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Subclinical myopathy and colorectal cancer: identification and role of new muscle damage and regeneration biomarkers

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Summary

Background

Skeletal muscle is the major reservoir of body proteins and it can be affected in conditions associated to altered protein turnover and metabolism such as cancer. Although severe wasting is seen primarily in patients with advanced malignancy, some of them present degree of wasting at the onset of disease. Autophagy has been recently described to play a relevant role in muscle wasting.

Materials and Methods

We performed morphometric studies and immunohistochemical analyses on intraoperative rectus abdominis muscle biopsies from 50 consecutive weight stable colorectal patients and 25 weight-stable patients operated for non-inflammatory benign diseases with no clinical signs of myopathies. Biochemical and molecular analyses have been performed in order to evaluate protein profile, the presence of autophagy induction and their correlation with clinical outcome.

Results

In cancer patients, we observed a subclinical myopathy characterized by an abnormal distribution of myonuclei relocated from the periphery inside the myofiber. The percentage of myofibers with abnormally located myonuclei was significantly higher in patients compared to controls. Analyses on serum samples showed that, in the absence of systemic inflammation, in the prevalence of cancer patients the levels of albumin and prealbumin were below the normal range and the mean value was significantly lower compared to that detected in controls. Molecular analyses showed an accumulation of p62, a typical marker of autophagy induction, significantly higher in cancer patients compared to controls. We found an inverse correlation between the number of abnormally nucleated myofibers and the presence of lymph node metastasis. Cancer relapse was correlated with low serum levels of prealbumin and high levels of p62 in myofibers of cancer patients.

Conclusions

Colorectal cancer patients have a subclinical myopathy characterized by myofibers with internally located myonuclei. In the absence of inflammation, cancer patients show low levels of prealbumin and albumin as markers of altered protein turnover and persistent high levels of p62 in myofibers as expression of autophagy induction with an impairment in physiological autophagic flux. Up to now our data indicate that skeletal muscle fibers show nuclear abnormalities that seems to be associated to a better prognosis, while the presence of an altered protein turnover at an early stage of disease, with an impairment in the physiological autophagic flux, that could be predictive of cancer relapse and onset of cancer cachexia.

Riassunto

Introduzione

Il muscolo scheletrico rappresenta la principale riserva proteica del corpo e può essere compromesso in varie affezioni metaboliche e di alterato turnover proteico, quale il cancro. Benchè una severa perdita di massa sia generalmente presente in quadri neoplastici avanzati, in alcuni casi può essere già evidente in una fase di malattia iniziale. L'autofagia è stata recentemente descritta come uno dei possibili fattori responsabili del processo catabolico.

Materiali e Metodi

50 pazienti sottoposti ad intervento chirurgico per neoplasia colorettale e 25 pazienti operati per patologia benigna non infiammatoria, in assenza di segni clinici di miopatia, sono stati sottoposti a biopsia muscolare su cui sono state eseguite analisi di carattere morfometrico ed istochimico. Sono state, inoltre, eseguite analisi biochimiche e molecolari al fine di valutare l'assetto proteico e lo stato di attivazione del processo autofagico e la loro correlazione con l'outcome clinico dei pazienti.

Risultati

Nei pazienti neoplastici abbiamo riscontrato la presenza di una miopatia subclinica, caratterizzata dalla presenza di fibre muscolari con un'anomala localizzazione del nucleo cellulare al centro della fibra, significativamente maggiore rispetto ai controlli. L'analisi dell'assetto proteico ha dimostrato valori sierici di albumina e prealbumina significativamente più bassi nei pazienti oncologici, mentre l'analisi molecolare ha documentato elevati livelli di p62 nelle fibre muscolari dei pazienti affetti da carcinoma colorettale, rispetto ai controlli.

La valutazione dell'outcome clinico ha dimostrato una correlazione inversa tra la percentuale di miofibre anomale e l'insorgenza di metastasi linfonodali, mentre bassi livelli sierici di prealbumina ed alti livelli di p62 nelle fibre muscolari sono risultati correlati con un aumentato rischio di ripresa di malattia

Conclusioni

I pazienti affetti da carcinoma colorettale presentano una miopatia subclinica già all'insorgenza della malattia, caratterizzata dalla presenza di fibre con

alterata posizione del nucleo nella cellula. In assenza di infiammazione sistemica e tissutale, i pazienti oncologici presentano bassi livelli sierici di albumina e prealbumina, come espressione di un alterato turnover proteico, nonché elevati livelli di p62 nelle fibre muscolari, a dimostrazione dell'attivazione del processo autofagico che risulta tuttavia compromesso. Tali dati suggeriscono, pertanto, un verosimile ruolo protettivo per le anomalie nucleari descritte, mentre un alterato turnover proteico ed una compromissione del normale flusso autofagico, in concomitanza dell'insorgenza della neoplasia, costituiscono un potenziale fattore predittivo negativo in termini di ripresa di malattia ed evoluzione verso uno stato cachettico.

INTRODUCTION

1. Cachexia

The etymology of the word cachexia points to its association with poor prognosis: it is derived from the Greek *kakos* and *hexia*—"bad condition" and has long been recognised as a key sign in many cancers. It is a multifactorial condition which comprises skeletal muscle and adipose tissue loss which may be compounded by anorexia, a dysregulated metabolic state with increased basal energy expenditure and is resistant to conventional nutritional support. The pathophysiological mechanisms have begun to be elucidated and this has led to developments in therapeutic avenues [1]. Cachexia correlates with poor performance status, poor quality of life, and a high mortality rate in cancer patients [2]. In a meta-analysis of studies pertaining to patients with advanced cancer and survival of less than 90 days, symptoms including weight loss and anorexia correlated with poor prognosis [3]. Loss of greater than 5–10% of body weight is usually taken as a defining point for cachexia, although the physiological changes may be present long before this cutoff point is reached. Furthermore, the degree of weight loss which significantly impacts on prognosis or performance has not been defined. A longitudinal study has shown that 2.5 kg weight change over 6–8 weeks is sufficient to produce significant changes in performance status [4]. Death usually occurs when there is 30% weight loss [5]. The prominent clinical feature of cachexia is weight loss
in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently associated with cachexia [6]. However, there is no clear consensus definition of this common problem in cancer patients leading to a poor understanding of the aetiology of the condition. Earlier
definitions of cachexia described "a wasting syndrome involving loss of muscle and fat directly caused by tumour factors, or indirectly caused by an aberrant host response to tumour presence" [7], however more recent definitions have downplayed the importance of fat loss and describe cachexia as "a complex

metabolic syndrome associated with underlying illness and characterised by loss of muscle with or without loss of fat mass" [6], thus highlighting the unique consequences of muscle wasting—the hallmark of cachexia. Without an established definition, future studies in this area will be hampered. A recent consensus definition has been proposed to include further factors to diagnose the cachexia syndrome such as involuntary weight loss, decreased muscle mass, anorexia, and biochemical alterations (*C*-Reactive Protein (CRP), albumin, haemoglobin [8]).

One such study looked at 170 pancreatic cancer patients with weight loss >5% and whether a triad of >10% weight loss, low food intake (<1500 kcal/day), and systemic inflammation (CRP > 10mg/dL) could better predict adverse functional outcome as well as poor prognosis versus weight loss alone [8]. When two of three of these criteria were present, (representing 60% of the patients) a cohort of patients with adverse function and prognosis were identified [8].

The prevalence of cachexia is thought to be up to 80% of upper gastrointestinal cancer patients and 60% of lung cancer patients at the time of diagnosis [9]. There are no clear figures for the estimated prevalence within specific cancer cohorts. When the electronic medical records of over 8500 patients with a wide variety of malignancies were analysed for the prevalence of cachexia amongst the cohort, the proportion varied according to which standard definition was used: 2.4% using the World Health Organisation's International Classification of Diseases (ICD) cachexia diagnostic code; 5.5% for the ICD diagnosis of cachexia, anorexia, abnormal weight, and feeding difficulties; 6.4% were prescribedmegestrol acetate, oxandrolone, somatropin, or dronabinol; 14.7% had >5% weight loss [10]. Despite methodological flaws, there was an interesting lack of overlap between the different criteria pointing to the underdiagnosis of cachexia in clinical practice.

Decreased muscle strength may help distinguish cachexia from other causes of anorexia and fatigue in cancer patients [11]. Decreasedmuscle strength could be used as a diagnostic criterion with greater sensitivity and specificity for cancer cachexia. Cancer patients who are losing weight and have a systemic

inflammatory response have poorer performance status [4]. Until a clear definition with well-defined cut-offs emerges, identification and treatment of cachectic patients as well as research in the area will remain limited. A new consensus definition for diagnostic purposes has been suggested and is outlined in Table 1 [6].

Table 1: Diagnostic criteria for cachexia syndrome [6].

