1) to confirm if ProC3 and IC6 are useful biomarkers of LBM in a population of recovering HNSCC patients and to elucidate if ProC3 and IC6 are biomarkers of LBM in a group of age-matched and gender-matched controls and (2) to investigate whether serum C6M is a biomarker of change in LBM in HNSCC patients subjected to resistance training.

Materials and methods

Setting and patients—head and neck squamous cell carcinoma patients

The data from patients enrolled in the DAHANCA 25B trial were included in this study. The DAHANCA 25B is a multicenter, randomized trial investigating the effect of 12 weeks of progressive resistance training on LBM in HNSCC patients. This study was approved by the Ethics Committee for the Central Denmark Region (id: 20110065), and the study was registered at clinicaltrials.gov (identifier: NCT01509430). All patients had completed radiotherapy with or without chemotherapy with curative intent according to the DAHANCA guidelines (www.dahanca.dk). Patients were included from the oncological departments at Aarhus University Hospital and Odense University Hospital, and all testing and blood sampling were conducted and repeated at the same local facilities for each patient. Full details on treatment are presented elsewhere.⁶ Baseline data on body composition from 41 patients from the DAHANCA 25B trials were included (Table 1). The subjects in the DAHANCA 25B trial were randomized and did 12 weeks of resistance training either immediately after completion of radiotherapy (early exercise, EE) or 12 weeks after radiotherapy (delayed exercise, DE). All executed the same 12 week progressive resistance training protocol.

Setting and patients—healthy individuals

Twenty-four healthy individuals were included in the DAHANCA 25B trial as controls. The recruitment of the healthy individuals took place concurrently with the inclusion of patients in the original DAHANCA 25B trial,^{6,12} and the procedure was conducted to

Table 1 Summary demographic data

| | Intervention group subjects | | | Controls |
|--------------------------|-----------------------------|-------------|-------------|-------------|
| | EE group | DE group | Pooled | |
| <i>n</i> | 20 | 21 | 41 | 21 |
| Age (years) | 55 ± 7 | 58 ± 7 | 56 ± 7 | 59 ± 6 |
| Gender (m/f) | 17 m/3f | 19 m/2f | 36 m/5f | 14 m/7f |
| Height (m) | 1.75 ± 0.09 | 1.77 ± 0.08 | 1.76 ± 0.08 | 1.76 ± 0.09 |
| Weight (kg) | 71.4 ± 17.2 | 73.9 ± 9.6 | 72.7 ± 13.7 | 76.3 ± 13.2 |
| BMI (kg/m ²) | 23.2 ± 4.1 | 23.6 ± 3.0 | 23.4 ± 3.6 | 24.4 ± 3.1 |
| LBM (kg) | 52.1 ± 9.9 | 54.3 ± 7.5 | 53.2 ± 8.7 | 54.9 ± 12.5 |
| Cancer stages | | | | |
| 1 | 3 (15%) | 1 (5%) | 4 (10%) | NA |
| 2 | 1 (5%) | 3 (14%) | 4 (10%) | NA |
| 3 | 2 (10%) | 2 (10%) | 4 (10%) | NA |
| 4 | 12 (60%) | 10 (48%) | 22 (54%) | NA |
| ND | 2 (10%) | 5 (24%) | 7 (17%) | NA |

Summary demographic data expressed as means ± standard deviations. Parts of these data have been published previously by Lønbro *et al.*⁶ ND means cancer stage is not defined for unknown primary tumours. BMI, body mass index; LBM, lean body mass; EE, early exercise; DE, delayed exercise; NA, not applicable.

match the population of HNSCC patients of the DAHANCA 25 trials with respect to age, gender and socio-economic status. Thus, asking all patients from the DAHANCA 25 trials to find family members, friends, or colleagues interested in participating provided twenty-four individuals. Three individuals were excluded in the present paper because of lack of serum samples, leading to a final number of control subjects of 21. Participants fulfilled the following inclusion criteria: (1) no current or previous malignancies, psychological, social, or geographical conditions that could prevent participation; (2) no self-reported excessive alcohol intake (men > 21 and women > 14 units/week); (3) no participation in resistance training of more than 1 h per week; and (4) a written consent. All interested individuals were contacted by telephone, and if they fulfilled the inclusion criteria, received both written and oral information before giving written consent. One of the interested individuals was excluded because of knee problems. The healthy controls were only sampled once, and not subjected to the resistance training protocol.

