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TESI DI LAUREA

RECTAL ADENOCARCINOMA: INFLUENCE OF BMI IN THE IMMUNOLOGICAL MICROENVIRONMENT OF PERITUMORAL HEALTHY MUCOSA - AN OBERSERVATIONAL STUDY

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INDEX

ABSTRACT

Background: Rectal cancer represents ~ 35% of the total colorectal cancer (CRC) incidence, the latter being the third most common cancer diagnosed globally. Although the aetiology of *sporadic* rectal cancer (≥70% of all cases) has not been fully understood, several risk factors have been proven to increase the likelihood of developing such disease, among which diet and obesity play a major role: the association between BMI and rectal cancer incidence has been extensively investigated and it has been proven that incidence increases with BMI. The importance of the tumor microenvironment in the development, growth, and progression of cancer is well recognized: the peritumoral microenvironment is known to act to suppress the immune response against cancer cells, and tumor-infiltrating lymphocytes have a crucial role in immune surveillance. Obesity leads to an imbalance in adipokines, gut dysbiosis, and endotoxemia, as well as IGF-1 activation pathways and free fatty acids release that can influence the immune microenvironment. The cross-talk between tumor cells and the immune microenvironment can be detected in the normal "healthy" mucosa surrounding cancer, according to the concept of "field of cancerization". Although previous studies describe the impact of overweight and obesity as risk factors for rectal cancer development, very few information is present regarding the influence that a high BMI could have on the immune microenvironment of peritumoral healthy mucosa.

Aim of the study: The aim of this study is to analyze the healthy rectal mucosa surrounding rectal cancer in overweight/obese patients who underwent surgery to evaluate the potential alteration of immune surveillance mechanisms of healthy rectal mucosa.

Materials and methods: This study is a sub-analysis of data from the IMMUNOREACT 1 and 2 trials (NCT04915326 and NCT04917263). This multicentric study collected healthy mucosa surrounding the neoplasms of patients with early rectal cancer. A panel of immune markers was retrospectively investigated at immunohistochemistry: CD3, CD4, CD8, CD8beta, Tbet, FoxP3,

PD-L1, MSH6, and PMS2 and CD80. A prospective analysis was performed with fluorescence-activated cell sorting (FACS) to determine the proportion of epithelial cells expressing CD80, CD86, CD40, HLA ABC or HLA DR and the proportion of activated CD8+ T cells, CD4+ Th1 cell, and T reg. Immune markers of healthy rectal mucosa were compared between patients under and over the BMI 25 (BMI<25 and BMI ≥25).

Results: A total of 213 patients with rectal cancer, whose data of body mass index were available, were analyzed: 103 in the retrospective cohort and 110 in the prospective cohort. In our study group, 88 patients were normal weight (BMI <25) while 125 were overweight or obese (BMI≥25). Obese/overweight patients with rectal cancer had a lower expression of HLA-ABC on the surface of their epithelial cells than those with BMI under 25 (CK+HLA-ABC+ MFI 549 (377, 792) vs 416 (261, 663), p= 0.069). Such result was particularly significative in patients undergoing neoadjuvant therapy, where overweight/obese ones had a lower frequency of high expression of HLA-ABC on epithelial cells than normal-weight patients. Moreover, overweight/obese patients had a lower infiltration of CD8beta+ T cells within the healthy mucosa surrounding the cancers than those with BMI under 25 (CD8beta+ T cell /field: 87 (IQR: 60-106) 16 (IQR: 5-68), p=0.04). Finally, the infiltration of CD8+ T-cells in the healthy mucosa inversely correlated with BMI (rho=-0.34, p=0.03).

Conclusions: Our findings suggest that in patients with rectal cancer, those who are obese/overweight have a lower activation of epithelial cells as antigen-presenting cells (APC cells) and a lower activation of cytotoxic T-cells (CD8+ T-cells) in their healthy mucosa surrounding rectal cancer. This could be in part explained by the chronic inflammatory state related to obesity and excessive fat accumulation, that has been proven to influence negatively on the ongoing immune perturbation within the tumoral and peritumoral microenvironment, potentially favoring tumor progression. Surely, more studies need to be de done to identify causal mechanisms of BMI-associated influence on the immune microenvironment of rectal cancer treated patients. These data could be useful to plan a tailored approach to overweight/obese patients with a rectal cancer diagnosis.