Weight loss of at least 5% in 12 months or less (or BMI <20 kg/m²)		
AND 3 of 5 From:	Decreased muscle strength	
	Fatigue	
	Anorexia	
	Low fat-free mass index	
	Abnormal biochemistry:	Increased inflammatory markers (CRP, IL-6)
		Anaemia (Hb < 12 g/dL)
		Low serum albumin (<3.2 g/dL)

Note: Fatigue is defined as physical and or mental weariness resulting from exertion; an inability to continue exercise at the same intensity with a resultant deterioration in performance.
Anorexia is defined as limited food intake (total caloric intake less than 20 kcal/kg body weight/day) or poor appetite.
Low-fat-free mass index represents lean tissue depletion (i.e., mid upper arm muscle circumference <10th percentile for age and gender' appendicle skeletal muscle index by DEXA <5.45 (kg/m²) in females and <7.25 in males).

2. Pathophysiology of Cachexia

Pathophysiological changes and clinical consequences of cachexia are summarised in Figure 1.

2.1 Metabolic Changes.

The metabolic changes found in cachexia resemble those of infection rather than starvation [12] and are multifactorial and complex. Weight loss of cancer cachexia is due to loss of both skeletal muscle and adipose tissue mass, whereas weight loss is mainly from adipose tissue stores in starvation [13]. In cachexia there is an increase in muscle protein catabolism leading to net loss of muscle mass. The ATP ubiquitin-dependent proteolytic pathway is the greatest contributor to proteolysis in cachexia [14, 15]. Other proteolytic pathways such as lysosomal cathepsins B, H, D, and L [16] and activity of the calcium/calpain pathway have also been implicated [17]. Increased intracellular

proteolytic activity usually manifests as loss of body weight. This proteolysis has been shown to occur even in the absence of weight loss in cancer patients.

Activation of proteolysis is an early event during tumour growth and it may be present for a long time prior to its clinical manifestation. Protein synthesis may be increased or unchanged [18].

Loss of adipose tissue mass is due to lipolysis [5]. This process is driven by lipid mobilising factor (LMF) and tumour (and host) factor zinc-alpha-2 glycoprotein which has a direct lipolytic effect and sensitises adipocytes to lipolytic stimuli and shows increased expression in cachexia [19].

A further compounding factor is the increased resting energy expenditure due to the dysregulation of energy metabolism. Cancer patients have a higher resting energy expenditure than non-cancer controls [20]. It has been speculated that this is due to altered gene expression of mitochondrial membrane uncoupling proteins which uncouple respiration from ATP production resulting in loss of energy as heat [5]. The metabolic changes seen in cachexia are a result of the interplay of tumour factors, host factors, and the interaction between the two.

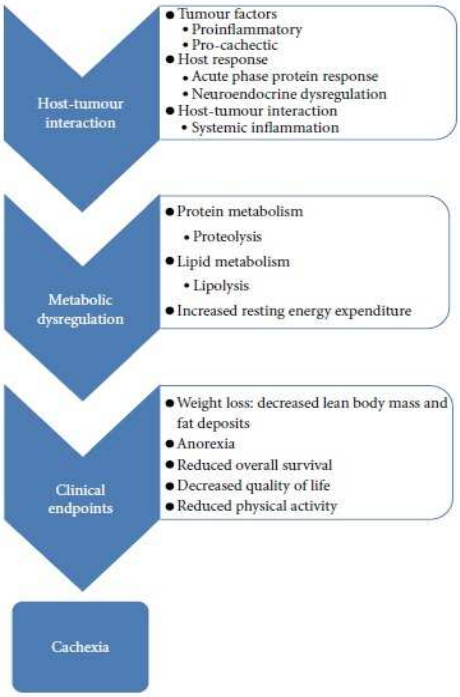


Figure 1: Clinical consequences of cancer cachexia.

2.2. Tumour Factors.

Tumour cells produce both proinflammatory and procachectic factors, which stimulate a host inflammatory response [1]. Tumour produced procachectic factors include proteolysis-inducing [21] and Lipid-mobilising factors [22]. PIF has been identified in the urine of weight losing patients with pancreatic, colon, lung, ovarian, breast, and liver cancers [22]. In animals, PIF signals via NFκB and STAT3 pathways. Stimulation of these pathways, induces proteolysis in muscles via the ubiquitin-proteasome pathway and in hepatocytes, results in production of IL-6, IL-8 and CRP. Tumour xenografts expressing human PIF do not induce cachexia in mice. Further attempts to correlate PIF levels and outcomes have not shown any correlation [23]. Therefore the proposed mechanisms of PIF have not yet been validated in humans. Parathyroid hormone-related peptide (PTHrP), another tumour-derived circulating factor, is associated with higher soluble tumour necrosis factor receptor levels and with lower albumin and transferrin levels. Lipid mobilising factor has been found in cancer patients losing weight but not in those with stable weight. It is thought that LMF sensitises adipocytes to lipolytic stimuli
by increasing cyclic AMP production [24]. LMF may bind to beta adrenergic receptors and causes either increased receptor number or increased G protein expression [25].

2.3. Host-Tumour Interaction.

Inflammatory cytokine production by the tumour microenvironment in response to tumour cells may drive the cachexia process. Rodent tumour models display increased systemic inflammatory cytokine production, which correlates with the amount of weight loss. The murine model of cancer cachexia associated with systemic inflammation suggests that there is an interplay between IL-1β and IL-6 within the tumour microenvironment, which leads to their amplification [26]. Reduction of IFN-γ by monoclonal antibody treatment reverses cachexia in the Lewis lung carcinoma in mice [27]. Pro-inflammatory cytokines produced include TNF-α, IL-1 and IL-6 [1]. It is not certain whether the cytokine production is primarily from tumour or host inflammatory cells. It has been

hypothesised that either tumour cell production of pro-inflammatory cytokines or the host inflammatory cell response to tumour cells is the source of the acute phase protein response seen in many malignancies and in cachexia. One study of oesophagogastric cancers showed cytokine protein concentrations of IL-1β, IL-6 and TNF-α are significantly elevated in tumour tissue. Tumour tissue concentrations of IL-1β protein correlated with serum CRP concentrations (r = 0.31, P = .05; linear regression) and tumours with diffuse or patchy inflammatory cellular infiltrate were associated with elevated serum CRP [27]. Similarly the production of IL-6 by Peripheral Blood Mononuclear Cells (PBMCs) in pancreatic cancer patients induced an acute phase protein response in another study [26]. Martignoni et al. have suggested that IL-6-overexpression in cachectic pancreatic cancer patients is related to the ability of IL-6 producing tumours to sensitise PBMC and induce IL-6 expression in PBMCs [27]. TNF-alpha and the tumour factor proteolysis-inducing factor are the major contenders for skeletal muscle atrophy in cachectic patient. They both increase protein degradation through the ubiquitin-proteasome pathway and depress protein synthesis through phosphorylation of eukaryotic initiation factor 2 alpha [19]. Studies have shown that proteolysis-inducing factor levels correlate with the appearance of cachexia, but there is some disagreement regarding a correlation between serum levels of TNF-alpha and weight loss. Furthermore, only antagonists to proteolysis-inducing factor prevent muscle loss in cancer patients, suggesting that tumour factors are the most important.

2.4. Host Response Factors

2.4.1. Acute Phase Protein Response.

Systemic changes in response to inflammation denoted the acute phase response [28]. Up to 50% of patients with solid epithelial cancers may have an elevated acute phase protein response [28]. This acute phase protein response (APPR) has been associated with hypermetabolism: in pancreatic cancer patients APPR correlated with elevated resting energy expenditure and reduced energy intake [29]. Other longitudinal studies have found a poorer prognosis in patients displaying this response, independent of weight loss [29]. *C*-reactive

protein (CRP) is the most prevalent method used to assess the magnitude of the systemic inflammatory response [28].

The modified Glasgow prognostic score (mGPS) (Table 2) combines CRP and albumin concentrations to create a simple scoring system which is a prognostic factor independent of stage and treatment and predicts survival [28, 29].

Biochemical measure	Score
C-reactive protein ≤10 mg/L + Albumin ≥35 g/L	0
C-reactive protein ≤10 mg/L + Albumin <35 g/L	0
C-reactive protein >10 mg/L	1
C-reactive protein >10 mg/L + Albumin <35 g/L	2

Table 2: Modified Glasgow Prognostic Score (mGPS): an inflammation-based prognostic score [28].

Raised CRP concentrations at the time of admission to hospital are indicative of an increased risk for all-cause mortality; there is a 22.8-fold increase in cancer mortality in patients with highly elevated CRP concentrations (>80mg/L) [30]. This response appears to be prevalent amongst cancer patients with elevated CRP measured in almost 80% of 106 patients with inoperable nonsmall cell lung cancer (NSCLC), 40% of whom had >5% weight loss [30]. In patients without weight loss, those who displayed evidence of a systemic inflammatory response reported more fatigue ($P < .05$) [30]. In patients with gastro-oesophageal cancer, the rate of weight loss correlates with serum concentrations of *C*-reactive protein [31]. Elevated CRP levels at the time of diagnosis has been found to be a predictor of poor prognosis in pancreatic, lung, melanoma, multiple myeloma, lymphoma, ovarian, renal, and gastrointestinal tumours. The exact mechanisms linking cachexia, APPR, and poor outcomes is not known. It may be that this systemic alteration in protein metabolism drives the proteolysis of skeletal muscle to fuel the switch to acute phase reactant production. The APPR requires large amounts of essential amino acids: 2.6 g of muscle protein must be catabolised to produce 1 g of fibrinogen [32].