Resistance training protocol

The progressive resistance training protocol consisted of 30 sessions dispersed evenly over 12 weeks in a local commercial training facility. All patients received individual instruction 2–3 times during the initial five training sessions and individual continuous follow-up as needed (3–7 times) during the remaining training period. After two introductory training sessions with two sets at a load corresponding to 15 repetitions maximum (RM, e.g. 15 RM indicating the heaviest load that can be lifted 15 times using proper technique), volume and load progressed throughout the training period from two sets of 12RM towards three sets of 8RM. For full details on the training protocol, see Lønbro *et al.*¹³. When the HNSCC patients were not assigned to resistance training, they were advised to return to voluntary levels of physical activity. The effects of the resistance training intervention have been reported previously⁶ but are summarized for reference. Changes described in the succeeding texts are for the defined periods, for example, from T1 to T2 or T2 to T3. During the T1 and T2 periods, the EE group increased their LBM by 4.3% ± SD (2.3 kg; $P < 0.001$; 95% CI 1.7; 3.0) after the resistance training, which was 1.5 ± 0.5 kg larger ($P = 0.005$; 95% CI 0.5; 2.5) than the 1.5% change in the DE group after a 12-week period of self-chosen physical activity. During the T2 and T3 periods, the DE group, LBM, increased by 4.2% ± SD (2.4 kg; $P < 0.001$; 95% CI 1.1; 3.1) after resistance training (during the T2 and T3 periods), which was 2.1 ± 0.5 kg larger ($P < 0.001$; 95% CI 1.1; 3.1) than the 0.5% change in EE after self-chosen physical activity during the same period. In summary, an overall increase of approximately 4% in LBM was seen after resistance training irrespective of whether training was performed early or late.

Sampling

The control subjects and both intervention groups were dual X-ray absorptiometry (DXA)-scanned and had blood drawn at baseline, designated as T1. The intervention groups were scanned and had blood drawn also at the 12-week time point, designated as T2, and the final 24-week time point, designated as T3.

Body composition

Whole body LBM was evaluated using DXA with narrow fan beam technology (Aarhus site: Lunar Prodigy Advance, GE Healthcare Technologies, Madison, WI, USA; Odense site: Hologic QDR-Series, Hologic Inc., Bedford, MA, USA). The provided LBM is without bone mass.

Biomarker assays

All biomarker assays were competitive ELISA assays based on proprietary antibodies against the relevant peptides. The assays have previously been described.^{7,8,14} The reported intra-assay and inter-assay coefficients of variations (CVs) are for ProC3: 4.11 and 11.03%; for IC6: 11.1 and 11.8%; and for C6M: 4.1 and 10.1%.^{7,8,14} Each ELISA assay was conducted as follows: Streptavidin-coated 96-well ELISA plates were incubated with a biotinylated coater in 100 µL coating buffer for 30 min at 20°C on a shaker (at 300 rpm). Next, the plate was incubated with 20 µL of sample or standard/calibrator and 100 µL of HRP-conjugated monoclonal antibody diluted in Ab incubation buffer for a defined period of time in a fixed-temperature cabinet. After each incubation step, the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec cat. no. 438OH) was added and the plate was incubated for 15 min at 20°C in the dark. The TMB reaction was stopped by adding 100 µL stopping solution (1% H₂SO₄) and measured spectrophotometrically at 450 nm with 650 nm as the reference. Standard curves were generated by serial dilution of the calibrator peptide in each assay, pipetted on every plate and automatically fitted using a four-parameter fit ($y = (A - D) / (1 + (x/C)^B) + D$) model in Softmax Pro version 5 (Molecular Devices, Sunnyvale, CA, USA). These fits were used to calculate biomarker levels through curve regression.

Statistical analysis

Initial analysis of the data revealed that the biomarker data were non-normally distributed and displayed an upward skew characteristic of many biological traits, and we therefore, log₂ transformed data in order to approach normality. The LBM distribution did not display any skewed distribution and was not transformed.

Biomarkers data from the control group and the HNSCC subjects were compared at baseline in a mixed models analysis of variance. In this analysis, all HNSCC subjects were pooled, as they were not separated by different intervention courses yet and thus biologically comparable.

