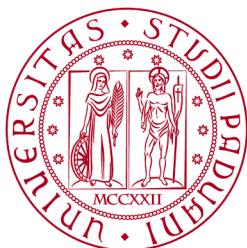


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:

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BMP AXIS IN CANCER CACHEXIA

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ABSTRACT

BACKGROUND

Cancer cachexia is a devastating metabolic syndrome characterized by systemic inflammation and massive muscle and adipose tissue wasting. Although cancer cachexia is responsible for about 25% of cancer deaths, no effective therapies are available, and the underlying mechanisms have not been fully elucidated.

Its occurrence complicates patients' management, reduces tolerance to treatments and negatively affects patient quality of life. Muscle wasting, mainly due to increased protein breakdown rates, is one of the most prominent features of cachexia. Blocking muscle loss in cachexia mouse models dramatically prolongs survival even of animals in which tumor growth is not inhibited.

Recent observations showed that bone morphogenetic protein (BMP) signaling, acting through Smad1, Smad5 and Smad8 (Smad1/5/8), is a master regulator of muscle homeostasis. BMP-Smad1/5/8 axis negatively regulates a novel ubiquitin ligase (MUSA1) required for muscle loss induced by denervation.

MATERIALS AND METHODS

First aim of the present work was to test if alterations of the BMP signaling pathway occur in cancer-induced muscle wasting in patients. For this purpose we checked the state of activation of the BMP pathway in muscle of cachectic vs non-cachectic patients affected by colon, pancreatic and esophagus cancer and in control subjects. We checked by Western Blot the phosphorylation levels of Smad1/5/8 and of Smad3 and by quantitative Real-Time PCR (qRT-PCR) the expression levels of different atrophy-related genes. The second aim was to evaluate the degree of muscle atrophy and distribution of muscle fibers in patients and control subjects using morphometric and immunohistochemical

analyses. We also performed analysis on distribution of NCAM positive muscle fibers to assess the effect of denervation on muscle tropism.

RESULTS

From December 2014 we collected 95 rectus abdominis muscle biopsies of cancer patients and 11 from control subjects. In line with the results we obtained in C26 mice model (a well-established cancer cachexia experimental model) Smad1/5/8 phosphorylation, readout of the state of activation of the BMP pathway, was nearly completely abrogated in the muscles of cancer cachectic patients compared to cancer non-cachectic ones. Interestingly, the level of phosphorylation of Smad3 was not significantly affected suggesting specific effects of cancer growth on BMP pathway. The expression levels of different atrophy-related genes including MUSA1 were induced in the cachectic muscles. Interestingly, several BMP related genes are also changing the expression during cancer growth. We also found a correlation between suppression of BMP pathway, expression of atrophy related genes and Noggin, known to block BMP pathway.

Morphometric analysis shown that patients with cancer cachexia have smaller myofiber diameter (in particular fast type fibers) in comparison to age-matched controls. In skeletal muscle from cancer patients (either cachectic or non-cachectic) we detected a prevalence of flat shaped, angulated and severely atrophic myofibers (i.e. morphological features of denervated myofibers), big fiber-type grouping (i.e. typical hallmark of denervation/reinnervation events) and numerous NCAM positive myofibers (i.e. specific marker of denervation).

CONCLUSIONS

These findings are consistent with the hypothesis that BMP inhibition is permissive to cachexia onset. Since the reactivation of the BMP-dependent signaling and MUSA1 suppression was sufficient to prevent tumor-induced muscle atrophy in our C26 mouse model (data not shown), the present data

suggest that the BMP axis can be an effective target for therapeutic approaches to counteract cachexia also in cancer patients.

The results of morphometric and immunohistochemical studies collected till now may suggest that denervation contributes to myofiber atrophy in cancer cachexia.

AIM OF THE STUDY

The aim of this study is to investigate the role of the BMP axis and its target gene MUSA1 in cancer cachexia in human cancer patients in order to understand if regulation of this pathway in muscle should be a rationale to prevent / counteract the loss of muscle tissue in cancer cachexia.

Secondary objectives are listed below:

- to assess the state of activation of the BMP pathway (level of phosphorylation of Smad1/5/8) and TGFb/myostatin pathway (level of phosphorylation of Smad3) in muscle biopsies from control patients, non-cachectic and cachectic cancer patients;
- to evaluate the expression of BMP dependent E3-ubiquitin ligase MUSA1 in muscle biopsies from control patients, non-cachectic and cachectic cancer patients and compare it with that of other ubiquitin ligases such as Atrogin1, MuRF1;
- to evaluate muscle morphology in the biopsies of control patients, pre-cachectic and cachectic cancer patients.

INTRODUCTION

1.1 CANCER CACHEXIA

1.1.1 DEFINITION

One in four of the general population will die from cancer, and cachexia affects the majority with advanced disease. The syndrome of cancer cachexia is multifactorial and cannot be fully reversed by traditional nutritional support. It is caused by a combination of reduced food intake and abnormal metabolism, seemingly induced by tumor- and host-derived factors. It is not known precisely how or why cancer so frequently develops in such a way as to induce cachexia.

Cachexia has commonly been considered a paraneoplastic syndrome in which tumor-derived factors induce widespread alterations in gene expression or metabolic flux that may function to release intermediate metabolites, which can then be used by the tumor for growth and expansion¹. In this model, cachexia can be considered a state of “autocannibalism” in which the tumor survives at the expense of the host. In contrast, many of the metabolic changes in cachexia are the result of activated immune and neuroendocrine responses that are common to trauma or sepsis and are thus more likely related to a generic

response to injury. In this model, cachexia may be regarded as the downside of a double-edged sword designed to respond to tumor-related noxious stimuli such as pain or tissue necrosis. However, the existence of tumors with similar growth patterns and identical origins, one of which induces cachexia while the other does not², implies that differences in a limited number of genetic events or gene expression may underlie the tumor phenotype associated with cachexia.

1.1.2 CLASSIFICATION AND STAGING

Cancer cachexia is a continuum (with three stages of clinical relevance: precachexia, cachexia, and refractory cachexia³).

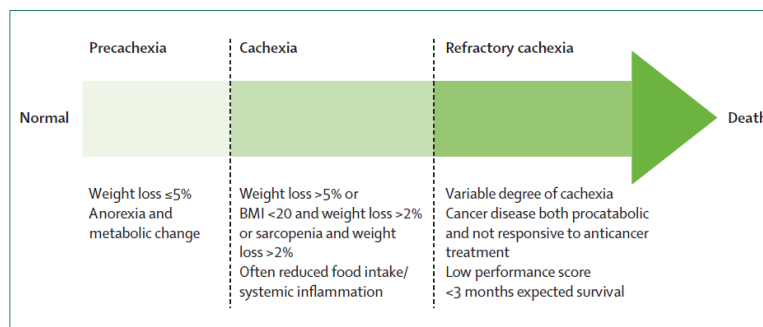


Figure 1 Stages of cancer cachexia

Not all patients traverse the entire spectrum. In precachexia, early clinical and metabolic signs (e.g., anorexia and impaired glucose tolerance) can precede substantial involuntary weight loss (i.e., $\leq 5\%$). The risk of progression varies and depends on factors such as cancer type and stage, the presence of systemic inflammation, low food intake and lack of response to anticancer therapy. Patients who have more than 5% loss of stable body weight over the past 6 months, or a body-mass index (BMI) less than 20 kg/m^2 and ongoing weight loss of more than 2%, or sarcopenia and ongoing weight loss of more than 2%, but have not entered the refractory stage, are classified as having cachexia. In refractory cachexia, the cachexia can be clinically refractory as a result of very

advanced cancer (preterminal) or the presence of rapidly progressive cancer unresponsive to anticancer therapy. This stage is associated with active catabolism or the presence of factors that render active management of weight-loss no longer possible or appropriate. Refractory cachexia is characterized by a low performance status (WHO score 3 or 4) and a life expectancy of less than 3 months. The burden and risks of artificial nutritional support are likely to outweigh any potential benefit. Therapeutic interventions focus typically on alleviating the consequences and complications of cachexia as symptom control (appetite stimulation, management of nausea or eating-related distress of patients and families).

The severity of depletion can be classified according to the rate of ongoing loss of weight in combination with the concurrent degree of depletion of energy stores and body protein mass (which can be compounded by a low initial reserve). Thus, a fall of 5 kg/m² in BMI from an initial value of 22 has more severe implications than the same loss from an initial value of 35. Furthermore, a patient with a BMI of 30 and a history of weight loss is more at risk if muscle wasting has led to sarcopenia, and less at risk if muscle protein mass remains intact.

1.1.3 CLINICAL PRESENTATION OF CACHEXIA

Cancer cachexia can vary according to tumor type, site, and mass⁴. The anatomical position of a tumor in the upper gastrointestinal tract may lead to obstruction and reduce food intake directly. The relationship between cachexia and tumor mass is complex. In many animal models the tumor grows quickly and reaches >10% of body mass acting as a “nitrogen trap”⁵. In humans, however, tumor burden is often <1% when there is profound cachexia, suggesting that the metabolic demands of the tumor are less important than distant metabolic effects induced by the tumor upon the host. For example, in pancreatic cancer,

high tumor IL-6 production has been associated with cachexia⁶, and increased levels of tumor-derived, or tumor-induced but host-derived, proinflammatory cytokines is perhaps the most common correlation between cancer and the prevalence of cachexia.

Even with the same tumor type and burden, one individual may become cachectic whereas another will not. Such variation may relate to host genotype. Genetic variation in immunity and associated signaling pathways is known to relate to outcomes in major sepsis⁷, and recent findings suggest a similar pattern in cancer cachexia. Single-nucleotide polymorphisms in the IL-1, IL-6, and IL-10 genes that are linked to production rates of these cytokines have been associated with the prevalence of cachexia in gastric or pancreatic cancer⁸. For example, the 1082G allele in the IL-10 promoter has been validated as a procachectic genotype in an independent cohort⁹. IL-10 has been shown to be elevated in a *Myc*/mTOR-driven murine model of cancer cachexia (Robert et al., 2012), as well as in cachectic patients with colorectal cancer (Shibata et al., 1996). Likewise, the C allele of the rs6136 polymorphism in the P-selectin gene has recently been associated with weight loss in a large heterogeneous group of cancer patients and validated in an independent cohort (Tan et al., 2012). Taken together, these findings are consistent with a key role for the immune system in the variable presentation of cachexia. However, currently no genome-wide studies in either animal models or patients have been performed.