2.4.2. Neuroendocrine Factors.

A number of neuroendocrine factors appear to be dysregulated in the cancer state resulting in insulin resistance, reduced anabolic activity, and elevated cortisol. This dysregulation may be driven by the systemic inflammatory response associated with cancer.

Inflammatory cytokines such as TNF-*α* and IL-6 have been implicated in insulin resistance [33]. The endogenous production of or response to anabolic growth factors in patients may be affected either by the tumour or the host response to the tumour and may contribute to cachexia. Testosterone or derivatives have been shown to increase protein synthesis and muscle mass. Emerging evidence implicates reduction in insulin-like growth factor 1 in cachectic states [34].

2.5. Anorexia and Cachexia: An Interdependent Relationship?

Whilst loss of appetite and resultant decrease in energy intake undoubtedly contribute to weight loss associated with cancer cachexia, whether anorexia occurs by an independent process or is a result of the inflammatory process of cachexia is not fully understood. Anorexia itself may have a number of components—nausea, altered taste sensation, swallowing difficulties, or depression. The failure of aggressive supplementary nutritional regimes to reverse weight loss in many patients points to primacy of the cachexia disease process [5] and in fact, this disease process may act to establish anorexia. It is thought that lack of appetite is secondary to factors produced by the tumour or the immune response to the tumour. Specifically, cytokines may inhibit the neuropeptide Y pathway ormimic negative feedback action of leptin on the hypothalamus, leading to anorexia [35]. In a study of patients with gastro-oesophageal malignancy (n = 220), 83% of whom had weight loss, multiple regression identified dietary intake (estimate of effect: 38%), serum CRP concentration (estimate of effect: 34%), and stage of disease (estimate of effect: 28%) as independent variables in weight loss in these patients [32]. If serum CRP is taken as a proxy measure of systemic inflammation due to cancer cachexia, this indicates that weight loss in cancer is not merely due to reduced calorie intake. Recently, understanding of the physiologicalmechanisms

of appetite regulation has been increasing. There are two sets of neurons within the arcuate nucleus of the hypothalamus identified to be involved: the melanocortin system and the neuropeptide Y system. Neuropeptide Y stimulates appetite on its own or via release of other orexigenic proteins. Neurons which release α-melanocyte-stimulating hormone (α-MSH) and signal via melanocortin-3 and 4 receptors (MC3R, MC4R) result in decrease in food-seeking behaviour, increased basal metabolic rate and decreased lean body mass [36]. These neurons are constitutively active as mutation in the MC4R results in childhood obesity. Agouti-related protein (AgRP) is produced by neurons (which also produce neuropeptide Y) and counteracts the action of MC4R-stimulating proteins promoting appetite [36]. These "appetite neurons" also express receptors for circulating leptin and interleukin-1β (IL-1β) [84], both of which downregulate appetite and receptors for ghrelin (the orexigenic protein, which increases AgRP) [37].

3. Consequences

Cachexia results in a state of active inflammation whereby tumour-derived factors and the aberrant host response to these factors result in a catabolic state. Whether this catabolic state is the ultimate cause of death in some patients is unknown although a substantial proportion of cancer patients die with symptoms of advanced cachexia [9]. Cachexia directly impacts overall survival, quality of life, and physical activity.

3.1. Survival.

Weight loss has been indicated as an important prognostic factor for cancer patients. A classic study by DeWys and colleagues underscores the impact and outcome of weight loss in cancer patients [2]. Using retrospective evaluation in a multicentre study of more than 3000 patients with different tumour types, these researchers reported moderate to severe weight loss in 30% to 70% of patients,

depending on the tumor type. The amount of weight loss depends upon tumor site, size, type, and stage. Age and treatment type also play a role. The

greatest incidence of weight loss was seen among patients with solid tumours, for example, gastric, pancreatic, lung, colorectal, and head and neck. Patients with solid tumours are often likely to lose 10% or more of their usual body weight. There is a lower risk of weight loss in patients with breast and hematological cancers. Within each tumour type, survival times were shorter for patients who had experienced weight loss than in those who did not. Not only did weight loss predict overall survival, but it also indicated a trend towards lower chemotherapy response rates. In more recent studies, similar findings of reduced survival have been reported. Buccheri and Ferrigno (2001) [38] reported in 388 NSCLC cases that total weight loss was the best indicator of prognosis. In ovarian cancer Hess et al. (2007) [39] found a significant relationship between weight change and survival—on multivariate analysis the risk of death increased by 7% for each 5% drop of body weight. In Gastro-oesophageal cancer Deans and Wigmore (2009) [31] reported that patients with the lowest rate of weight loss had a median survival of 30.2 months versus 7.5 months in those with the highest rate of weight loss. One proposed mechanism to explain why patients with weight loss have a poorer survival is the increased incidence of complications from surgical, radiotherapeutic, and chemotherapeutic treatments. In patients with weight loss: chemotherapy doses were lower; they developed more frequent and more severe dose limiting toxicity and received, on average, one month less chemotherapy ($P < .001$ in all). Weight loss correlated with shorter failure-free survival, overall survival, decreased response, quality of life, and performance status ($P < .001$ in all). Whether reduced survival is due to a more aggressive tumour profile in patients with weight loss or due to suboptimal treatment related to weight loss, remains unknown.

3.2. Quality of Life.

Cachexia contributes substantially to morbidity in cancer patients. It is associated with symptoms such as fatigue, weakness, poor physical performance, and thus leads to a lower self-rated quality of life. Indeed, when the impact of various factors is

related to self-rated quality of life scores, the proportion determined by weight loss is 30% and by nutritional intake 20%, compared to cancer location (30%), disease duration (3%), and stage (1%) [40]. Patients who continue to lose weight while receiving palliative chemotherapy have reduced global quality of life and performance scores when compared to those whose weight loss stabilises .

3.3. Physical Activity.

Physical activity has been described as a novel, objective, and robust functional outcome measure that is frequently impaired in cachectic states [41]. Activity levels are influenced by several conventional quality of life domains. Measurement of physical activity has long represented a challenge for researchers using timeconsuming and expensive tools such as doubly labelled water and indirect calorimetry. However research using these methods has revealed that although resting energy expenditure may be elevated in cachectic patients, total energy expenditure is reduced because weight-losing cancer patients reduce the magnitude of their energy deficit through reductions in physical activity. In a more recent study by Dahele et al. (2007) [42] using advanced ambulatory pedometer technology, cancer patients receiving palliative chemotherapy were shown to spend significantly more time lying and sitting, and significantly less time in quiet standing or stepping compared with controls, taking on average 43% less steps than healthy controls. It is known that bed rest leads to a decrease in skeletal muscle mass in healthy patients, due to reduced protein synthesis [43]. Thus, loss of physical function results in decreases in performance status, ability to perform activities of daily living, decreased social interactions, and alterations in body image, all of which manifest as reduced quality of life [44]. Interventions which
increase physical activity would be anticipated to be highly beneficial. Antineoplastic therapies such as surgery, radiotherapy and chemotherapy, may also impact on the development of systemic inflammation and particularly may impact on swallowing difficulties and anorexia due to nausea [45].

4. Colorectal Cancer

4.1 *Morphology and Histology of Colon and Rectum*

Approximately 60% of colorectal cancer cases arise in the distal part of colon (including splenic flexure, descending colon, sigmoid and rectosigmoid colon and rectum) in countries where colonic cancer incidence is high, whereas proximal (including cecum, ascending colon, hepatic flexure and transverse colon) cases predominate in countries with low incidence [46]. The anatomy of colon and rectum are illustrated in Figure 2.

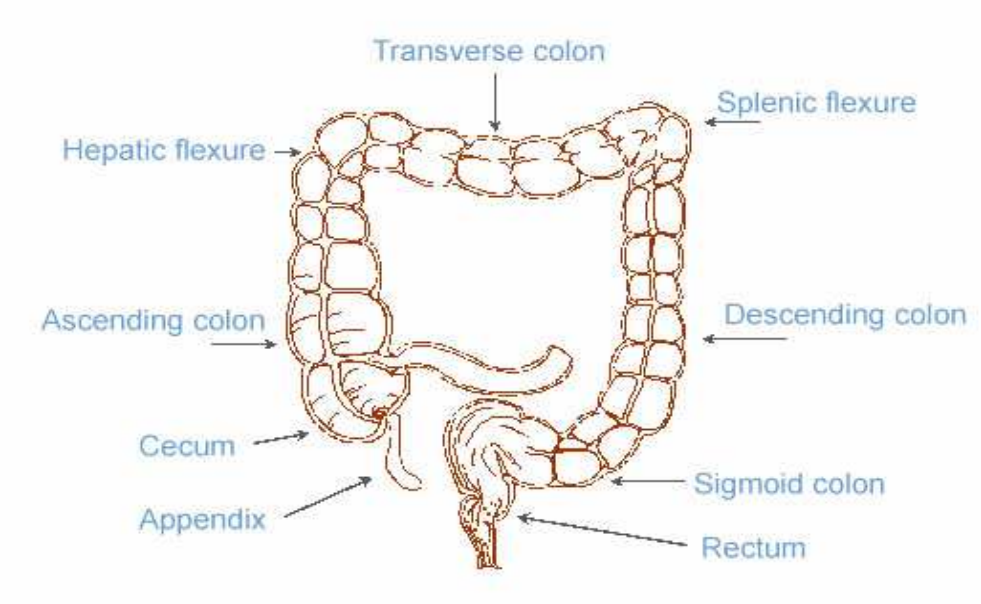


Figure 2: Anatomy of colon and rectum in humans.