RIASSUNTO

Presupposti dello studio: Il cancro del retto rappresenta ~ il 35% della totale incidenza del cancro colorettale (CCR), terzo tumore più frequentemente diagnosticato a livello globale. Sebbene l'eziologia del carcinoma rettale sporadico non sia ancora completamente chiara, è stato dimostrato che diversi fattori di rischio aumentano la probabilità di sviluppare tale patologia, tra i quali dieta e obesità giocano un ruolo fondamentale: l'associazione tra BMI e incidenza del cancro del retto è stata ampiamente studiata ed è stato dimostrato che essa aumenta all'aumentare del BMI. L'importanza del microambiente tumorale e peritumorale nella promozione, crescita e progressione del cancro è ben riconosciuta: il microambiente immunitario è noto, infatti, per poter essere influenzato in diversi modi, arrivando a sopprimere la risposta immunitaria contro le cellule tumorali (*favorendo l'"immunoevasione" delle stesse*) o a controllarla positivamente ("*immunosorveglianza"*). In proposito, i linfociti infiltranti il tumore (TILs) hanno un ruolo cruciale nella sorveglianza immunitaria. L'obesità si associa a squilibrio adipochinico, disbiosi intestinale ed endotossiemia, nonché all'iperattivazione delle vie di signalling dell'IGF-1 e all'eccessivo rilascio di acidi grassi liberi (FFA) che possono influenzare il microambiente immunitario. Il cross-talk tra cellule tumorali e microambiente immunitario può essere rilevato nella normale mucosa "sana" che circonda il cancro, secondo il concetto di "campo di cancerizzazione". Sebbene studi precedenti descrivano l'impatto del sovrappeso e dell'obesità come fattori di rischio per lo sviluppo del cancro del retto, sono poche le informazioni relative alla possibile influenza di un elevato BMI sul microambiente immunitario della mucosa sana peritumorale.

Scopo dello studio: Lo scopo di questo studio è quello di analizzare la mucosa rettale sana che circonda il tumore del retto nei pazienti sovrappeso/obesi sottoposti a intervento chirurgico, con l'obiettivo di valutare la potenziale alterazione dei meccanismi di sorveglianza immunitaria della mucosa rettale sana stessa.

Materiali e metodi: Questo studio nasce come sub-analisi dei dati dei due più grandi progetti "IMMUNOREACT 1 e 2" (NCT04915326 e NCT04917263). Trattasi di uno studio multicentrico che ha raccolto la mucosa sana circondante le neoplasie di pazienti con cancro del retto in fase iniziale (*early rectal cancer*). Un pannello di marcatori immunitari è stato retrospettivamente analizzato mediante utilizzo di

tecniche di immunoistochimica (IHC): CD3, CD4, CD8, CD8beta, Tbet, FoxP3, PD-L1, MSH6, PMS2 e CD80. È stata inoltre eseguita un'analisi prospettica mediante tecniche di citofluorimetria a flusso (FACS) per determinare la proporzione di cellule epiteliali esprimenti CD80, CD86, CD40, HLA-ABC o HLA-DR e la proporzione di cellule T CD8+ attivate, cellule Th1 CD4+ e T reg. I marcatori immunitari della mucosa rettale sana sono stati confrontati tra pazienti con BMI inferiore e superiore a 25 (BMI <25 e BMI ≥25).

Risultati: Sono stati analizzati 213 pazienti operati per tumore del retto, di cui erano disponibili i dati di indice di massa corporea: 103 nella coorte retrospettiva e 110 nella coorte prospettica. Nel nostro gruppo di studio, 88 pazienti erano normo/sottopeso (BMI <25) mentre 125 erano sovrappeso o obesi (BMI ≥25). I pazienti obesi/sovrappeso con tumore del retto presentavano una minore espressione di HLA-ABC sulla superficie delle cellule epiteliali rispetto a quelli con BMI< 25 (CK+HLA-ABC+ MFI 549 (377, 792) vs 416 (261, 663), p= 0,069). Questo risultato è risultato particolarmente significativo nei pazienti sottoposti a terapia neoadiuvante, dove quelli sovrappeso/obesi avevano una marcata minore espressione di HLA-ABC sulle cellule epiteliali rispetto ai normo/sottopeso. Inoltre, i pazienti sovrappeso/obesi presentavano una minore infiltrazione di cellule T CD8beta+ all'interno della mucosa sana peritumorale rispetto al gruppo con BMI <25 (cellule T CD8beta+ /HPF: 87 (IQR: 60-106) 16 (IQR: 5-68), p=0,04). In ultimo, l'infiltrazione di cellule T CD8+ nella mucosa sana è risultata inversamente correlata al BMI (rho=-0,34, p=0,03).

Conclusioni: I nostri risultati suggeriscono che nei pazienti con cancro del retto, quelli obesi/sovrappeso hanno una minore attivazione delle cellule epiteliali come cellule presentanti l'antigene (APC) e una minore attivazione delle cellule T-citotossiche (cellule T CD8+) nella mucosa sana circostante il tumore. Questo potrebbe essere, in parte, spiegato dallo stato di infiammazione cronica associato all'obesità e all'eccessivo accumulo di grasso, che si è dimostrato poter influenzare negativamente la risposta immunitaria in corso nel microambiente peritumorale, potenzialmente favorendo la progressione del tumore. Sicuramente è necessario condurre ulteriori studi per identificare i meccanismi causali sottostanti l’impatto del BMI sul microambiente immunitario nei pazienti con RC. Questi dati, tuttavia, potrebbero essere utili per pianificare un approccio personalizzato ai pazienti sovrappeso/obesi con diagnosi di cancro del retto.