The classical presentation of cachexia is of an extremely thin and wasted individual. However, heterogeneity in this clinical presentation is introduced by the current epidemic of obesity. When healthy individuals develop a chronic disease, the higher risk associated with obesity is reversed and obesity becomes “protective,” perhaps due to increased adipose and lean tissue reserves. This is known as the obesity paradox. Mean BMI of advanced cancer patients is now commonly measured at >25. There is, however, a subgroup of these overweight patients who hide gross muscle wasting under a mantle of adipose tissue. Approximately 40% of overweight or obese patients with advanced pancreatic

cancer have significant skeletal muscle wasting and this “myopenic or sarcopenic obesity” is an independent risk factor for accelerated demise¹⁰.

As skeletal muscle is a key target in cachexia, it is also relevant to consider heterogeneity as a result of sexual dimorphism. Men have greater muscle mass than women and one might assume that this greater “reserve” would be protective. However, weight loss and loss of muscle mass are greater in male than female cancer patients¹¹, and this may further relate to a high prevalence of hypogonadism in males¹². In fact, male lung cancer patients have shorter survival than women¹³.

1.2 MUSCLE ATROPHY IN CANCER CACHEXIA

Loss of skeletal muscle mass in cancer cachexia is generally due to reduced protein synthesis, increased degradation, or a relative imbalance of the two¹⁴. The signaling pathways that are thought to control these processes are shown in figure below.

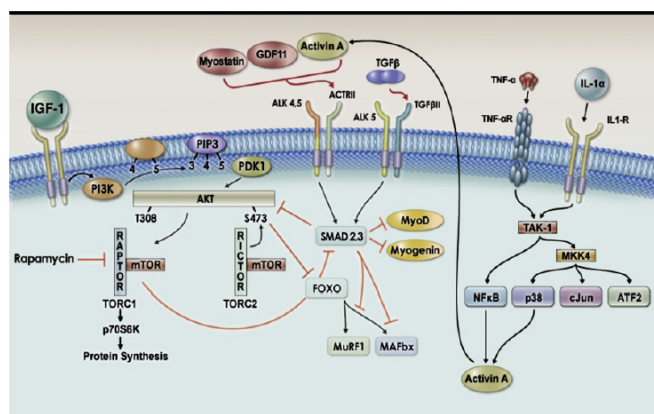


Figure 1 Atrophy and hypertrophy pathways in skeletal muscle skeletal muscle mass is controlled by the balance between atrophy and hypertrophy pathways.

One prominent subset of the pro-cachectic molecular mechanisms can be traced as follows: proinflammatory cytokines such as TNF α , TWEAK, or IL-1 signal into two established pathways, the NF- κ B pathway (weakly in the case of TWEAK) and p38 MAP kinase. These two signaling mediators are required to upregulate the expression of the key E3 ligases (muscle RING finger-containing protein 1, MuRF1, and muscle atrophy F box protein, MAFbx, otherwise known as Atrogin-1), which mediate sarcomeric breakdown and inhibition of protein synthesis¹⁴. MuRF1 is upregulated in multiple settings of muscle atrophy¹⁵. This E3 ubiquitin ligase is responsible for mediating the ubiquitination of the thick filament of the sarcomere—MyHC¹⁶, and other thick filament components¹⁷. The cytokine TWEAK, in particular, induces MuRF1 upregulation via NF- κ B, resulting in MyHC loss¹⁸. Inhibition of classical NF- κ B is sufficient to significantly decrease tumor-induced muscle loss, at least in mice, in part, by inhibiting the upregulation of MuRF1¹⁹.

The RING finger in MuRF1 binds zinc, which is required for its activity, as is the case with all RING finger-containing E3s²⁰. One recent study demonstrates that zinc accumulates during cachexia, speculating that this may help induce ubiquitination by zinc-dependent E3s²¹. MAFbx/Atrogin-1 also serves as a high-fidelity marker of acute muscle atrophy, being upregulated in multiple settings of cachexia²², in addition to immobilization, denervation, and glucocorticoid excess²³. MAFbx up-regulation occurs via p38 activation (Li et al., 2005) and by the induction of the C/EBP β transcription factor, which itself is activated through p38 phosphorylation²⁴. MAFbx induces the ubiquitination of an eIF3f, which is part of the protein translation machinery²⁵. However, it is not clear if this is sufficient to decrease protein synthesis. Some studies in cachectic tumor-bearing rats indicate that if amino acids are provided there is an increase in protein synthesis, but the breakdown of proteins outpaces this increase²⁶. If the tumor-bearing rats were nutritionally deprived of amino acids, a concomitant decrease in protein synthesis and an increase in protein turnover would be observed. The demonstration that both MuRF1 and MAFbx contribute to skeletal muscle atrophy was provided by studies of knockout animals—in the absence of either

MuRF1 or MAFbx, rates of atrophy are diminished. Thus, inflammatory cytokines secreted by tumors directly induce signaling pathways that upregulate enzymes that induce skeletal muscle protein turnover.

The E3 ligase Fbxo40 may be able to contribute to atrophy by inducing the ubiquitination of IRS1 and thereby short-circuiting the IGF1/IGF1R/IRS1 pathways and downstream protein synthesis activation²⁷. Fbxo40 is able to cause IRS1 to be degraded upon its phosphorylation by the IGF1R. Under settings where protein synthesis is ongoing, this would not be expected to decrease signaling in a sustained fashion, because the degraded IRS1 could simply be re-synthesized, and signaling could thereby continue. However, under conditions where protein synthesis is blocked, IRS1 would not be replenished, and IGF1 signaling would thereby be silenced. Some indication that this might happen is supported by a recent study suggesting that Fbxo40 is upregulated upon denervation. It remains to be determined whether this happens in other settings of muscle atrophy²⁸. Hypertrophy Signaling and Links with Atrophy Pathways

Opposing skeletal muscle atrophy are those pathways that induce muscle hypertrophy. One of the best-characterized mechanisms for inducing hypertrophy is through IGF1 (insulin-like growth factor 1) signaling. IGF1 is upregulated in skeletal muscle normally during resistance exercise²⁹, helping to explain why there is asymmetric hypertrophy of particular muscles depending on work and resistance. The pathway that mediates hypertrophy downstream of IGF1 activation is IRS1/PI3K/Akt³⁰. Under atrophy conditions, autophagy is induced in addition to ubiquitin-mediated proteolysis. However while blockade of ubiquitin signaling, for example using the proteasome inhibitor Velcade, has been demonstrated to result in healthy preserved muscle³¹, developmental blockade of autophagy results in pathologic muscle³². In addition to activating TORC1 signaling, Akt also phosphorylates the Foxo (or Forkhead) family of transcription factors. Foxo1 and Foxo3 play a key role in inducing transcriptional upregulation of MuRF1 and MAFbx³³. Apparently these transcription factors are required, since IGF1/Akt mediated Foxo phosphorylation and subsequent inhibition of Foxo transport to the nucleus is sufficient to block the upregulation

of the E3 ligases. In addition, transgenic overexpression of Foxo3 in skeletal muscle is sufficient to induce dramatic skeletal muscle wasting³⁴, while recent evidence supports that inhibition of Foxo spares muscle loss in a mouse model of cancer cachexia³⁵.

Links with Protein Synthesis and Degradation

The mass of protein within a muscle is regulated by the net interplay between protein synthesis and degradation. In rodent models of cancer-associated muscle wasting, both decreased synthesis and increased degradation have been described³⁶. There have only been a few direct measurements of protein synthesis in humans with cancer cachexia. Protein synthesis has been shown to be decreased³⁷ with only indirect evidence available on the issue of protein degradation.

Transcriptional activation of ubiquitin proteasome pathway (UPP) components has been found in the skeletal muscle of both rodent models³⁸ and patients with pancreatic, upper gastrointestinal (UGI), or liver cancer, and this was associated with increased protein or proteolytic activity *in vitro*³⁹. It has also been reported that skeletal muscle calpain, MAFbx, and MuRF1 mRNA remained unchanged in the skeletal muscle of gastric cancer patients, while calpain activity was elevated even in the absence of weight loss⁴⁰. In lung cancer patients, while UPP genes in skeletal muscle are not increased, elevation of cathepsin B mRNA has been observed⁴¹.

Using genome-wide transcript analysis of sequential quadriceps muscle biopsies in patients before and after curative surgery for upper gastrointestinal cancer, a recent study has shown that 1,868 genes were regulated in the cachectic state. Ontology analysis demonstrated that these gene products belonged to both anabolic and catabolic biological processes, which were overwhelmingly downregulated in the cachectic state. No literature-derived genes from preclinical cancer cachexia models (e.g., atrogenes) were found to be elevated in cachectic muscle⁴². These findings are consistent with the notion of a

predominant decrease in synthesis in the early phase of cancer cachexia. Interestingly, it has been suggested that MAFbx expression is a poor index of muscle proteolysis and may instead be predominantly linked with controlling protein synthesis⁴³.

In addition to regulating UPP, Foxo transcription factors directly regulate genes coding for the autophagy pathway, which like the ubiquitin system also contributes to the degradation of muscle proteins to promote atrophy. In autophagy, organelles are sequestered in autophagosome vacuoles that fuse with lysosomes and become digested by lysosomal enzymes⁴⁴. Autophagy genes and the lysosomal proteolytic system are activated during denervation and cancer, and in both cases contribute to atrophy through the activity of Foxo. A unique finding determined that analogous to Akt, Foxo3 is negatively regulated by PGC-1a⁴⁵. PGC-1a is itself downregulated in muscles from tumor-bearing mice and other wasting conditions, and trans- genic expression of PGC-1a rescues muscle loss in part by inhibiting Foxo3 and through the production of metabolic products. PGC-1a is highly induced during exercise, and one of the primary roles of this factor is to regulate mitochondrial biogenesis and oxidative phosphorylation in myofibers, characteristic of type I, fatigue-resistant, muscles⁴⁶. Interestingly, in cancer cachexia, muscle atrophy is selective to type II fast twitch myofibers, while type I oxidative fibers are relatively spared of similar catabolic effects⁴⁷. Therefore, it is possible that this sparing effect results from PGC-1a inhibition of Foxo3 activity in type I myofibers. Attempts to screen for PGC-1a activators represents a promising therapeutic avenue to prevent muscle atrophy in cancer or other catabolic conditions.