It has been suggested that the risk of colorectal cancer conferred by various environmental (and genetic) factors is different for proximal and distal tumours. Various physiological and histological differences exist between the proximal and distal part of a normal colon, which may predispose tumours originating at these sites to develop along different pathways. It may be convenient to categorize colorectal cancers into either proximal or distal location, but it is important to note that this is a simplification of colorectal carcinogenesis, and that underlying molecular features are responsible for determining tumour phenotype.

These features may very likely show considerable overlap between right- and left-sided colorectal cancers. The principal functions of colon are recovery of water and propulsion of solid faeces to the rectum prior

to defaecation. The luminal surface of the intestine are composed of a columnar epithelial mucosa, with finger-like projections (villi) and glandular invaginations (crypts). Mucosa consists mainly of two cells types: the absorptive cells recovering water and some salts from the liquid residue of the contents of the small intestine, and the mucus-secreting goblet cells lubricating the passage of faeces. Goblet cells predominate at the base of the villi, whereas the luminal surface is almost entirely lined by columnar
absorptive cells. The cells of the intestinal epithelium are progressively more differentiated as they age and pass along the crypt–villus axis. The rectum is the short dilated terminal portion of colon. The rectal mucosa is similar to that of colon except from more numerous goblet cells.

The proximal colon originates from the embryonic midgut and is perfused by the superior mesenteric artery, surrounded by a multilayered capillary network, whereas the distal colon derives from the hindgut and is served by the inferior mesenteric artery, surrounded by a single-layered capillary network. The multilayered capillary network in proximal colon is possibly related to the greater water absorption and electrolyte transport capacity. The average villi length is greater in the distal colon than in the proximal colon. The apoptotic index is lower in the right colon compared to the left colonic mucosa [47].

Gastrointestinal stem cells undergo multi-potent division to produce the entire specialised cell repertoire of the gastrointestinal tract. The numbers and location of stem cells in the intestinal crypts and gastric glands have never been conclusively proven, and, consequently, the clonal origins of these structures under normal circumstances and in neoplasia are clouded issues. Intestinal stem cells are primitive cells located in a specialised compartment consisting of epithelial and mesenchymal cells and extra-cellular substrates that lack expression of any definitive markers of lineage commitment and are therefore difficult to define and to characterise morphologically. It is believed that the surrounding mesenchymal cells regulate stem cell behaviour through paracrine secretion of growth factors and cytokines [48]. The number of stem cells within the compartment is believed to be between four and six [48], but the exact number has never been conclusively proven and, consequently, is the topic of

debate still. It has been postulated that stem cell number may fluctuate throughout the crypt cycle and that the stem cell number varies throughout different regions of the gastrointestinal tract [49]. Monoclonal intestinal crypts have been demonstrated following irradiation, showing that a single multipotent surviving stem cell can regenerate an entire crypt, thus confirming the hypothesis, that the epithelial cell lineages of the gastrointestinal tract are clonal populations derived from a single stem cell, albeit in damaged mucosa. No evidence of any crypts with a mixed phenotype was observed in 2260 crypts located at the periphery of a patch, indicating that colonic crypts are indeed monoclonally derived, which is consistent with results obtained previously [50]. However, conflicting data have emerged from different studies, and the pathways and mechanisms of gastrointestinal neoplasia are thus far uncertain.

The turnover of cells in the gastrointestinal tract is high throughout life with the differentiating cells shed into the lumen and replaced every 2–7 days under normal circumstances. Thus, lifespan of the cells are not sufficient to accumulate the mutations necessary for malignant change, why the perpetual stem cell is widely believed to be the target of mutational changes. A stem cell division can produce one stem cell and one daughter cell (asymmetric division), two stem cells by self-replication (symmetric division) or a stem cell loss, where both daughter cells go on to differentiate (symmetric division) [51].

The majority of divisions are thought to be asymmetric. According to the so-called immortal strand hypothesis there may be a retention of the template DNA strand within the stem cell located in the niche [52], which allows any DNA replication errors to pass into the differentiating, shortlived daughter cell affording a mechanism of stem cell genome protection [52]. If indeed stem cells are the original targets for the mutation(s) required to initiate a neoplasm, then whether such a cell acts alone or in cooperation

with other mutated stem cells becomes important.

The stem cell compartment is believed to be at the origin of the crypt–villus axis. However, as mentioned before the location of the gastrointestinal stem cells is debated. Studies by Wright have suggested a location in the mid crypt of the ascending colon and in the base of the crypt of the descending colon [53],

whereas different observations have been made in other studies. It has been suggested that a crypt would be incited to go into fission when it reached a threshold size. However, the stem cell number is now thought to be the important factor [53].

4.2 *Morphology and Histology of Polyps in Colon and Rectum*

A polyp is defined as a mass that protrudes into the lumen of the colon. Polyps may be non-neoplastic or neoplastic. The non-neoplastic polyps are hyperplastic, inflammatory, juvenile or hamartomatous and lack dyplastic features. Adenomatous polyps are benign neoplasms that, by definition, display some dysplasia. The degree of dysplasia may be graded into mild, moderate and severe on the basis of cytological and structural features. Adenomatous polyps are generally believed to be precursors of most colorectal adenocarcinomas, which is supported by epidemiological, genetic and pathological studies. Patients with adenomatous polyps have a higher risk of colon cancer over the general population and the risk increases if the polyps are multiple [54].

Neoplastic polyps are histological divided into three sub-groups: tubular adenomas, villous adenomas and mixed or tubulo-villous adenomas. The risk of malignant transformation is low in tubular adenomas (2-3%) and high in pure villous adenomas (15-25%), while the mixed adenomas have an intermediate risk of malignant transformation. The risk of developing subsequent cancer is generally believed to be higher in patients with polyps larger than 1cm in diameter [55]. The initiated polyp may be present and proliferate for 10-15 years before undergoing malignant transformation [56]. The earliest and smallest recognizable histopathological entity may be an aberrant crypt focus (ACF). Two types of ACFs have been observed in humans: The common one called the hyperplastic or non-dysplastic crypt being a hypercellular crypt with normal individual cells which is unlikely to lead to clinically significant lesions, and the less common one called dysplastic ACFs, which are believed to be the precursors of the adenomas and carcinomas [57].

There are currently two proposed morphological pathways of spontaneous development of adenomas, the "bottom up" and the "'top down" pathways (illustrated in Figure 3). The gastrointestinal stem cells are important players in each of them. In the "bottom-up model" a stem cell situated in the base of the crypt acquires mutations in the tumoursuppressor gene adenomatous polyposis coli (APC), which thereby impairs the function of the APC protein (a). The mutated cell proliferates and produces neoplastic daughter cells, which migrate upwards to colonise the entire crypt (b) and form a monocryptal adenoma [157]. Further expansion is achieved by crypt fission (c) [158], where crypts undergo bifurcation (division into two) followed by longitudinal division, with the ultimate formation of two daughter crypts. Thus, this model involves monocryptal adenomas, where the dysplastic cells occupy an entire single crypt. These lesions are observed to be common in FAP [58].