INTRODUCTION

1. RECTAL CANCER

1.1. EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most common cancer diagnosed globally, comprising ~ 11% of all cancer diagnoses (1) and the second leading cause of cancer death among American adults and in Western countries (2) - numbers for men and women being combined.
Moreover, according to ESMO 2017 data (3), rectal cancer represents ~ 35% of the total colorectal cancer incidence, reflecting 15–25 cases/100.000 population per year (mean European incidence) and is predicted to increase further in both genders. Although colon cancer and rectal cancer are often epidemiologically and pathologically grouped together, it is important to notice that they are different tumors that develop from different parts of the GI tract; this has huge implications in terms of diagnosis, staging and therefore treatment. Rectal cancer differs from the former because of the anatomical narrow confines of the pelvis and the proximity of the genitourinary organs and nerves and the anal sphincter mechanism. It is important to have a clear anatomical definition of the rectum. Any tumor whose distal margin is seen at 15cm or less from the anal verge, using a rigid sigmoidoscope, should be classified as rectal (4). Furthermore, evidence is accumulating that rectal cancer is distinct from colon cancer in terms of different etiologies and risk factors (5), possibly reflecting different environmental exposures, *as discussed more ahead*.

CRC incidence has been steadily rising worldwide, especially in developing countries that are adopting the “western” way of life: not only it is well established that CRC is more incident among men than women, but also ~3–4 times more common in developed than in developing nations (3).
Age-standardized world incidence rates per 100.000 of CRC among men is 30.1 in high-HDI (*human development index*) nations, while it is 8.4 in low-HDI nations (the same statistics for women are 20.9 and 5.9, respectively).

For rectal cancer, regions of highest incidence are Eastern Europe, Australia/New Zealand, and Eastern Asia (3). North America also features among the highest incidence rates for both cancers, colon and rectal. In countries undergoing a major developmental transition, incidence rates tend to rise uniformly with increasing HDI, suggesting a causal relationship (3). (Figure 1)