For changes over time, biomarker measurements were expressed as ratios relative to the baseline and subjected to two-way mixed models of analysis. Where significant group effects were seen, manual testing, subjected to Bonferroni correction was performed. ProC3, IC6, and IC6/C6M were correlated using linear regression to LBM assessed by DXA providing Pearson correlation coefficients. The C6M before and after resistance exercise periods were correlated to the change in LBM during resistance training rehabilitation using linear regression in order to identify whether individual biomarker levels were related to individual changes in LBM

Data were organized and transformed in Microsoft Excel for Mac 2011 (Microsoft Corporation, Redmond, WA, USA). The analysis of variance was performed in SAS/STAT software package 9.3 (SAS Institute). Correlations and Linear regression was performed using Prism (v6.00 for Mac and Windows). A significance threshold of 0.05 was selected for statistical testing.

Results

Biomarker levels at baseline

For the purpose of baseline comparisons, we pooled the intervention groups' baseline measurements prior to statistical analysis. We found no significant differences between the intervention group and the control group for IC6 and ProC3, nor for the ratio between IC6 and C6M, whereas for C6M, a highly significant difference ($P < 0.001$) was observed (Figure 1).

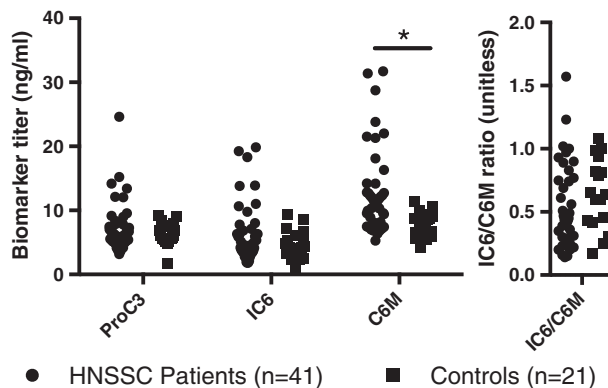
Biomarker levels across times

In the two-way mixed models analysis, significant main effects for time and borderline significant group effects could be observed for the IC6 biomarker (time: $P = 0.0161$; group: $P = 0.0569$) and the IC6/C6M ratio (time: $P = 0.0282$; group: $P = 0.0503$). However, post hoc testing revealed no significant differences at any particular time points (Figure 2)

Correlation between biomarkers and lean body mass at baseline

The linear regression analysis revealed significant correlations between IC6 or the IC6/C6M ratio and LBM in the healthy controls (Table 2 and Figure 3). Furthermore, we found a borderline

Figure 1 Biomarker levels at baseline—HNSCC vs. controls. Back-transformer biomarker levels at baseline between the HNSCC intervention group (pooled, termed 'HNSCC patients' in the figure, $n = 41$) and the matched healthy controls ($n = 21$). Dots represent individual values. An asterisk (*) denotes significant difference between the intervention group and the control group ($P < 0.05$). HNSCC, head and neck squamous cell carcinoma.



significant ($P = 0.057$) correlation between the ProC3 biomarker and LBM. However, we found no correlation on either a biomarker or an LBM in HNSCC patients at baseline (Tables 2 and 3).

C6M during resistance training periods

As we have previously shown that the C6M biomarker appeared to be related to the rate of LBM regain during remobilization following immobilization, we compared the levels of the C6M with the exercise-induced changes in LBM.⁸ We did this by comparing biomarker levels obtained before and after the resistance training periods in each group individually and pooled (T1 and T2 for the EE group and T2 and T3 for DE group) to the change in LBM for the same periods and found no significant correlations (Table 3). It does not appear as if the C6M biomarker is a biomarker of anabolic response to resistance training in recovering HNSCC patients.