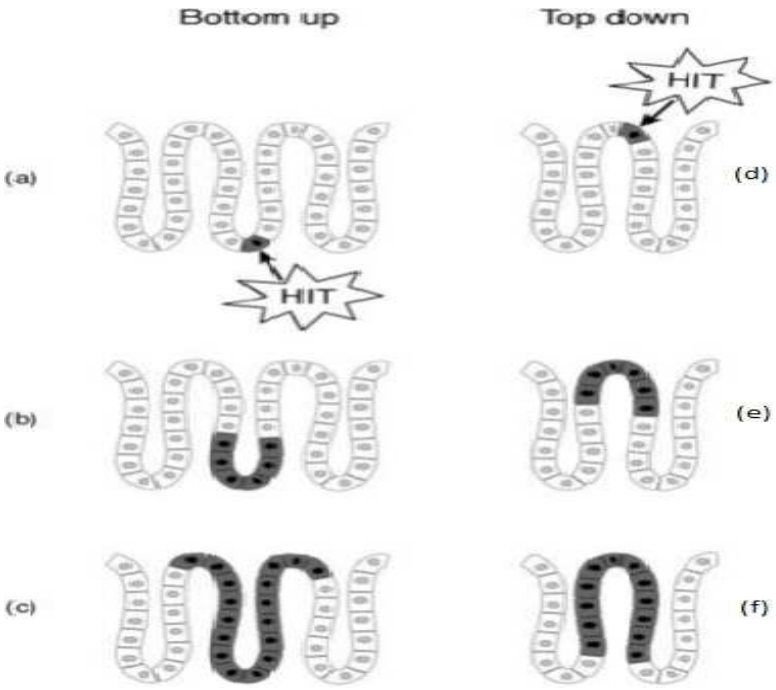


Figure 3: Top-down or bottom-up growth of colorectal adenomas. Adapted from [59]

The "top-down model" is based on observations of dysplastic cells only located at the luminal surface of the crypts (d), along with migration of adenomatous cells from the surface to the base of the crypt (e). In this model an initial stem cell mutation is proposed to occur in the epithelial mucosa situated in the intra-cryptal zone, between two crypt orifices, with subsequent stem cell division producing a mutant clone which expands laterally and downwards into the crypt, and thereby displacing the normal epithelial cells (f) [59]. Analysis of four single-nucleotide polymorphisms (SNP) within the APC gene in tissue from sporadic adenomas showed loss of heterogeneity (LOH) of APC in cells in the upper portion of the crypts, while no LOH was observed in the histological normal crypt bases [59]. Cells towards the top of the crypt display high proliferation activity [60]. These observations led to two hypotheses for a top-down model instead of the conventional bottom-up model: The stem cell could be located in the intra-cryptal zone, or if located in the base of the crypt the APC mutation in the stem cell would prevent it from a terminal differentiation and alter the cell's migration dynamics, migrate to the luminal surface and allowing it to remain in the mucosa before expanding laterally and downwards [61]. Both models ("top-down" and "bottom-up") may possibly occur. However, the bulk of evidence indicates, that the gastrointestinal stem cells are located in the base of the crypt [51], with no indication of a stem cell population in the intra-cryptal zone, and so the modified top-down hypothesis is proposed; that a stem cell in the crypt base acquires a mutation and subsequently migrates to the intra-cryptal zone, whereupon it undergoes neoplastic expansion. A crypt cycle, the time from a crypt "born" by crypt fission until they divide by crypt fission themselves, takes approximately 9-18 years in the human colon [62]. Studies on the methylation patterns of adjacent crypts showed significant inter-crypt variation, both in adjacent crypts and in those up to 15 cm apart, which may be a consequence of the time taken for crypts to divide, allowing neighbouring crypts to develop different methylation patterns during the process [63].

Identification of the origins, location, and molecular regulators of the intestinal stem cell will provide a clearer understanding of the genetic pathways and cell

signaling involved in the neoplastic changes in colorectal carcinogenesis. The stepwise pattern of mutational activation of oncogenes and inactivation of tumour suppressor genes that causes adenomas to develop to adenocarcinoma are called the adenoma–carcinoma sequence.

4.3 *The Adenoma-Carcinoma Sequence*

The progression of normal tissue through dysplasia to tumour tissue involves numerous steps. It is estimated that a typical colorectal tumour contains at least 11,000 genomic alterations. Two distinct pathways have been suggested in colorectal carcinogenesis. One involves chromosomal instability, which is characterized by allelic losses in chromosome 5q (APC), 17p (p53) and 18q (DCC/SMAD4), and the other involves microsatellite instability (MSI). The initial mutations in most of the cases occur at the APC tumour-suppressor gene locus (5 q21- q22). Loss of APC tumour suppressor gene function is thought to be one of the first genetic changes in colorectal adenoma development. APC encodes a large multifunctional cytoplasmic protein [64], which is an essential component of a "destruction complex" in the Wnt pathway involved in the binding and down-regulation of beta-catenin and thereby preventing excessive cell proliferation. Additionally, APC are involved in regulation of apoptosis, cell-cycle progression and chromosomal stability (reviewed in [65]. Hence, the importance of the APC protein in a number of different regulatory functions in cells in colon means, that mutation in the APC gene alone may be sufficient to provide a stem cell with a selective growth advantage by allowing unregulated activation of Wnt signalling. Hundreds of specific APC mutations have been characterised, and the position of the mutation appears to dictate the severity and onset of the hereditary syndrome FAP [66]. Patients with FAP have an autosomally dominant inherited germline mutation of APC and are therefore susceptible to mutation of the remaining wild-type APC allele [67]. FAP is characterized by the presence of hundreds of polyps in the large bowel. These arise first in the rectum and distal colon before extending to more proximal segments. Close to 100% of FAP individuals will develop colorectal cancer in the

distal colon. The mutations and genetic occurrences in the adenoma-carcinoma sequence are summarized and illustrated in Figure 4.

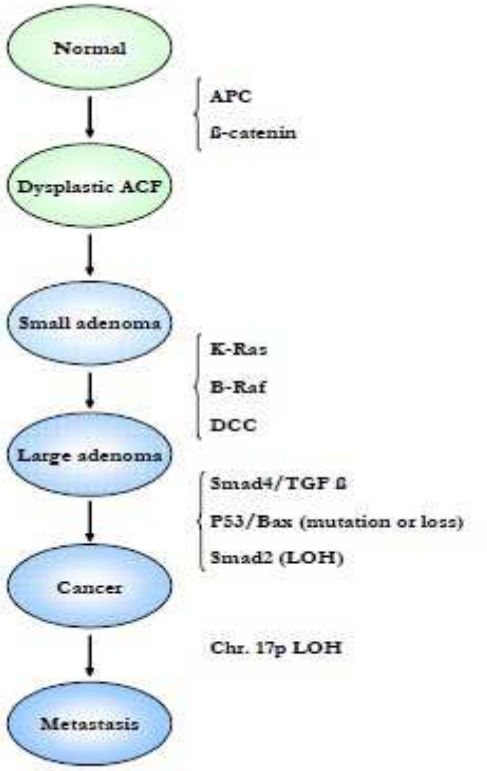


Figure 4: The possible genetic occurrences in the adenoma-carcinoma sequence.

Mutations in APC are found in 63% of sporadic adenomas and up to 80% of sporadic colorectal tumours [68]. Mutations in beta-catenin, that prevents the breakdown of the protein, can also promote adenoma initiation; however, small adenomas with beta-catenin mutations alone do not progress to larger adenomas or carcinomas as frequently as adenomas with APC mutations [69]. The P53 gene, located on chromosome 17p, is a tumour suppressor gene and is frequently lost in colorectal malignancy. The gene encodes for a DNA-binding phosphoprotein that prevents progress past the G1-phase of the cell cycle if DNA damage has occurred [70]. It is also characterized as a transcription factor, activating and promoting expression of genes involved in growth inhibition. The protein p53 is involved in several essential cell functions including control of the cell cycle, DNA repair and apoptosis, and thus is called the "guardian of the

genome". The half-life of wild type p53 protein and mutant p53 protein is approximately 20 minutes and 24 hours, respectively. The extended half-life of mutant p53 allows it to accumulate in the nucleus and be over-expressed in tumours [70]. Mutations of p53 are found in more than 50% of all human cancers and in more than 75% of colorectal adenocarcinomas [69].

It is debated whether the gene "deleted in colorectal carcinogenesis" (DCC) is a candidate tumoursuppressor gene. The DCC gene is deleted in more than 70% of colorectal carcinomas [71]. A second candidate tumour-suppressor gene, DPC4/Smad4, located in the same region on 18q21, is deleted in up to a third of the cases. The protein family SMAD are intracellular proteins that mediate the effects of signaling from extracellular transforming growth factor beta (TGF-U) and TGF-U-related factors [72].

Microsatellite instability (MSI) is explained by defects in DNA mismatch repair (MMR) genes, encoding proteins involved in recognition and repair of single base lesions and larger strand slippage mismatches in DNA replication. In sporadic colorectal cancer MSI usually arises due to epigenetic silencing of the DNA mismatch repair gene MutL homologue 1 MLH1 by methylation of cytosine and guanine residues in CpG-rich promoter regions [73], which prevents the gene-regions from being transcribed. MSI causes the Lynch syndrome primarily by a germline mutation in the mismatch repair genes MutS homologue 2 (MSH2) and MLH1. The life time risk of developing colorectal cancer is up to 75% higher in children with Lynch syndrome compared with the general population. Approximately 70% of large bowel tumours in patients with Lynch syndrome arise in the right/proximal colon [74].

The two pathways in the adenoma-carcinoma sequence, involving the chromosomal instability and microsatellite instability, seems well characterized. However, recent molecular studies have shown that colorectal carcinogenesis is not necessarily clearly divided into these two pathways, and may include other routes like the transforming growth factor beta (TGF-U)/SMAD-pathway, the serrated pathway and the epigenetic pathway. The TGF-U family are known inhibitors of gastrointestinal epithelial cell proliferation. Under normal circumstances TGF-U are involved in phosphorylation of two cytoplasmic

proteins, Smad2 and Smad3, following a formation of a heteromeric complex with Smad4. This complex translocates to the nucleus where it induces TGF-U target gene transcription [75]. Disruption of the TGF-U/Smad signalling pathway causes up-regulation of epithelial cell proliferation which may lead to tumorigenesis.