Since even in cachectic patients there is the experience of both a decrease in lean body mass (atrophy) and muscle weakness (loss of strength / functional parameters), we decided to investigate whether also in cachectic patients there is an involvement of the nerve or denervation phenomena that can contribute to muscle wasting that characterizes these patients.

1.3 BMP AXIS IN CANCER CACHEXIA

Skeletal muscle is a major site of metabolic activity and is the most abundant tissue in the human body. During development, the growth of skeletal muscle mass depends on protein and cellular turnover⁴⁸. In adulthood, skeletal muscle adapts its size and function to different physiological requirements and pathophysiological conditions, primarily by affecting pathways regulating protein turnover. In particular, the size of post-mitotic cells is determined by a balance between new protein accumulation and the degradation of existing proteins⁴⁹. Few pathways have been identified that regulate muscle growth in adulthood, and, among these, myostatin, a transforming growth factor (TGF)- β family member has a key role as a negative regulator^{50,51}. Myostatin signals via the activin type II receptors (ActRIIA and ActRIIB) and activin type I receptors (ALK4 and ALK5) to phosphorylate responsive Smad proteins (Smad2 and Smad3, Smad2/3), which enables the Smad proteins to form a transcriptional complex with Smad4 to transcribe target genes. Several genetic and biochemical studies have shown that inhibition of the myostatin-ActRIIB-ALK4/ ALK5-Smad2/3 pathway promotes muscle hypertrophy in adulthood^{52,53}. However, the target genes and mechanisms that drive muscle hypertrophy downstream of myostatin inhibition remain unclear.

BMPs are cytokines of the TGF- β superfamily that bind to dedicated BMP receptors (for example, ALK3) that in turn phosphorylate BMP-responsive Smad proteins (Smad1/5/8)⁵⁴. Similar to Smad2 and Smad3, BMP-dependent Smad proteins also form a transcriptional complex with Smad4 that translocates to the nucleus and regulates the transcription of target genes, including the inhibitor of DNA binding (ID) gene family. ID proteins (ID1, ID2, ID3 and ID4) belong to the E-protein family and control cell growth and differentiation⁵⁵. BMP signaling is mostly known for its roles in embryonic development and in bone/cartilage formation⁵⁴. In adult skeletal muscle, activation of BMPs has been associated with ectopic bone and cartilage formation and with diseases such as

fibrodysplasia ossificans progressiva⁵⁶. The role of endogenous BMP signaling in the homeostasis of adult tissues and its relationship with myostatin signaling remain largely unknown.

The study published by Sartori et al. in 2013 provides several new insights into signaling by TGF- β pathways and their contribution to the regulation of muscle mass in adulthood. BMP signaling resulted as the dominant pathway controlling muscle mass, even more so than myostatin. In particular, the hypertrophic phenotype caused by myostatin inhibition in fact results from unrestrained BMP signaling. This fits with a model in which a decrease in the levels of phosphorylated Smad2/3 leads to the release of Smad4, which is recruited into BMP signaling to promote hypertrophy and counteract atrophy. Conversely, when the BMP pathway is blocked or myostatin expression is increased, more Smad4 is available for phosphorylated Smad2/3, leading to an atrophy response. Therefore, under normal circumstances, a balance between these competing pathways is required to maintain muscle mass. The dramatic loss of muscle mass after denervation in *Smad4* knockout mice and following overexpression of noggin, that inhibits BMP pathway, strongly suggests that BMP signaling is also required to prevent excessive muscle loss under pathological conditions. Similar conclusions have been reached by an independent study on BMP signaling in skeletal muscle⁵⁷. The molecular mechanism underlying the anti-atrophic action of the BMP pathway relies on the negative effect it exerts on the expression of a newly characterized ubiquitin ligase, named MUSA1. This E3 ligase is not only required for protein breakdown in atrophying muscles but also exacerbates muscle atrophy in *Smad4*-deficient mice. Similar to other E3 ligases, MUSA1 undergoes autoubiquitination. Thus, it is reasonable to suggest that increased ligase activity of MUSA1 during denervation would amplify its autoubiquitination activity, resulting in increased proteasome-dependent degradation. Therefore, transcriptional upregulation is particularly important, mostly to replenish the loss of MUSA1 protein that occurs in denervated muscles as a consequence of increased MUSA1 activity. BMP signaling is indispensable for the regulation of adult muscle mass in normal and pathological situations,

highlighting its importance in developing new strategies for control.

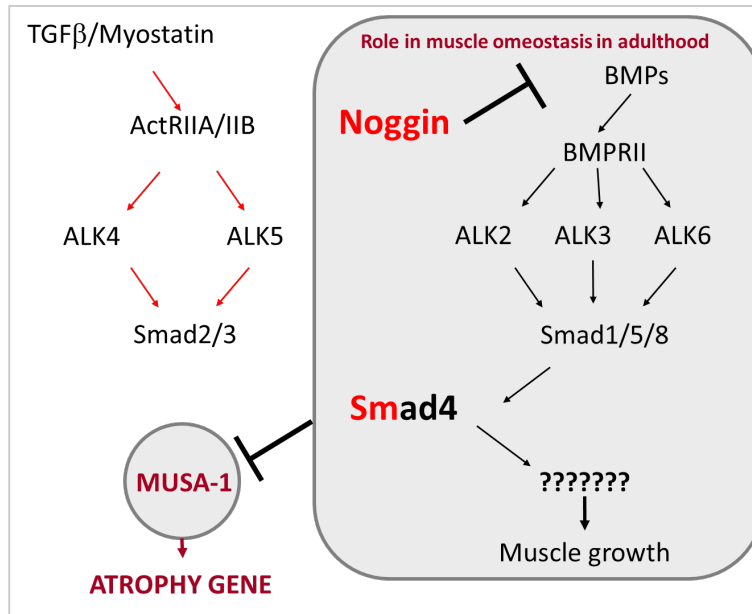


Figure 2 BMP pathway

1.4 ROLE OF DENERVATION IN MUSCULAR ATROPHY

Many of publications on relation between denervation and muscular atrophy are about denervation in aging. Aging is characterized by a gradual decline that impairs cell homeostasis and functional reserves. Histologic studies of skeletal muscle have shown that denervation is among the numerous mechanisms that contribute to tissue atrophy and degeneration in aging⁵⁸. The term “disseminated neurogenic atrophy” was coined to describe the progressive accumulation and clustering of small angulated fibers with aging; this phenomenon is associated with progressive loss of alpha motoneurons⁵⁹. Electrophysiologic studies have confirmed that there is a decrease in the number

of motor units with a concomitant increase in their size with age. These results suggest that some reinnervation events follow muscle fiber denervation⁶⁰. Further evidence supporting the occurrence of rounds of denervation and reinnervation includes the increased clustering of myofiber types in the motor units of rodents and other mammals as they age⁶¹. In adult humans, the incomplete reinnervation of fibers by surviving motor neurons, may contribute to loss of muscle strength and mass as people grow older⁶². These rearrangement processes are generally accompanied by a progressive increase in the proportion of slow muscle fibers, although there is some evidence to the contrary⁶³. Some of the discrepancies have been dispelled by comparisons of muscle from normally active and immobile older patients that show that muscle wasting in “normally active” seniors is accompanied by a shift toward a slow-twitch phenotype, whereas inactive seniors demonstrates a shift toward fast-twitch isoform expression. This latter case is common in “unloaded” muscle undergoing atrophy, for example, during limb suspension, immobilization, paralysis, and spaceflight⁶⁴. To complicate the situation further, conflicting results regarding fast to slow myosin transition arise in endurance training studies using animal models and in clinical trials of humans involving either voluntary exercise or electrical stimulation both directly to denervated muscle and indirectly to muscle through nerve stimulation^{65,66}. Furthermore, increased exercise that is sustained for decades protects against age-related loss of motor units and, thereby, of lean muscle mass⁶⁷. However, the degree to which denervation causes loss of myofibers is an open question because reinnervation events may compensate for motor neuron loss during aging as well as with spinal cord injury and/or axonal abnormalities of peripheral nerves. Whether the aging-related shifts are under neural control or the result of the direct influence of use/disuse on myogenic processes remains to be clarified. Histologic studies of skeletal muscle have shown that denervation is among the numerous mechanisms that contribute to tissue atrophy and degeneration in aging.

METHODS

2.1 STUDY DESIGN AND SELECTION OF PATIENTS

This is a spontaneous pathophysiological and observational single center study, on hospitalized patients with esophageal, colorectal and pancreatic cancer and control patients. The study enrolls patients with colorectal, esophago-gastric and pancreatic cancer surgically treated at 3rd Surgical Clinic of Padua University Hospital.

All patients join the project according to the guidelines of the "Declaration of Helsinki" and the research project has been approved by Ethical Committee for Clinical Experimentation of Provincia di Padova (protocol number 3674/AO/15).

The patients has been divided into cachectic and non-cachectic according to the definition of Fearon et al.⁶⁸ .: unintentional weight loss of greater than 5% of the weight over a period of 6 months or a weight loss of > 2% in the case of a patient with BMI <20 or with a diagnosis of sarcopenia. For each patient, the nutritional status is detected by calculating BMI, weight loss, food intake assessment, the dosage of inflammatory markers and biohumoral markers of protein turnover. For each patient it will also be recorded the administered cytoreductive therapy and home therapy.

The project includes the collection of a blood sample and one muscle biopsies (rectus abdominis). Blood and muscle tissue sampling is taken simultaneously at surgery.

All patients will then be inserted in a clinical and instrumental follow-up program of at least 60 months with recording data related to disease-free survival, overall survival and response to therapy.