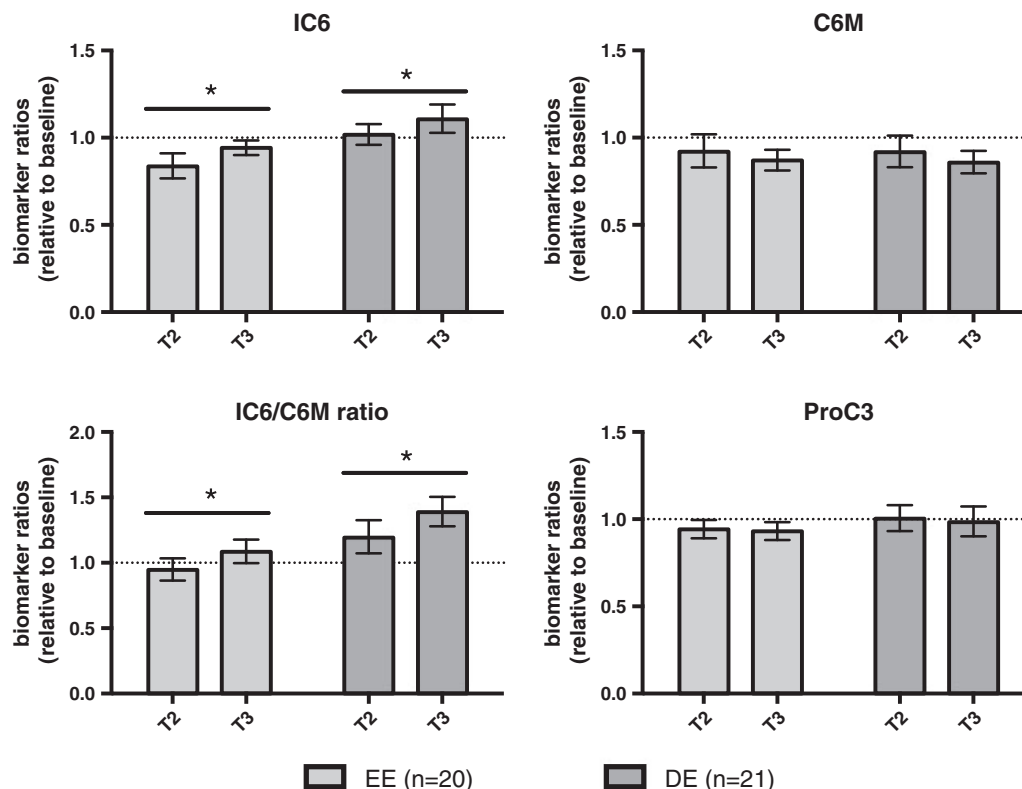
Discussion

In the present study, we measured biomarker levels in recovering HNSCC patients subjected to resistance training following primary treatment as well as in age-matched and gender matched controls. The purpose was to validate our previously reported findings, that is, that certain novel collagen-based peptide biomarkers are biomarkers of LBM or change herein.

Biomarkers at baseline

We found that levels of the C6M biomarker were significantly higher in HNSCC subjects than in the matched controls, that is, before they were exposed to resistance training (Figure 1).

Figure 2 Biomarker levels across times and groups. Biomarker levels at all time points expressed as ratios relation to baseline (T1) in early exercise (EE, *n* = 20) and delayed exercise (DE, *n* = 21) groups. Data are shown as geometric means and back-transformed standard error. Bar denotes significant time effects (*P* < 0.05). EE, early exercise; DE, delayed exercise.



In this study design, we cannot determine if this is derived from muscle or from other bodily compartments. In previous studies, this biomarker has shown also to be produced during tissue fibrosis in the lungs or in the liver.¹⁴ Therefore, we hypothesize that the elevated levels of the C6M in the intervention groups are consequences of either the disease or the treatment, rather than due to a contribution from muscle.

IC6/C6M, but upon post hoc analysis, no individual differences could be observed between time points (Figure 2). Therefore, we were not able to confirm the part of our hypothesis concerning our biomarkers possible being markers of change in muscle mass in HNSCC patients.