Smad2 and Smad4 are frequently inactivated in human cancers confirming their function as tumor suppressor genes [72]. The serrated pathway is characterized by early involvement of oncogenic mutations in the BRAF or KRAS genes and excess CpG island methylation. K-Ras and B-Raf are participants in a pathway regulating cell growth, differentiation and apoptosis (the MAPK-ERK pathway) [76].

Recently, a wealth of studies has implicated alterations in the epigenome, as also being important in cancer formation [77]. Epigenetics refers to heritable modifications to DNA that regulate gene expression without involvement of change in the DNA sequence. These modifications are amendments or chemical modifications to the DNA that includes global hypomethylation at repititive sequences in satellite or pericentromeric regions, focal hypermethylation at CpG islands, histone modifications by deacetylation and methylation of amino acids in the histone tails (reviewed in [78]) and DNA alkylation by methylation of guanine [79]. A new aspect of recent studies of epigenetic alterations in cancer is the observation that some genes that are involved in DNA repair (mismatch repair) are commonly found to be aberrantly methylated in the early stages of tumors [80].

5. Aim of the study

In our previous studies on paraneoplastic polydermatomyositis (81-83), we screened a small series of skeletal muscle biopsies from asymptomatic patients at early diagnosis for CCR, identifying an unexpected population of muscle fibers with nuclei misplaced from their physiological position (82). Aim of the present study is to confirm this observation in a larger group of CCR patients, also verifying whether these features are associated to the clinical-serological profile at diagnosis and during the follow-up, possibly providing new diagnostic and prognostic biomarkers for CCR and cancer-associated muscle wasting and explaining molecular pathways responsible of these cellular alterations.

6. Materials and Methods

6.1 Patients and Controls

Patients undergoing laparoscopic or laparotomic resection for colorectal cancer, referring to the Coloproctological Surgery Unit of Padua University between July 2008 and August 2010, were selected within endoscopic screening protocols and recruited for the study.

Inclusion criteria were: early diagnosis of colorectal cancer, no neoadjuvant treatments, no weight loss or other clinical signs of myopathy, no previous cancers. Exclusion criteria were: neoadjuvant treatment; previous neoplasms, systemic or radiant treatment before surgery, a weight loss >10% (with respect to habitual weight during the last 6 months); clinical signs of myopathy, presence of muscle pain, weakness or fatigue.

Patients undergoing surgery for benign, non-inflammatory conditions were also recruited as a control group. All patients included in the study signed an informed consent.

Demographic parameters (age, gender), Body Mass Index (BMI), clinical details (including previous surgeries, treatment with drugs known to induce myopathy, and concomitant diseases) were recorded at diagnosis and during the follow-up.

Follow-up was performed according to Institute protocols: in the first two years clinical and serological evaluations were performed every six months, also with chest X-Ray, colonoscopy and Liver ultrasonography or CT-scan. Starting from the third year of follow-up clinical, serological and radiological evaluations were performed annually, in absence of cancer relapse.

Operative time, surgical technique, site of the tumor and the anatomopathological characteristics of the surgically removed neoplasia (PTNM classification, tumor pattern; mitotic index, necrosis, lymphomonocytic infiltrate, vascular and perineural invasion, grading) were recorded.

6.2 Skeletal muscle biopsies and blood samplings

At the beginning of surgery all patients and controls underwent open biopsy of the *rectus abdominis* muscle. All biopsies were immediately frozen and stored in liquid nitrogen until use.

A venous blood sample was taken before surgery, collected serum was frozen in 500 microliters aliquots and stored at -80°C until use.

6.3 Serological analyses

Peripheral red and white blood cell counts, and biochemical parameters, including transthyretin, serum albumin, C reactive protein (CRP), muscle creatin kinase (CK) and cancer associated biomarkers (CEA, CA 19.9, AFP) were measured using routine laboratory methods.

6.4 Histological and histochemical analyses

Serial cross sections (8 μm 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 Hematoxilin and Eosin (HE) stained cross sections using Scion Image software for Windows, version Beta 4.0.2, (2000 Scion Corporation, Inc.; 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 at the periphery of the muscle fiber, within the extracellular matrix. The number of muscle fibers with internally located nuclei was expressed as the percentage of the number of abnormally nucleated fibers/number of total fibers of biopsy's area.

6.5 Immunofluorescence analyses

For immunofluorescence analyses in order to detect fast and slow myosin heavy chains proteins, regenerating muscle fibers (MHC-emb and N-CAM), nuclei

expressing marker of muscle stem cells (Pax7), or plasma membrane associated protein (laminin), unfixed sections were labelled either for 1 hour at room temperature (RT) or overnight at 4°C, with mouse monoclonal anti-embryonic, fast or slow myosin heavy chain antibodies (Novocastra, Newcastle-upon-Tyne, U.K.), rabbit polyclonal anti-N-CAM antibody (Chemicon, Italy), anti-Pax7 mouse monoclonal antibody (DSHB, Iowa), or anti-laminin rabbit polyclonal antibody (Sigma, Italy) 1:100 diluted in PBS, respectively. Sections were then incubated for 1 hour at RT with Cy3 or Alexa Fluor® 488 dye conjugated antibodies against rabbit (Chemicon, Italy) or mouse IgG (Life technologies, Italy). Sections were then mounted on glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies).

On the acquired images the number of MHC-emb and N-CAM positive muscle fibers were counted and expressed as the number of positive fibers per mm^2 of total biopsy's area (each mm^2 corresponds approximately to 500 muscle fibers).

6.6 Western Blot

Antibodies against LC3 (rabbit, polyclonal,1:1,000 in 2.5% milk in Tris-Buffered Saline Tween 20 (TBST);Novus Biologicals), p62 (mouse, monoclonal, 1:1,000 in 2.5% milk in TBST; BD Biosciences), and α-tubulin (mouse, monoclonal, 1:1,000 in 2.5% milk TBST; Sigma) were used. The ECL Advance Western Blotting Detection Kit (Amersham Biosciences) was used for signal detection.

6.7 Statistical analysis

Continuous variables have been compared with non-parametric Wilcoxon test with exspression of Median value and Interquartile Range (IQR). Categorical variables have been compared with Fisher exact test. Association between continuous variables was assesed with Spearman's Rho coefficient. Statistical significance was defined as $p < 0,05$. All statistical analyses were performed with SAS 9.1.

7. Results

We screened *rectus abdominis* muscle biopsies from 50 weight stable, non cachetic patients affected with CCR at diagnosis and from 25 healthy subjects undergoing surgery for benign non-inflammatory conditions as controls (Tab. 3).

Table 3. Demographic characteristics of the recruited patients; site and TNM classification of resected specimens from CCR patients.

Controls (n=25)	
Median age (IQR)	61 (35-66)
Sex (M:F)	18:7
CCR patients (n=50)	
Median age (IQR)	67 (61-70)
Sex (M:F)	28:22
Site of neoplasm	No. (%)
Right colon	8 (16)
Left colon	28 (56)
Rectum	13 (26)
Colon-rectum	1 (2)
PTNM classification	No. (%)
pTis/T1/T2	23 (46)
pT3/T4	27 (54)
pN0	36 (72)
pN1-2	14 (28)
pM0	47 (94)
pM1	3 (6)

F, female; M, male; IQR, interquartile range;

In serial sections, muscle fibers with nuclei mispositioned in the cytoplasm were observed (Fig.5) and nuclei ultrastructure was confirmed by Electron Microscopy (Fig.6).

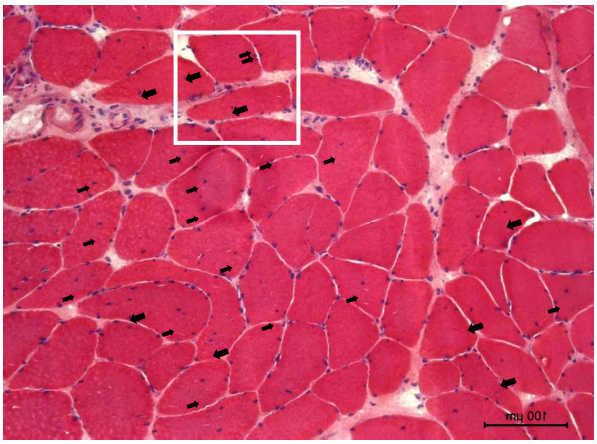


Fig.5 Muscle sections showed fibers with nuclei placed at sarcolemma and between myofibrills (arrowed in black).