The inclusion and exclusion criteria are listed in tables below:

Table 1 Selection criteria

SELECTION CRITERIA
- Males and female gender, older than 18 year, who have given their informed consent to the study
- Patients with esophageal, colorectal or pancreatic cancer at any stage according to TNM classification 7th
- - Patients undergoing exploratory surgery or resective surgery via midline laparotomy or thoracotomy
- The control subjects were patients of both genders, almost aged 18, suffering from non-neoplastic and non-inflammatory diseases candidates for surgery by median laparotomy or thoracotomy

Table 2 Exclusion criteria

EXCLUSION CRITERIA
- Patients younger than 18 years
- Patients with pancreatic, esophageal or colorectal cancer who are not candidates for surgery (e.g. High anesthetic risk)
- Patients affected by chronic inflammatory diseases or myopathies
- Patients affected by hemorrhagic diathesis and coagulation disorders
- Patients affected by severe cardiovascular diseases or diabetes

The study also enrolls control healthy donors undergoing elective laparotomy for non-neoplastic and non-inflammatory diseases, matched by age and gender to the cancer patients.

2.2 EXECUTION OF MUSCLE BIOPSIES AND SAMPLE CONSERVATION

The biopsies were performed during elective surgery by cold section of a rectus abdominal fragment of about 1x0.5 cm. Each fragment was immediately split in two pieces. One was immediately frozen and conserved in liquid nitrogen for biochemical and molecular and gene expression analysis, the other was frozen in isopentane cooled in liquid nitrogen and stored at -80C until use for morphological and histological analyses.

2.3 EVALUATION OF ATROGENES EXPRESSION

2.2.1 GENE EXPRESSION ANALYSIS

Total RNA was prepared from skeletal muscles using Promega SV Total RNA Isolation kit. Complementary DNA generated with Invitrogen SuperScript III Reverse Transcriptase was analyzed by quantitative real-time RT-PCR using SYBR Green chemistry (Applied Biosystems). The internal gene reference used in our real time PCR was alpha1 skeletal muscle actin, whose abundance did not change under the experimental conditions.

A relative quantification method was used to evaluate the differences in gene expression, as described by Pfaffl (Pfaffl, 2001).

Gene-specific primer pairs were selected with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi); sequences of distinct exons were chosen to avoid amplifying contaminant genomic DNA.

2.4 EVALUATION OF BMP PATHWAY ACTIVATION

Western blot

A small piece of muscle for every biopsies was powdered by tissue lyser in 100 μ l of the following lysis buffer:

- 50 mM Tris, pH 7.5
- 150 mM NaCl
- 5 mM MgCl₂
- 1 mM DTT
- 10% Glycerol (Sigma - Aldrich)
- 1 % Triton (Sigma - Aldrich)
- 1% SDS
- 1x Complete Protease Inhibitor Cocktail (Roche)
- 1 mM PMSF (Phenylmethanesulfonyl fluoride)
- 1 mM Na₃VO₄
- 5 mM NaF
- 3 mM beta-glycerophosphate

Then the solution was incubated at 1200 rpm for 10 min at +70°C. After centrifugation at 13,000 rpm for 10 min at +4°C, we measured the protein concentration of the surnatant using BCA™ Protein Assay kit (PIERCE) following

the manufacture protocol. 50 ug of the extracted proteins from rectus abdominis patients muscle were solubilized in 20 µl of LDS Sample Buffer 1X (NuPAGE® Life Technologies) and 50mM DTT. The samples were denaturated at 95°C for 5 minutes at 1250 rpm and loaded on SDS 4-12 % precast polyacrylamide gels (NuPAGE® Novex-Bis-Tris gels) (Invitrogen). The electrophoresis was run in 1x MOPS Running buffer (Invitrogen) for 1hour and 30 minutes at 150 V constant at r.t.

After electrophoretic run, proteins were transferred from gels to nitrocellulose membranes. The transfer was obtained by applying a current of 400mA for 1 hour and 30 minutes at +4°C. The following primary antibodies were used:

Anti phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467) (D5B10) Rabbit mAb #13820 by Cell Signaling Technology, anti phospho-Smad3 (Ser423 + Ser425) Rabbit mAb [EP823Y] (ab52903) by Epitomics and anti GAPDH mouse monoclonal (ab8245) by Abcam.

Immunoreaction was revealed by enhanced chemiluminescent method (SuperSignal® West Pico Chemiluminescent Substrate, Pierce). Blots were stripped using Restore™ Western Blot Stripping Buffer (Pierce) for 10 minutes at r.t. and reprobed if necessary.

2.5 MORPHOLOGICAL AND IMMUNO-HISTOCHEMICAL ANALYSIS

Serial cross-sections (8 um thickness) from frozen muscle biopsies were mounted on polysine glass slides, air-dried and used for further analyses. For morphometric analysis, the mean muscle fiber diameter was evaluated in H&E-stained cross-sections in accordance to Refs.8 and 9 using Scion Image software for Windows, version Beta 4. 0.2 (2000, Scion Corporation, Inc.; <http://www.scioncorp.com>). Slides images were acquired using a Zeiss microscope connected to a Leica DC 300F camera at low magnification; identical

conditions were used to acquire reference ruler images. On the acquired images, nuclei were counted and categorized as located inside or outside the muscle fiber, within the extracellular matrix.

To give an expression of the number of very small fibers, the atrophy factor was calculated in muscle biopsies as described by Dubowitz (1985). To put the results in a proportional basis, the total number of fibers having diameter between 40 and 30 μm , 30 and 20 μm , 20 and 10 μm , and less than 10 μm was counted and multiplied by one, two, three, and four, respectively. The products were then added together and normalized to the total number of analyzed myofibers.

To evaluate muscle fiber type distribution, conventional techniques were used to stain serial cross-sections for myofibrillar ATPases as described⁶⁹. Slow-twitch muscle fibers are visualized as dark after pre-incubation at pH 4.35, while light fibers are of fast type.

For immunofluorescence analyses, unfixed muscle sections were labeled for 1 hour at room temperature using rabbit polyclonal antibody directed against neural cell adhesion molecule (N-CAM) (Chemicon, Millipore, Milan, Italy), 1:200 diluted in phosphatebuffered saline (PBS) as described⁹. Sections were rinsed 3 X 5 minutes in PBS, and then incubated for 1 hour at room temperature with Cy3-labeled conjugates directed against rabbit IgG (SigmaAldrich) 1 : 200 diluted in 10% goat serum/ PBS. Negative controls were performed by omitting the primary antibodies on samples. Coverslips were mounted onto glass slides using ProLong Gold anti-fade reagent with DAPI to counterstain nuclei (Life Technologies) and observed under the fluorescent microscope. Images were acquired as described above, and the number of N-CAM-positive myofibers were counted and expressed as the percentage of positive myofibers relative to the total number of fibers within the analyzed section.

2.6 DATA COLLECTION AND STATISTICAL ANALYSIS

The statistical significance of data collected was determined using a Student's *t* test (Microcal Origin_ 6.0; Microcal Software, Inc.) and a chi-squared test (Microsoft_ Office Excel_ 2007; Microsoft Corporation). Statistical analysis of morphometric datasets was performed with GraphPad Prism v5.0 software; statistical significance of average numbers was determined using Wilcoxon matched pairs test. Values of $P < 0.05$ were considered significant.

Statistical analysis of densitometric and gene expression datasets was performed with GraphPad Prism v6.0 software. Data were analyzed by two-tailed Student's *t* test or one-way ANOVA and were applied upon verification of the test assumptions. For all graphs, data are represented as mean \pm SD.

RESULTS

3.1 STUDY POPULATION

From December 2014 to 2016 we collected rectus abdominis muscle biopsies from 93 patients suffering from gastro-enteric neoplasms, treated at 3rd Surgical clinic of University of Padua. They underwent median laparotomy surgery. In the same period we collected biopsies from 11 control subjects who underwent laparotomy for non-neoplastic and non-inflammatory diseases.

The average age of patients was 67.8 years, 56 (60.2 %) of them were men and 37 (39.8 %) were women. The average age of control subjects was 67.4 years.

Twenty-five (26.9 %) of them were affected by colorectal cancer, 34 (36.6 %) of them by pancreatic cancer, 27 of them were affected by esophago-gastric cancer (29.03 %) and 7 (7.5 %) by other cancer type.

Table 3 Study population – demographic features

CANCER PATIENTS (tot 93)		
	<i>n</i>	%
<i>Colo-rectal</i>	25	26,9
<i>Pancreatic</i>	34	36,6
<i>Esophago-gastric</i>	27	29
<i>Other</i>	7	7,5
<i>MALE</i>	56	60,2
<i>FEMALE</i>	37	39,7
<i>AGE (mean)</i>	67,8	

Mean BMI was 24.1 kg/m² (15.2 – 32.1) and 16 patients has a BMI lower than 20. The average loss of weight in the 6 months before observation was 6.5

% . Thirty-two patients (34.4 %) didn't lose weight during observation. According to Fearon definition of cancer cachexia, 39 patients (41,9%) were cachectic, 38 patients (40,9%) were non-cachectic. Twenty-five percent of colorectal patients were cachectic, versus 58,8% of pancreatic cancer patients and 60% of other cancer type patients (*p* n.s.).

At the beginning of the study we performed comprehensive preliminary analysis of all patients, however, we immediately found, among patients with gastric and esophageal cancer, a high variability and heterogeneity in the results obtained possibly due to the presence of severe dysphagia and the presence of nutritional support, as like as naso-gastric tube. We have therefore decided to consider the data of these patients as a possible source of BIAS for the study and therefore to exclude them from the study including in the final data analysis, only colorectal and pancreatic cancer patients, (59 patients overall) two malignancies in which cachexia reaches 80% incidence. The analysis of all biopsies collected is still in progress and the results obtained by the samples analyzed so far are shown.

3.2 BMP PATHWAY ACTIVATION ANALYSIS

Since inhibition or reduction of BMP signaling in mice induces an exacerbated muscle loss that mimics cachexia, in the first phase of the study we monitored the status of activation of BMP pathway in muscles of cancer patients by examining the phosphorylation levels of Smad1/5/8, the downstream effectors of BMP. Very preliminary results indicate that the BMP pathway is downregulated in muscles of cachectic patients compared to age-matched non cachectic ones in both colorectal and pancreatic patients.