Biomarkers across time

The changes in biomarkers across times were of modest magnitudes and manifested as significant time effects for IC6 and

Biomarker correlations with lean body mass

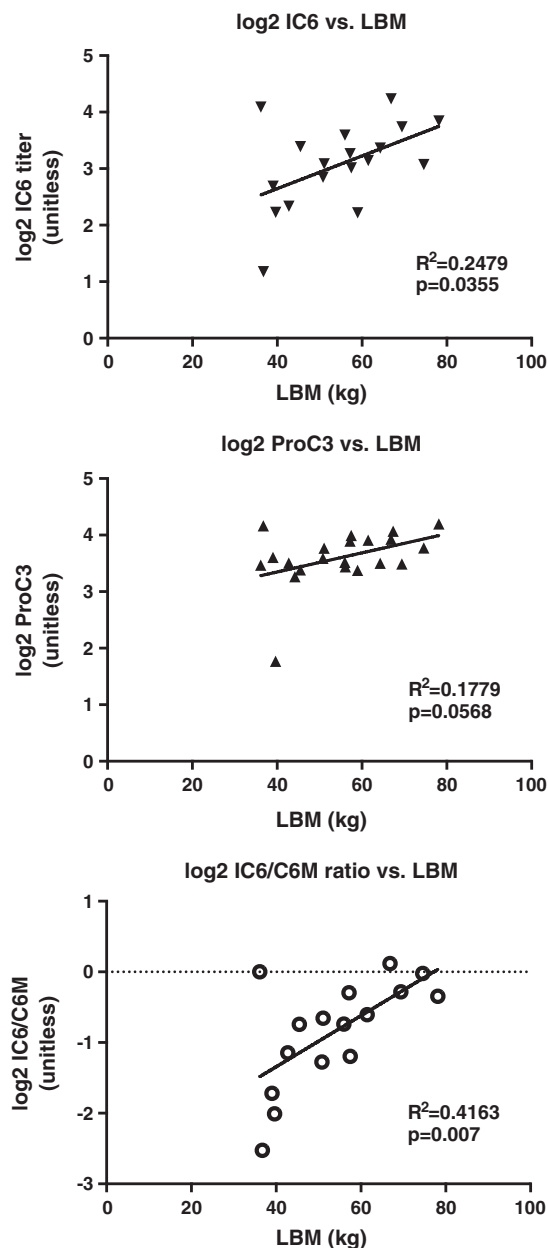
We have shown that the biomarkers IC6 and ProC3 and the ratio between IC6 and C6M all correlate with LBM at baseline in healthy humans of both genders and of ages in the range 27–69 (59 ± 6), but this was not the case in a cohort of recovering HNSCC patients (Figure 3). As for IC6 and ProC3, these findings are not only in agreement with our previous findings for these biomarkers but also expand the findings to a group of both genders and a larger age distribution, as the previous finding were confined to healthy young men.^{7,8} As the ProC3 is the pro-peptide of Type III collagen, it is produced and secreted during Collagen III synthesis. Our group has previously reported that this biomarker correlates with muscle mass in healthy adults in a steady-state condition.⁷ This indicates that a significant portion of the ProC3 neoepitope fragment in serum is possibly derived from continuous Collagen III

Table 2 Correlation matrix for selected biomarkers vs. LBM at baseline

| | EE/DE group pooled <i>n</i> = 41 | | Control group <i>n</i> = 21 | |
|--------------|-------------------------------------|----------|--------------------------------|----------|
| | <i>R</i> ² | <i>P</i> | <i>R</i> ² | <i>P</i> |
| Log2 ProC3 | 0.0034 | 0.717 | 0.1779 | 0.057 |
| Log2 IC6 | 0.0239 | 0.335 | 0.2479 | 0.036* |
| Log2 IC6/C6M | 0.0297 | 0.288 | 0.4163 | 0.007* |

The data shown are for the 'goodness of fit' coefficients provided by the linear regression analysis and their respective *P*-values. *denotes *P* < 0.05. EE, early exercise; DE, delayed exercise; LBM, lean body mass.

Figure 3 Selected biomarker correlations vs. LBM (control group). Correlations between log₂-transformed IC6, ProC3, and IC6/C6M biomarkers and LBM from healthy controls (*n* = 21) using a parametric linear regression, with curves showing the best fits and corresponding linear regression Pearson correlation coefficients and *P*-values. EE, early exercise; DE, delayed exercise; LBM, lean body mass.



production in muscle. Commercially available radio immuno-sorbent assays against the same peptide (PIIINP) (but using different antibodies) have detected increases in response to testosterone and/or human growth hormone therapy.^{15–17} As these treatments are known to increase muscle mass and intramuscular connective tissue in which collagen III is