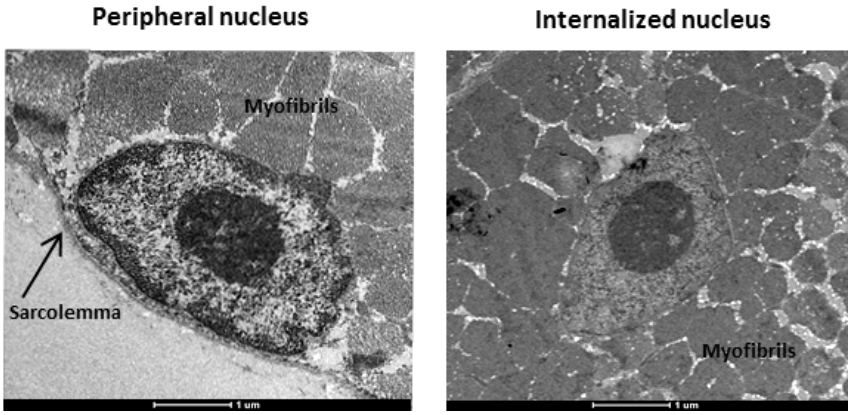


Fig.6 Nuclei ultrastructures were confirmed by Electron Microscopy.

Both, at univariate and multivariate analyses, the percentage of fibers with internal nuclei was significantly higher in CCR patients compared to controls (Fig.7).

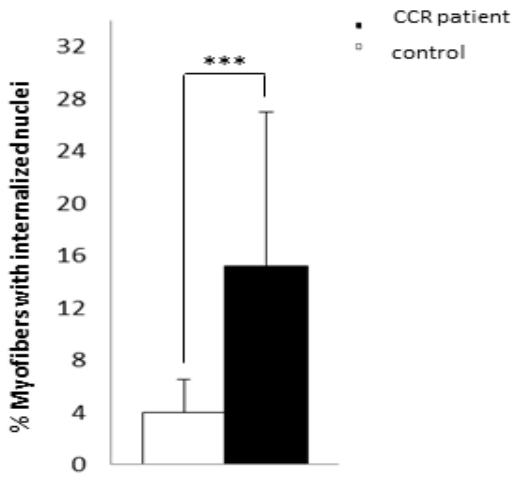


Fig. 7 The percentage of fibers with internally located nuclei was significantly higher in CCR patients compared to controls

Internal nuclei were negative for CD45 (leukocyte common antigen) (Fig.8) and Pax7, a nuclear transcription factor, expressed by quiescent muscle stem cells (satellite cells) that become activated contributing to postnatal muscle growth and repair (Fig.9).

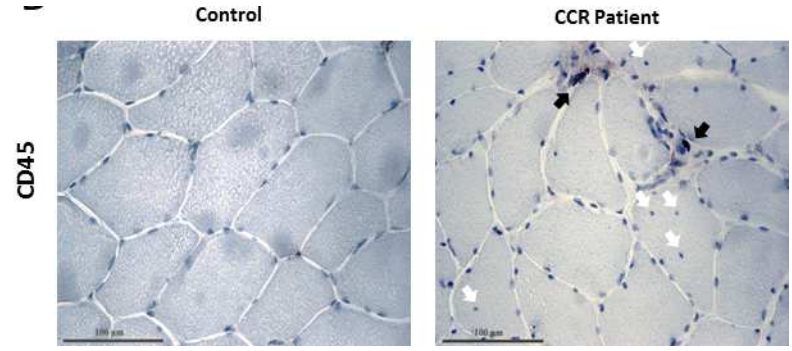


Fig.8 Seldom immuno-inflammatory cells were detected inside small vessels (brown stained), while misplaced nuclei were negative (white arrowed).

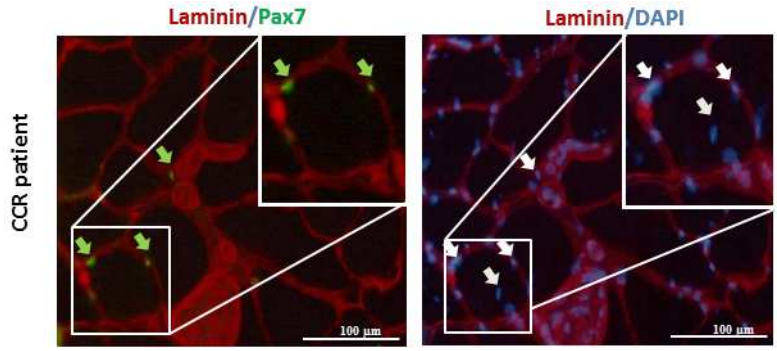


Fig.9 In CCR patients some muscle stem cells (green arrowed) physiologically positioned at peryphery were found, but not inside the fiber.

Some fibers expressing the embryonic isoform of Myosin Heavy Chain (MHC-emb) and Neural Cell Adhesion Molecule (N-CAM), two biomarkers of muscle regeneration, were identified at higher percentage in CCR patients compared to controls, even though not significantly for MHC-emb, while significantly for N-CAM.

	Controls	**CCR Patients**	**p**
Fiber diameter (mean±SD, μm)	50,0±7,9	53,0±8,7	n.s.
Abnormally nucleated fibers (median±SD, %)	4,0±2,5	15,2±11,8	**0,0002**
MHC-emb positive fibers (mean, %)	11,0	12,0	n.s.
N-CAM positive fibers (mean, %)	16,0	40,0	**0,04**

MHC-emb embryonic myosin heavy chain; N-CAM neural cell adhesion molecule; p statistical significance at univariate analyses; n.s. not significant; SD standard deviation. Significant values are indicated in bold.

Tab.4 Histopathological features of rectus abdominis muscle biopsies.

However, this significance was lost at multivariate analyses normalized by age. Atrophy of muscle fibers in CCR biopsies, wasn't observed, and no correlation was found between regenerating and internally nucleated fibers. At univariate analysis, the percentage of internally nucleated fibers was inversely correlated either with lymph-node metastasis or with the number of metastatic lymph nodes at diagnosis (ρ=-0.30; p=0.03). This significant correlation was confirmed also at multivariate analysis (p=0.02) (Tab.5).

		Abnormally Nucleated fibers (%) Median (IQR)	p
Age (ρ)		-0,14	0,33
Sex	M	16,0 (8,7-22,6)	0,11
	F	9,1 (5,1-18,9)	
BMI (ρ)		0,21	0,14
pT	1-2	15,0 (6,0-20,0)	0,96
	3-4	12,6 (6,6-21,2)	
pN	0	15,5 (8,2-22,6)	**0,05**
	1-2	7,1 (4,6-18,3)	
N+ (ρ)		-0,30	**0,03**
pM	0	12,8 (6,0-21,5)	0,95
	1	19 (10,9-19,7)	
Perivascular Invasion			
	Present	12,8 (8,4-21,0)	0,13
	Absent	6,6 (4,9-18,8)	

Tab 5. Correlation between abnormally nucleated muscle fibers (%) in CCR patients and clinical and phenotypical characteristics of the tumor (BMI, Body Mass Index; IQR, interquartile range. Significant values are indicated in bold).

Preoperative serum levels of cancer-associated biomarkers CEA, CA19.9 and AFP, C reactive protein (CRP) and muscle creatine kinase enzyme (CK) were above the normal range in some CCR patients, with no correlation with the percentage of abnormally nucleated or regenerating fibers. In the prevalence of CCR patients, preoperative serum levels of transthyretin and albumin were below the normal range, with mean values significantly lower compared to controls (albumin: 34.82 ± 5.8 g/L vs 45.2 ± 5.3, $p< 0.01$; transthyretin: 174.38±57.86 mg/L vs 264.00±69.73, $p<0.001$), but not in correlation with the percentage of internally nucleated fibers (Fig.10).

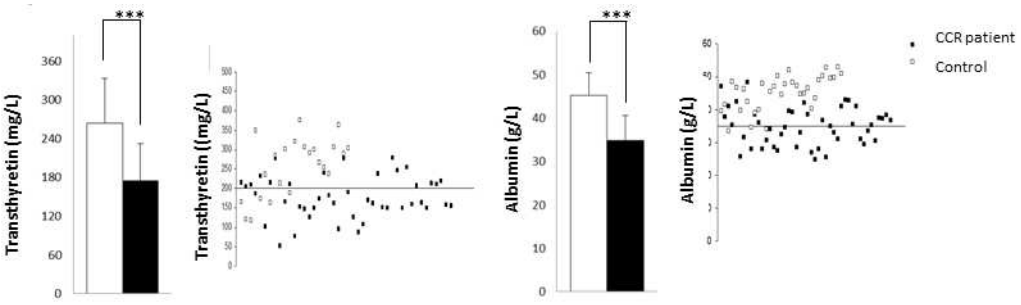
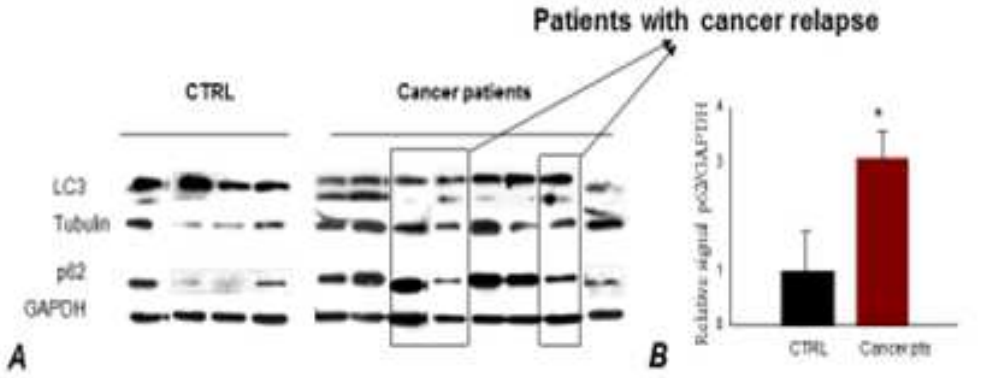


Fig.10 Preoperative serum levels of transthyretin and albumin were significantly lower in CCR patients.