The suppression of BMP pathway is in line with the hypothesis that this pathway is required to prevent excessive muscle loss and to maintain muscle mass.

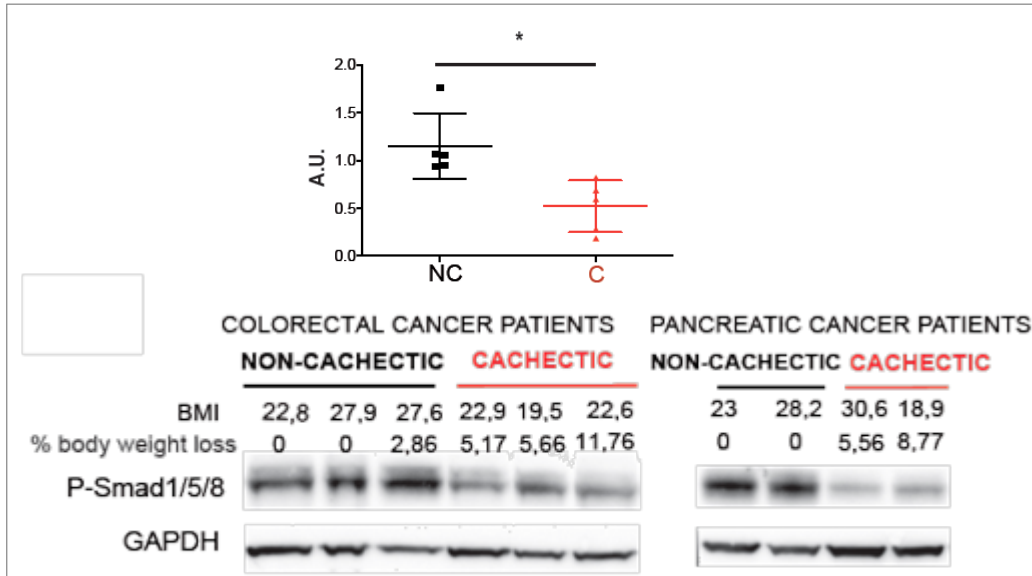


Figure 3 Activation of BMP pathway in colorectal and pancreatic cancer patients. Densitometric quantification (above) and western blot (below) of phosphorylation levels of Smad1/5/8 (BMP's downstream targets) in rectus abdominis muscle of non-cachectic (NC) and cachectic (C) cancer patients (very preliminary results).

On the contrary, we didn't find any significant changes of pSmad3 (downstream effector of Myostatin/TGFb pathway) levels between cachectic and non-cachectic patients.

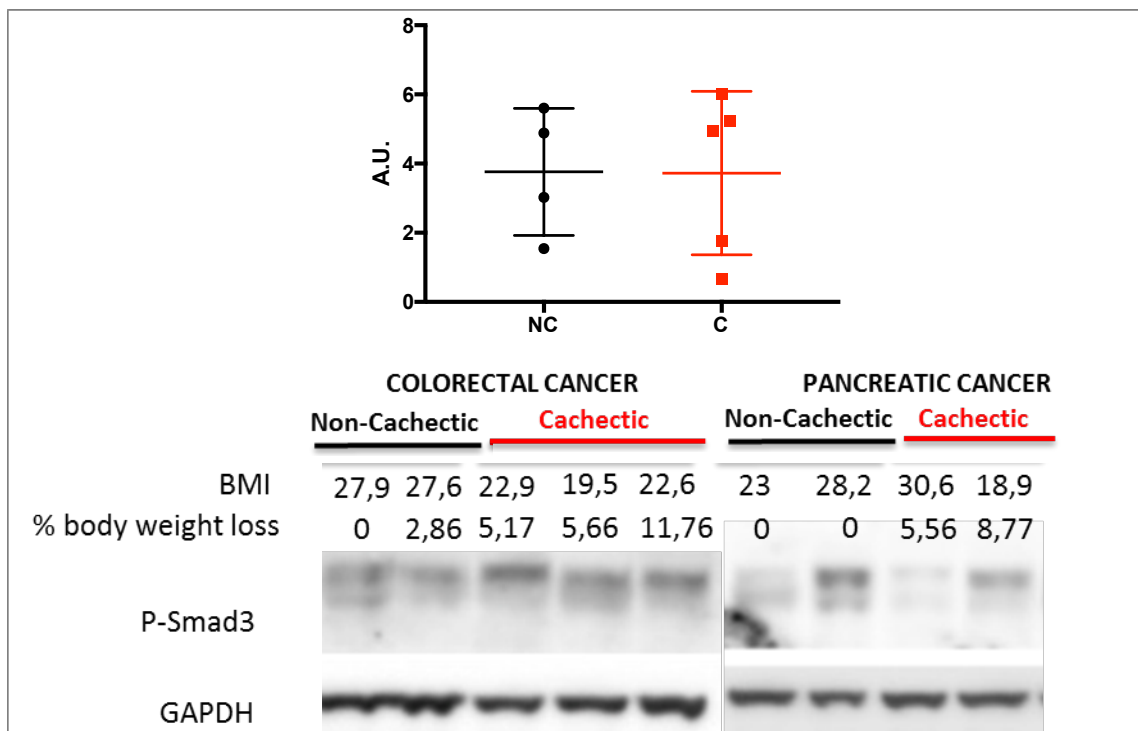


Figure 4 Activation of Myostatin/TGFb pathway in colorectal and pancreatic cancer patients. Densitometric quantification (above) and western blot (below) of phosphorylation levels of Smad 3 (Myostatin/TGFb downstream targets) in rectus abdominis muscle of non-cachectic (NC) and cachectic (C) cancer patients (very preliminary results).

We need to stress the fact that these are very preliminary results, we need to increase both the number of cancer patients and of control patients analyzed in Western Blot analysis. We had little material from control patients, that we used for real time PCR only and we are collecting other control patients' byopsies.

3.3 NOGGIN AND ATROGENES EXPRESSION

To explain why Smad1/5/8 are inhibited we monitored the expression levels of Noggin, a well-known inhibitor of BMP pathway. Noggin is an extracellular antagonist of BMP ligands preventing their binding to the receptors. The expression of Noggin was highly increased in muscles of cachectic patients compared to non-cachectic and control ones thus potentially explaining why BMP pathway is blocked during cancer progression.

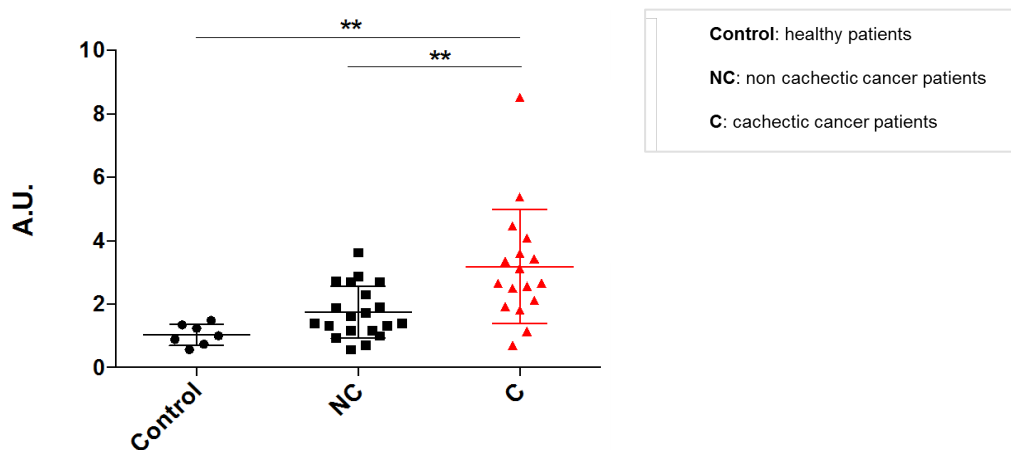


Figure 7 Real time PCR analysis of Noggin expression levels in rectus abdominis muscles of healthy control individuals, non-cachectic (NC) and cachectic (C) colorectal and pancreatic cancer patients. n=7, 20 and 17 respectively, **p<0.01

Interestingly, there is a significant positive correlation between the expression of Noggin in muscle tissue and the percentage of body weight loss in the patients analyzed.

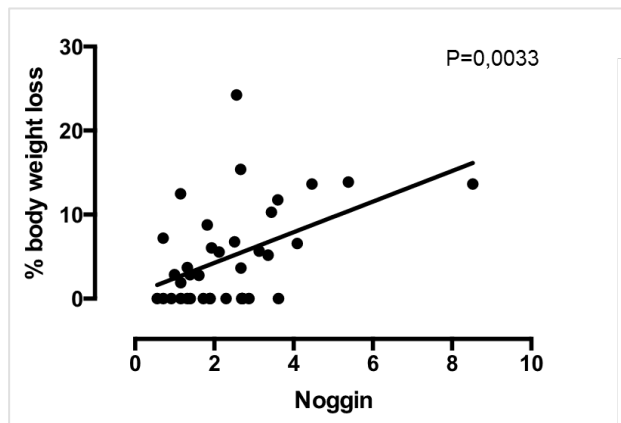


Figure 8 Noggin expression and percentage of body weight loss correlation study.

Since we have shown that the BMP pathway negatively regulates the expression of a novel ubiquitin ligase (MUSA1), we tested whether MUSA1 was also induced in cancer cachexia and we compared its expression with the expression of the well-known atrophy-related muscle-specific ubiquitin ligases atrogin1 and MuRF1. While we could not detect any significant difference in the expression of atrogin1, both MuRF1 and MUSA1 were significantly up-regulated in the muscles of cachectic patients compared to non-cachectic ones and to control subjects.