Table 3 Correlation matrix for C6M vs RT-induced change in LBM

| EE group <i>n</i> = 20 | R^2 | <i>P</i> |
|--|--------|----------|
| C6M at T1 (before RT) vs. Δ LBM | 0.001 | 0.896 |
| C6M at T2 (after RT) vs. Δ LBM | 0.0097 | 0.698 |
| DE group | | |
| <i>n</i> = 21 | | |
| C6M at T2 (before RT) vs. Δ LBM | 0.007 | 0.764 |
| C6M at T3 (after RT) vs. Δ LBM | 0.028 | 0.551 |
| EE + DE pooled | | |
| <i>n</i> = 41 | | |
| C6M before RT vs. Δ LBM | 0.003 | 0.769 |
| C6M after RT vs. Δ LBM | 0.017 | 0.474 |

Correlation matrix for C6M levels prior to and after RT periods, for the EE and DE groups as well as for both groups pooled. EE, early exercise; DE, delayed exercise; LBM, lean body mass.

highly expressed, this substantiates the notion that a significant portion of the collagen III propeptide in serum is derived from muscle. The reason that we could not find any correlation between the biomarkers and LBM in the HNSCC subjects is most likely that either the cancer pathology or treatment have caused changes in extracellular matrix turnover that cause changes in the circulating levels of biomarkers that are separated from LBM or muscle mass.

Biomarker correlations to change in lean body mass

In our previous studies, we showed that the levels of the C6M biomarker was inversely related to subsequent LBM regain during retraining following immobilization.^{8,18} We compared the changes in LBM for the resistance training periods with the C6M levels before and after the corresponding period. We could not reproduce this finding in the present study (Table 3). Several factors may explain this discrepancy: Firstly, the time frames and sampling time points (3 months between measurements) in the present study could be wrong for this type of correlation. Secondly, we cannot exclude the possibility that because the initial study was of a limited sample size, the correlation between C6M and muscle regain could be a type II error. This is always a risk when doing correlation analyses on small sample sizes. In conclusion, the current study does not suggest that this particular cohort C6M is indicative of subsequent exercise-induced anabolism.

Limitations

Even though Collagen types III and VI have both been shown to be important constituents of skeletal muscle extracellular matrix, we cannot exclude the possibility that they are derived from extra-cellular matrix processing in other tissues. This is most likely also the reason that we did not see any relations

between biomarkers and LBM or change thereof in the present study. This is of course a fundamental issue related to serological biomarkers, and this is particularly true for cancer patients having received chemotherapy and radiotherapy, as both of these can affect connective tissue turnover and fibrosis processes, possibly providing new sources of biomarker peptides for the circulation.^{19,20} In order to circumvent this problem, we need to further characterize where and how these biomarker peptides are produced and metabolized.

The correlations between biomarkers and muscle mass are, although interesting, probably of limited clinical utility so far, as more precise methods are available to characterize muscle mass. Also, the correlations between LBM and the biomarkers are probably not strong enough to allow for assessing changes in muscle mass from repeated biomarker samplings, as the correlation between the biomarker is not strong enough to reveal small differences in muscle mass.

It should also be stated the DAHANCA 25B is not a big cohort, and as we did not perform a power analysis, we cannot exclude the possibility that a larger cohort would have revealed correlations that we did not find in this study.

In our study the experiment was conducted at two separate sites, using two separate DXA scanners. It has previously been reported that comparing results between DXA scanners without compensation for this difference is problematic at best.^{21,22} However, a newer study comparing similar types of scanner with those used in this study showed the discrepancies in measured LBM between them to be of a statistically insignificant size.²³

Conclusion

In conclusion, we were able to reproduce and thus validate part of our previous findings in showing that the ProC3 and

IC6 biomarkers are biomarkers of LBM in healthy adults, thereby expanding them to both genders and to a wider age range. However, we were not able to show that they are biomarkers of LBM in recovering head and neck cancer patients. Nor, were we able to confirm our hypothesis that C6M would be a biomarker of anabolic response to training during recovery from head and neck cancer.

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The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle. (von Haehling S., Morley J. E., Coats A. J. S., Anker S. D. Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle. *J Cachexia Sarcopenia Muscle* 2010; 1:7–8).

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Conflict of interest

M.K. is an employee of and shareholder in Nordic Bioscience, K.H. and A.N. are employees of Nordic Bioscience. The remaining authors state that they have no conflicts of interest.

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