Molecular analyses showed a modulation of LC3 lipidation and an accumulation of p62 in skeletal muscle biopsies from CCR patients at Western Blot; densitometry of p62 levels showed significant accumulation of p62 in tumor patients compared to controls ($p < 0.05$), with higher risk of relapse even if in absence of statistical significance (Fig.11).



(A)Western blot showing modulation of LC3 lipidation and accumulation of p62 in skeletal muscle biopsies from colon carcinoma patients and healthy subjects (CTRL).

(B)Densitometry of p62 levels, showing significant accumulation of p62 in tumor patient in respect to healthy subjects (CTRL).

Fig.11 Modulation of autophagy flux in skeletal muscle of colorectal cancer patients.

Mean follow-up of CCR patients was 38±12 months: deceases occurred in 18% (9/50) and cancer recurrences in 32% (16/50). 50% of CCR patients (UICC Stage III-IV) was treated with adjuvant chemotherapy. No significant differences between the percentage of internally nucleated fibers, and preoperative serological parameters between treated and untreated CCR patients, were observed (Tab.6).

	CHT (n=25)	No CHT (n=25)	p
Myofiber diameter (mean±SD, μm)	55,1±8,3	51,0±8,7	n.s.
Abnormally nucleated fibers (mean±SD, %)	15,0±10,4	16,4±12,8	n.s.
Transthyretin (mean±SD, mg/L)	182,7±51,2	171,6±57,5	n.s
Albumin (mean±SD, g/L)	41,1±4,6	41,4±4,2	n.s.

CHT chemotherapy; SD standard deviation.

Tab.6 Histopathological features and serological markers of protein turnover in CCR patients treated and not treated with neo-adjuvant neo-adjuvant chemotherapy after surgery.

Cancer relapse was significantly correlated with lymph-node metastasis, and serum levels of transthyretin and albumin, as well as the percentage of internally nucleated myofibers at diagnosis were lower in those patients with cancer recurrence, compared to those without relapse, even though not significantly (Tab.7). None of the CCR patients developed cachexia during the follow-up.

	Relapse (n=16)	No Relapse (n=34)	p
Abnormally nucleated fibers (mean±SD, %)	13,2±10,4	16,2±11,5	0,42
Lymph-node metastasis (mean, %)	46	19	**0,02**
Transthyretin (mean±SD, mg/L)	156,5±44,2	190,6±57,2	0,08
Albumin (mean±SD, g/L)	33,1±6,5	36,1±5,6	0,16

SD standard deviation. Significant values are indicated in bold.

Tab.7 Correlation between clinical outcome and abnormally nucleated fibers and serological markers of protein turnover in CCR patients.

8. Discussion

Colorectal cancer is the second cause of cancer death in Europe and the third most often diagnosed neoplasia in the United States [84].

In our previous studies we observed a subclinical myopathy in a small group of patients affected with newly diagnosed colorectal cancer [81-83]. Surprisingly, the observed myopathic features were never been observed in the skeletal muscle of patients at this stage of disease, and were very similar to those detected in patients affected with cancer-associated myositis [83].

This preliminary and unexpected observation raised the question whether these morphological changes of the skeletal muscle in cancer patients were associated to the clinical and serological profile of the patients, possibly providing new diagnostic and prognostic markers of disease.

In order to address this question, we collected additional skeletal muscle biopsies from the *rectus abdominis* of patients affected with colorectal cancer at diagnosis undergoing surgery for tumor colorectal resection and subjects affected with benign non-inflammatory conditions as controls.

In the skeletal muscle from cancer patients, a significantly higher percentage of myofibers with abnormal distribution of the myonuclei and of regenerating myofibers expressing the MHC-emb and N-CAM was found in comparison to control biopsies (Tab.4). In particular, up to the 60% of the patients had a percentage of internally nucleated myofibers higher than the reported normal value (5%) [81,85-86].

Skeletal muscle during ageing undergoes several morphological changes characterized by muscle fiber atrophy, mainly of the fast type fibers, changes in fiber type distribution with a shift to a slower contractile phenotype, significant increase of fat and connective tissues, and motor unit remodeling documented by the presence of type grouping [87-89]. These age-related changes in muscle histology are predominantly observed starting from the 50 years of the subject, and are associated with a decrease in muscle force and contractile properties [90-91]. Beside the median age of 67 years of the cancer patients enrolled in the present study, in the skeletal muscle from these subjects we did not

observed the classical histopathological features of ageing except for the presence of slow type grouping as a typical sign of re-innervation in response to denervation events.

The shifting towards slow type fiber distribution in aged skeletal muscle is a general issue [89-93], but in the skeletal muscles from these group of cancer patients we observed a higher percentage of fast type myofibers compared to controls, even though the difference was not statistically significant (p= 0.06). The interesting point is that the abnormally nucleated myofibers were predominantly of fast type, indicating that this phenomenon is restricted to a specific type of muscle fibers.

Even if the age of our patients could be a critical point because of the associated muscle histopathology, no correlation was found between the percentage of internally nucleated myofibers and the age of the patients, both at univariate and multivariate analyses (Tab.5). Beside this, the multivariate analyses normalized by age, showed that the percentage of N-CAM positive myofibers was not significantly higher in patients compared to controls, indicating that this feature is more closely related to the age of the patients, reasonably as results of the denervation events associated to the age-related motor unit remodeling [86-89,92,93]. Both denervated and regenerating myofibers overexpress N-CAM molecule. In the skeletal muscles from our cancer patients, features of denervation and muscle fiber regeneration have been observed, but these aspects were not observed in association with the abnormal redistribution of myonuclei within the muscle fibers, indicating that these seem to be independent phenomena.

The negative expression of muscle stem cell marker by the misplaced nuclei, and the absence of correlation with regeneration events, indicates that they are post mitotic nuclei in mature and adult muscle fibers (Fig. 9) [83]. In the absence of local and systemic inflammation, this phenomenon can possibly occur in response to factors released by tumor microenvironment.

The intriguing inverse correlation observed between the percentage of abnormally nucleated fibers and lymph-nodes metastasis at diagnosis (Tab.5), indicate that these features could represent an adaptive response or an initial

defense mechanism of skeletal muscle to the onset and spreading of the tumor. This hypothesis is also supported by the observation that patients in which the percentage of fibers with nuclear abnormalities was higher at diagnosis, had better prognosis in terms of cancer relapse. Furthermore, recent studies focused on the contribution of autophagy in muscle wasting in cancer patients [94-99]; in particular, an activation of autophagy, probably induced by TNF-α, with an impairment of autophagic flux, has been demonstrated [96-98], with a relationship between tumorigenesis, cachexia and high levels of p62, a typical marker of autophagy induction [99]. According to these findings we demonstrated in C26-bearing cachectic mice an accumulation of p62, and these significant higher accumulation has been confirmed in skeletal muscle biopsies from cancer patients compared to controls (Fig. 11).

Furthermore, we have seen that promoting autophagy with exercise and two different drugs could ameliorate muscle homeostasis in cachectic mice, suggesting that exercise, as well as autophagy triggering drugs, could reveal a potential use in order to improve cancer cachexia, as described by some authors [100-101].

Transthyretin has been described as a prognostic marker of cancer relapse in patients affected with non-small-cell lung and CCR [102-103]; in line with these data, in our study patients who developed relapse, had lower preoperative levels of transthyretin compared to those without relapse, in trend of statistical significance.

These findings need more exhaustive investigations focused on the underlying molecular mechanisms, but they raise new perspectives on skeletal muscle physiopathology in CCR: the muscle can be not only a pathological target of the disease, but also as a tissue responding to the onset of the disease, contributing with many other factors, to the final outcome towards healing or dissemination of cancer.

We can conclude that in CCR patients at diagnosis, skeletal muscle fibers show nuclear abnormalities that seems to be associated to a better prognosis, and by altered protein turnover biomarkers, with an impairment in the physiological autophagic flux, which seem to be correlated with poor prognosis, defining subgroups of patients that could need different follow-up programs.

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"Digestive Disease Week" DDW. Orlando, Fl 18th-21st May 2013 with oral presentation.

"Digestive Disease Week DDW". Chicago, Il 03rd-06th May 2014 with oral presentation.

"XXXVII Congress of the Italian Society for Surgical Oncology" SICO. Padua, 19th-20th June 2014 with poster presentation.

"Digestive Disease Week" DDW. Washington, DC 16th-19st May 2015 with oral presentation.