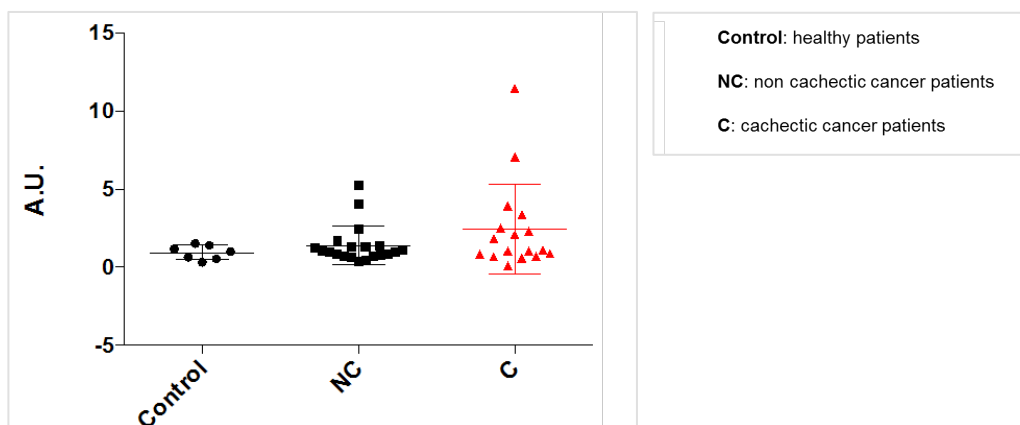


Figure 9 Atrogin-1 expression revealed by qRT-PCR in muscles of healthy control subjects, non cachectic (NC) and cachectic (C) colorectal and pancreatic cancer patients. n=7, 20 and 17 respectively.

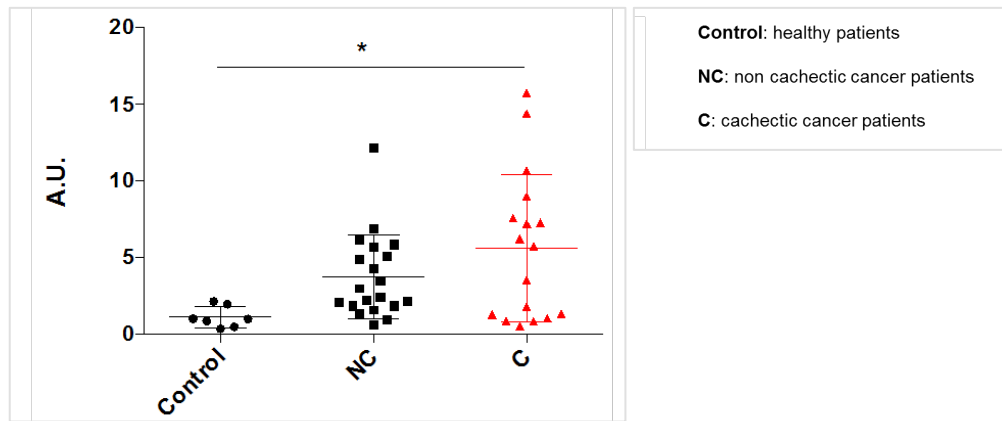


Figure 10 MuRF-1 expression revealed by qRT-PCR in muscles of healthy control subjects, non cachectic (NC) and cachectic (C) colorectal and pancreatic cancer patients. n=7, 20 and 17 respectively. *p<0.05

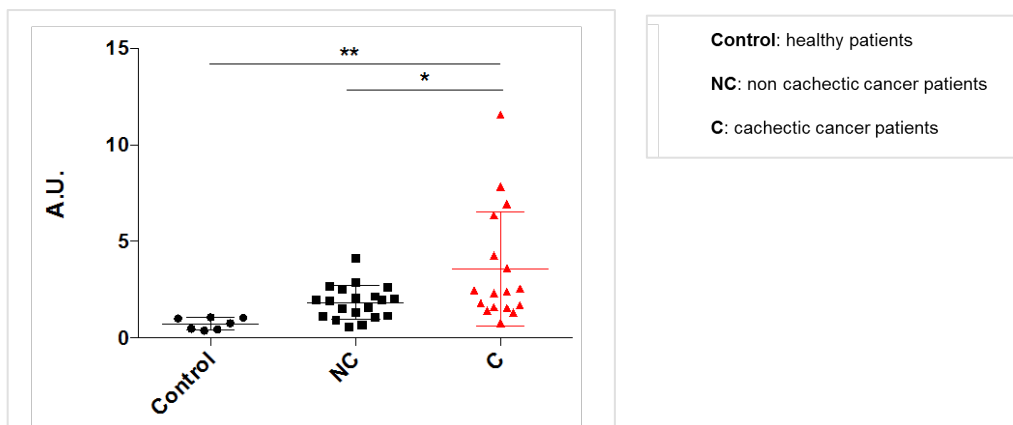


Figure 11 MUSA-1 expression revealed by qRT-PCR in muscles of healthy control subjects, non cachectic (NC) and cachectic (C) colorectal and pancreatic cancer patients. n=7, 20 and 17 respectively. *p<0.05, **p<0.01

These results have statistical significance especially in the case of MUSA1, whose up-regulation is in line with the observation that BMP pathway is inhibited during cancer progression. Moreover, the analysis of correlation between the percentage of body weight loss and the atrogenes expression demonstrates a significant positive correlation between the expression levels of MUSA-1 and the percentage of body weight loss. On the contrary, no significant correlation was found between the percentage of body weight loss and the expression of the other examined atrogenes, namely Atrogin1 and MuRF1.

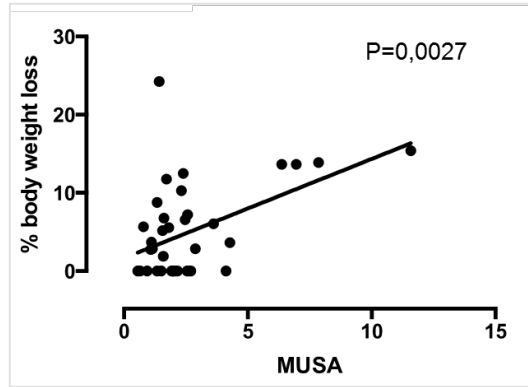


Figure 12 MUSA-1 expression and % of body weight loss correlation

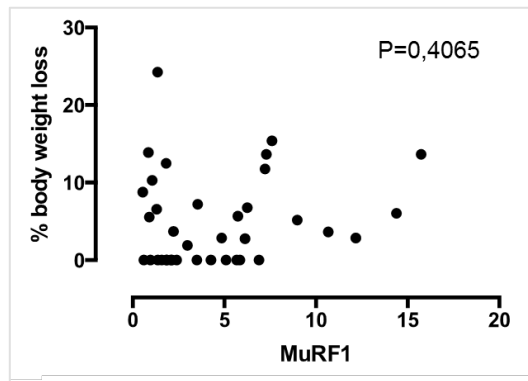


Figure 13 MuRF-1 expression and % of body weight loss correlation

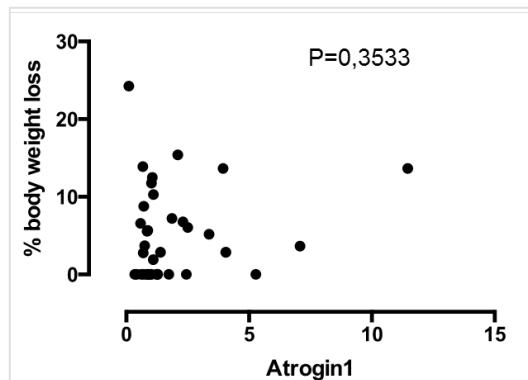


Figure 14 MuRF-1 expression and % of body weight loss correlation

Interestingly, there is also a significant positive correlation between Noggin and MUSA1 expression in the muscles of the patients analyzed.

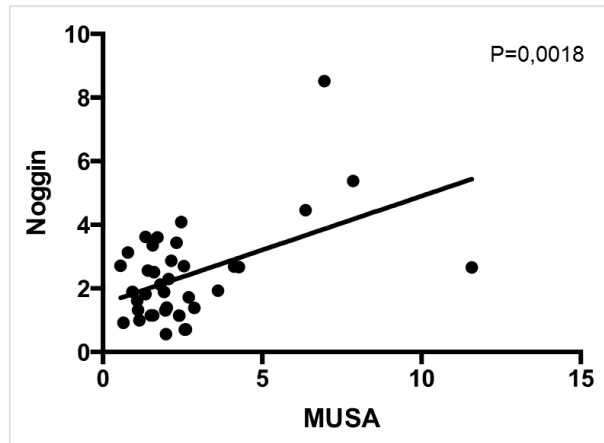


Figure 15 Correlation between Noggin and MUSA1 expression

3.4 MORPHOMETRIC AND IMMUNO-HISTOCHEMICAL ANALYSIS

Histological analyses of muscle biopsies from cancer patients showed numerous severely atrophic, flat shaped and angulated fibers, as typical signs of denervation. These morphological aspects were only seldom detected in muscle biopsies from control subjects, indicating that denervation events were less frequent.

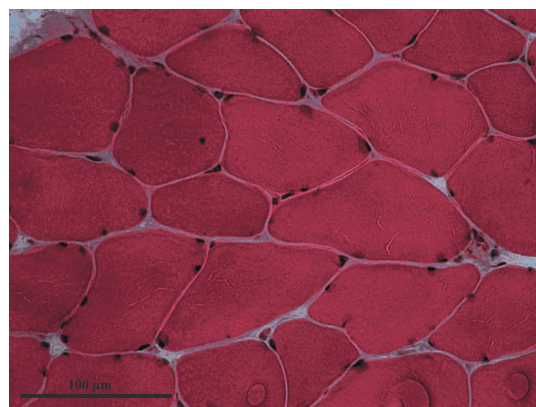


Figure 16 H&E staining of muscle biopsy in control subjects

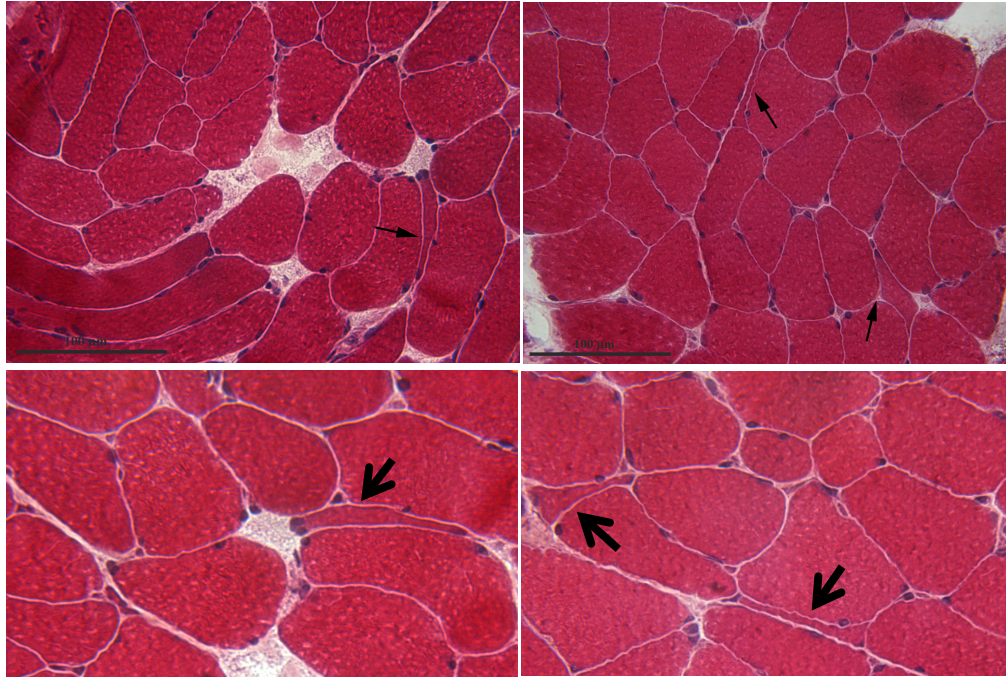


Figure 17 H&E staining in muscles of cancer patients reveals the presence of flat shaped, angulated and severely atrophic myofibers as indicated by arrows.

The calculation of the mean myofiber diameter showed a general shift to smaller values in muscle biopsies from cancer patients in comparison to age matched controls, as indicated by the myofiber diameter distribution reported in the graph here below. This shift is particularly evident in cachectic patients as revealed by the atrophy index. Cachectic patients are characterized by higher atrophy index compared to non-cachectic and age matched control subjects.

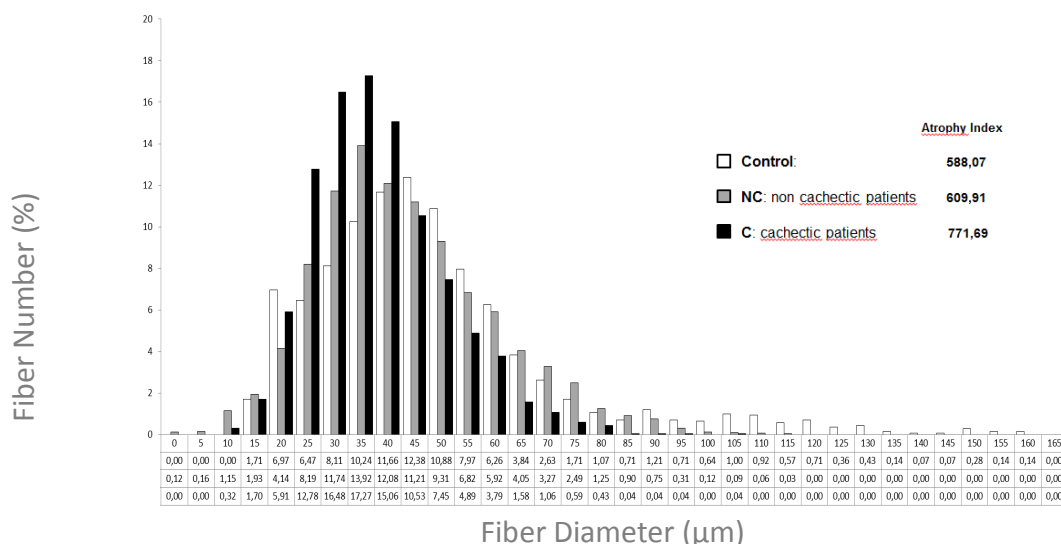


Figure 18 Frequency histograms showing the distribution of myofiber diameter in control, non cachectic (NC) and cachectic (C) cancer patients. The respective atrophy index values are also reported.

In cancer patients the mean myofiber diameter was significantly smaller in comparison to control subjects and cachectic patients had the smallest myofiber diameters when compared to non-cachectic ones.

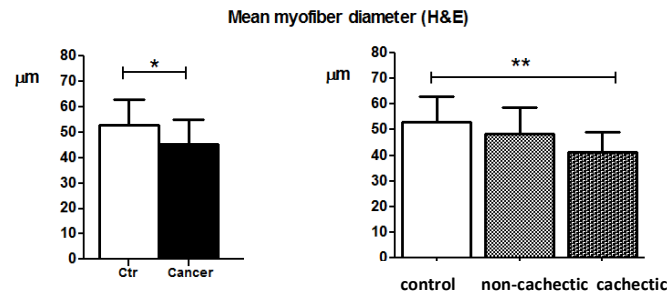


Figure 19 Mean myofiber diameter evaluation.

Histochemical analyses testing for the activity of the myofibrillar ATPase revealed numerous fiber type grouping identified on the basis that at least 1 muscle fiber is completely surrounded by fibers of the same phenotype. Fiber-type groupings are typical hallmark of denervation/reinnervation events of surviving muscle fibers by the neighbours motor neurons.

The ATPase histochemistry here below show the slow type fibers dark stained: slow type groupings were predominantly detected in muscles biopsies from cancer patients in comparison to control subjects.

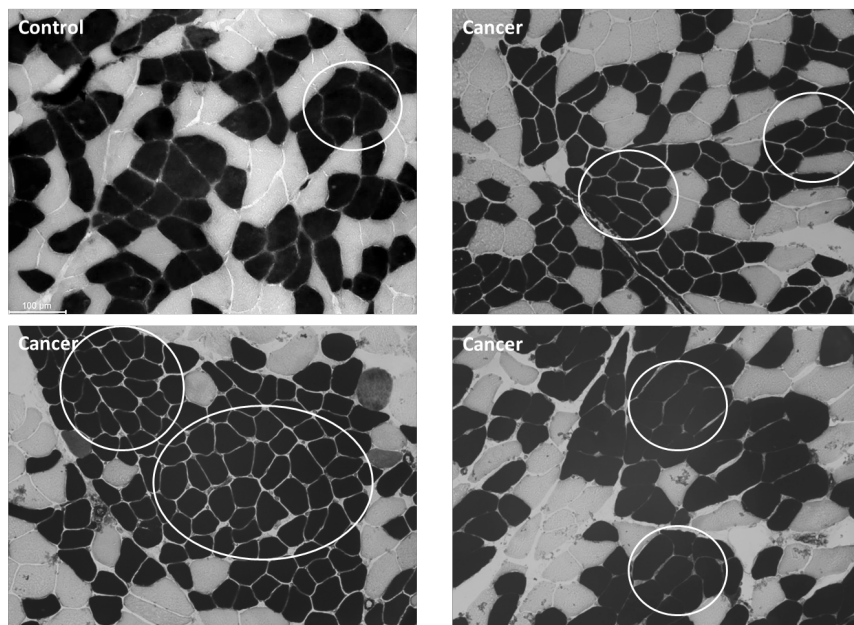


Figure 20 ATPase staining revealed the presence of slow fiber type grouping in muscles of cancer patients.

To confirm the presence of denervated myofibers, we then checked the expression of specific marker of denervation. The neural cell adhesion molecule (N-CAM) is normally enriched in the post-synaptic membranes of the neuromuscular junction, but it is largely absent in adult myofibers. However, N-CAM is re-expressed along the entire muscle fiber after the loss of innervation, thus denervated fibers express N-CAM along the sarcolemma and/or inside the sarcoplasm. Immunofluorescence analyses revealed the presence of several N-CAM positive fibers, particularly in the muscle of cancer patients.

The percentage of N-CAM expressing myofibers was significantly increased in muscles biopsies of cachectic patients in comparison to non-cachectic and age matched control ones as shown in the images and charts here below.

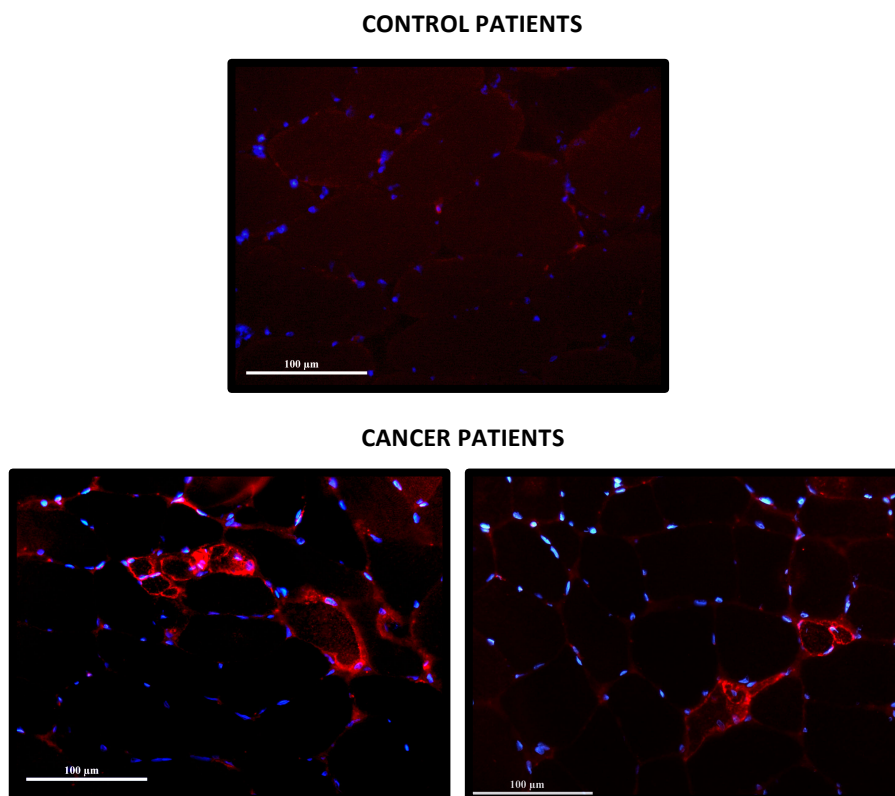


Figure 21 Immunofluorescence observations of NCAM positive fibers in muscle of control and cancer patients

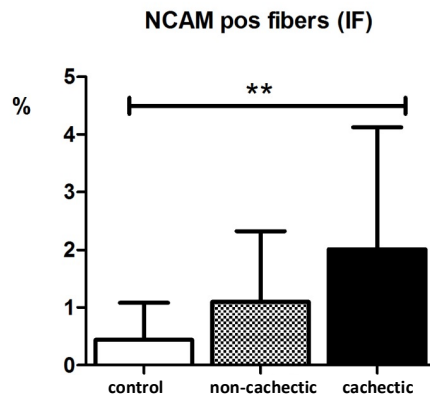


Figure 22 Percentage of NCAM positive fibers (below) in muscle of control, non-cachectic and cachectic patients.

We also checked by real time PCR the expression in the muscles of two other marker of denervation (MuSK and AchRg). Even if the result presented is still not statistically significant due to the high heterogeneity of values obtained, there is an interesting trend toward higher levels of expression of both markers in the muscles of cancer patients compared to control subjects.

The Increase of AChRg and MuSK transcripts suggests an ongoing degeneration of the neuromuscular junction in the muscles of cancer patients.

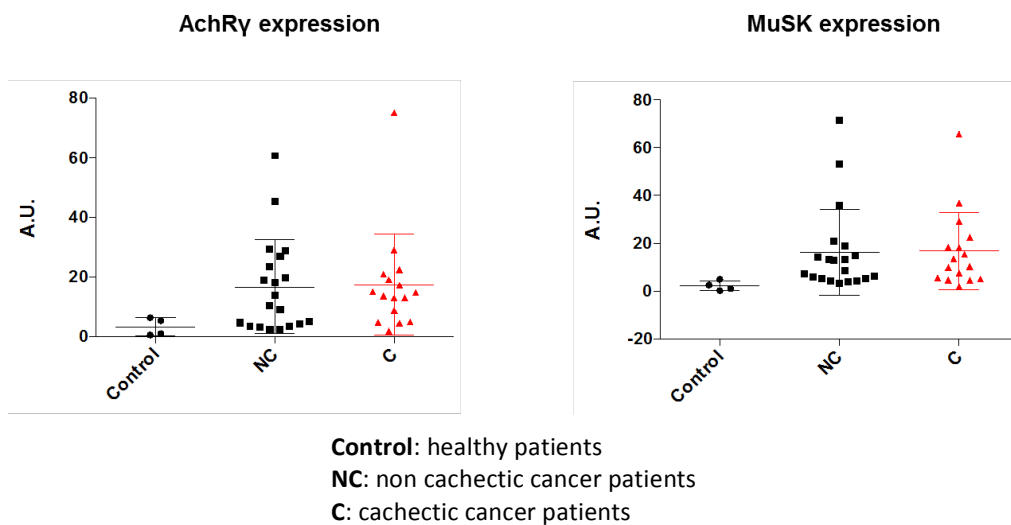


Figure 23 Expression levels of AchR and Musk

The results collected till now may suggest that denervation contributes to myofiber atrophy in cancer cachexia. This is completely new pathogenetic mechanism underlying muscle atrophy during cancer progression that we are analyzing in more detail. To do that, we are increasing the number of patients analyzed for markers of denervation (MuSK and AchRg) and we are analyzing other transcriptional markers of denervation (myogenin, NCAM, Runx1) and circulating markers of denervation (CAF). We should also carry on studies on functional parameters as strength tests and electromyography.

chapter 4

DISCUSSION

Cancer cachexia affects 80% of cancer patients. It is an independent prognostic factor and it's directly responsible for death in 20-30% of cancer patients. Clinical and animal studies using mice models of cancer cachexia demonstrated that reverting the muscle mass loss phenotype increase life-span in mice models and improve outcome in terms of prognosis of cancer patients, independently from tumor growth.

Muscle loss is mainly consequent to hyper-activation of protein breakdown and/or inhibition of protein synthesis. Therefore, to prevent cachexia scientists are developing drugs that promote protein synthesis or block protein degradation. Proteolysis in muscle is controlled by the ubiquitin proteasome and autophagy lysosome systems. Enhancement of protein breakdown requires a transcriptional dependent program that induces the expression of a subset of genes, the atrophy-related genes that encode rate limiting enzymes of the degradation systems. Few pathways have been found to simultaneously regulate atrophy program and protein synthesis. Among these, Myostatin/TGF β signaling is emerging as a critical pathway and therefore, is a promising pharmacological target of cachexia. Indeed, inhibition of myostatin dramatically prolongs survival,

even of animals in which tumor growth is not inhibited and fat loss and production of proinflammatory cytokines are not reduced⁷⁰. However, myostatin inhibitors display important side effects when tested in humans⁷¹. Therefore, it is mandatory to search for more specific targets of TGF β superfamily.

Sartori et al. has cleared that the BMP pathway has a fundamental role limiting protein degradation in mice models of muscle atrophy through the inhibition of the synthesis of a newly detected E3 ubiquitin ligase, MUSA-1, and that its inhibition is a necessary condition for the onset of muscle atrophy, regardless of the state of activation of myostatin pathway. Inhibition of BMP pathway induces an excessive muscle loss and tremendous weakness during catabolic conditions such as absence of nutrients or denervation, well copying the cachexia phenotype.

The preliminary results of our study confirm that BMP pathway is downregulated in muscles of cachectic cancer patients compared to non-cachectic ones. On the contrary we didn't detect any difference in the state of activation of the myostatin/TGF β pathway. These observations support the hypothesis that a decrease of BMP signal is a permissive condition for the onset of muscle loss and of cachexia syndrome in cancer patients. Moreover, the E3 ubiquitin ligases MuRF1 and MUSA1 are significantly upregulated in muscles of human cachectic patients compared to age-matched non cachectic and control ones. To explain why Smad1/5/8 are inhibited and, consistently, MUSA1 is up-regulated, we monitored the expression levels of Noggin, a well-known inhibitor of BMP pathway. We found that the expression of Noggin was highly increased in muscles of cachectic patients compared to non-cachectic and control ones thus potentially explaining why BMP pathway is blocked during cancer progression. Interestingly, there is positive correlation between the muscle expression of MUSA1 (but not Atrogin1 and MuRF1) and Noggin and the percentage of body weight loss in the patients.

Previous studies (Sartori et al) demonstrated that the overexpression of Noggin was sufficient to induce muscle atrophy and to exacerbate muscle loss during denervation.

Numerous myofibers with typical features of denervation were predominantly detected in skeletal muscle biopsies from (either cachectic or non-cachectic) together with a higher prevalence of N-CAM positive myofibers (i.e. denervated myofibers) and several fiber type grouping (i.e. features of denervation/reinnervation events).

Moreover, cancer patients, in particular cachectic ones, showed the smallest myofiber diameter and the highest atrophy index when compared to non-cachectic and control subjects, indicating that muscle atrophy was a predominant feature in cachectic patients.

These results suggest that denervation may contribute to myofiber atrophy in cancer cachexia. To better clarify this point we also checked the expression in the muscles of two other marker of denervation (MuSK and AchRg) and, even if the result presented is still not statistically significant, there is an interesting trend toward higher levels of expression of both markers in the muscles of cancer patients compared to control subjects suggesting an ongoing degeneration of the neuromuscular junction in the muscles of cancer patients. This result is the more interesting because so far no one has identified denervation as a pathogenetic mechanism underlying muscle atrophy in cancer patients. Serological testing for NMJ degeneration/denervation biomarkers in the different groups of patients (control and cancer, cachectic and non-cachectic, colorectal and pancreatic) are ongoing analyses that will further sustain this hypothesis.

Moreover, data obtained in this study will be integrated by increasing the number of patients and of control subjects analyzed. They will be then correlated with the clinical characteristics of the study population trying to figure out if there is a prognostic value associated with the activation of the different pathways and identify if, in precachectic cancer patients, the activation profile of the BMP pathway and the ubiquitin ligase is different, to understand what is the timing adjustment of BMP. The expression levels of different BMP ligands/receptors will be evaluated in the muscles of cancer patients at different stages of cachexia.

The ultimate goal of this research project is the development of a therapeutic strategy aimed to counteract the occurrence and to limit the progression of muscle atrophy in cancer patients. Preliminary results obtained in preclinical studies (data not shown) demonstrated that the reactivation of the BMP pathway by the overexpression of a constitutive active form of the type I BMP receptor is able to prevent atrophy in tumor bearing mice. However, these results do not discriminate which BMP ligand is involved in this beneficial effects on muscle mass. The identification of the critical BMP or of the critical combination of BMPs would have important consequences in terms of therapeutic approach for cancer patients. For this reason we are screening different BMP ligand in order to determine which one is important to prevent muscle loss. The critical one will be systemically administered in tumor bearing animals and the effects on animal survival and cachexia syndrome will be monitored. Preliminary results point to BMP7 as a very interesting candidate.

Moreover, we are developing, in collaboration with a drug designer team, a peptide able to competitively inhibit the binding of Noggin with BMP7. Synthesizing a peptide of this type, however, is technically complex, because of chemical instability of the peptides obtained, and it is economically onerous. For this reason, at the same time we are trying to develop a monoclonal antibody against Noggin.

CONCLUSIONS

The results obtained in this study will clarify the role played by Noggin/BMP/MUSA1 axis in cancer-induced muscle loss and its potential implication as a therapeutic approach to counteract cachexia syndrome and improve survival. Moreover we have identified denervation as a new pathogenetic trait underlying muscle atrophy in cancer patients. The impact of preventing cachexia is an important aspect for patient treatment and survival. Our data will identify BMPs as biomarkers suitable to identify patients at risk to develop cachexia syndrome improving patient management and the quality of life.

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