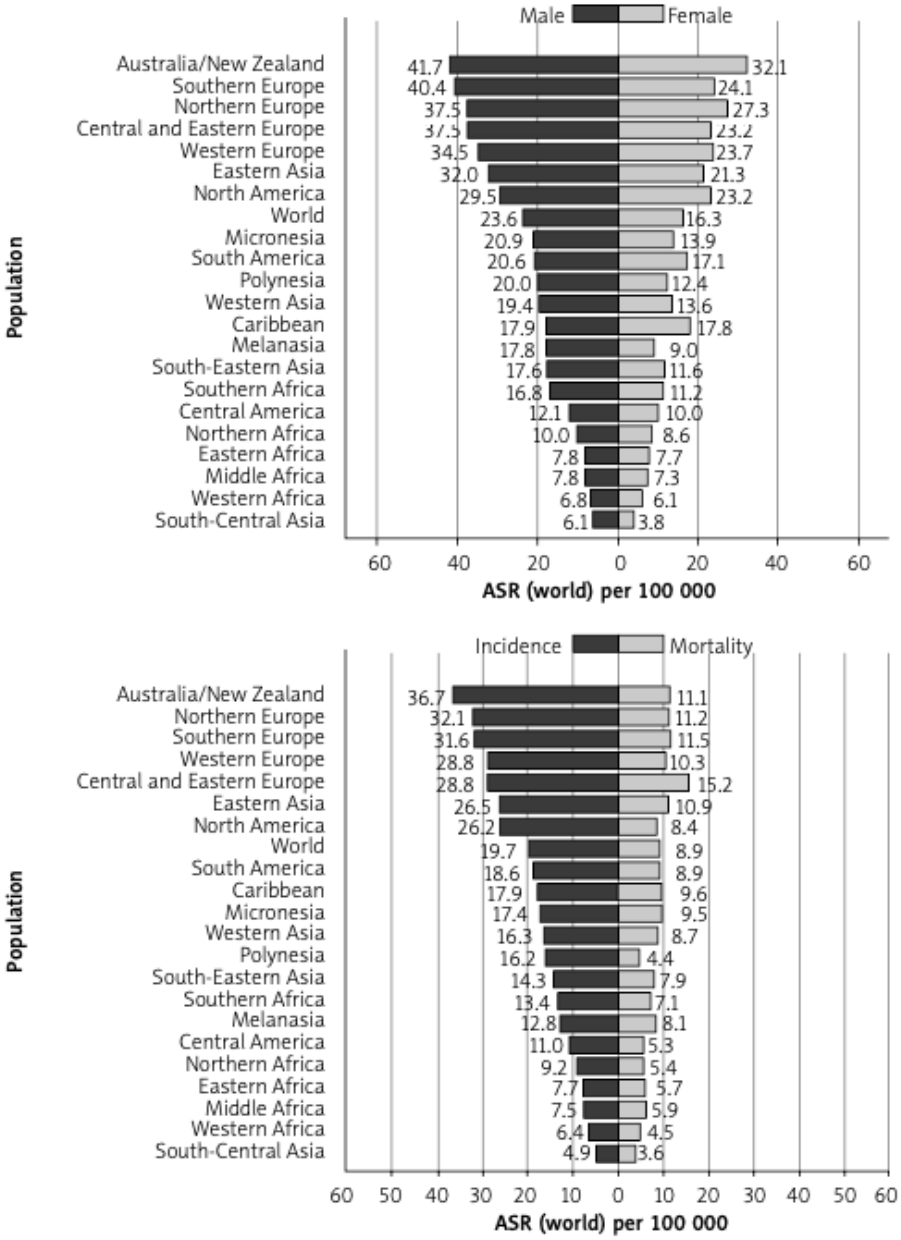


Figure 1. Bar chart showing age-standardized (world) incidence rate of CRC by sex in 2018 *(above)* and age-standardized (world) incidence and mortality rates of CRC in 2018 *(below)*. (From *Global Cancer Observatory: Cancer Today*. IARC, 2018 *(6)*)

1.1.1 Trends

The relation between trends in rectal incidence and mortality can be characterized

into three distinct global categories: the first, comprised of medium HDI nations undergoing economic transition (*"semi-periphery nations*", such as Brazil, Russia, China, Latin America, the Philippines, the Baltics), has witnessed an increase in both incidence and mortality in the past decade (3). The second, that includes mostly high-HDI (such as Canada, the UK, Denmark, and Singapore), has seen an increase in incidence but drop in mortality due to improved treatment options (3). Finally, the last group, made up of the highest HDI nations (such as the US, Iceland, Japan, and France *and others*) has witnessed a drop in both mortality and incidence due to successes in prevention and treatment (3). Nevertheless, while those over 50 years of age have seen decreases in colon and rectal cancer incidence in the US over the past decades, those aged 20–49 years have seen a dramatic growing incidence (3): in the latter, the incidence rates of was 9.3/100.000 in 1975 and now is up to 13.7/100.000 in 2015, a percentage change of 47.31%, whereas incidence rates in age groups 50/+ has steadily decreased: researchers believe this may be a reflection of a more sedentary lifestyle and have since recommended lowering the screening age to 45 years in order to detect cases in younger adults earlier (3).

The global burden of colon & rectal cancer is expected to increase by 60%, to over 2.2 million new cases and 1.1 million annual deaths, by the year 2030 (3), as a product of the economic development of transitioning and low-to-medium-HDI nations, as well as generational changes in developed nations. Increases in the incidence seem to uniformly follow the economic development: such growth is hypothesized to be a product of environmental changes, such as more sedentary lifestyle, greater obesity, processed food, alcohol, and meat consumption, and greater overall longevity. (7)

1.2. AETIOLOGY AND RISK FACTORS

Up to now, the aetiology of rectal cancer has not been fully understood. It most commonly occurs *sporadically* (~70%) as a "complex or multifactorial disease", whilst it is *familial* in approximatively 30% of all cases, of which ~5% are *hereditary,* arising in the setting of well-defined hereditary syndromes associated with highly penetrant inherited gene mutations (including: Lynch syndrome, familial adenomatous polyposis, MUTYH-associated polyposis, and certain hamartomatous polyposis conditions).

Risk factors for (colo)rectal cancer must be divided in two broad categories: modifiable and non-modifiable, among which – as reported by the *World Cancer Research Fund* and the *American Institute for Cancer Research* in their extensive revision on the scientific literature (8) – diet and obesity play a major role, rendering the disease by some means preventable through lifestyle modification.

Furthermore, evidence is accumulating that rectal cancer is distinct from colon in terms of exogenous risk factors, possibly reflecting different environmental exposures. After a systematic literature review of 752 publications, a panel of experts made the following conclusions (9):

- physical activity protects against CRC, although the evidence is stronger for colon than for rectal cancer.
- consumption of red meat and processed meat, moderate/heavy consumption of alcohol (more than 30 g per day of ethanol), body fatness and abdominal fatness are risk factors for CRC.
- evidence that high BMI, body or abdominal fatness and diabetes type 2 are risk factors for rectal >> colon cancer.

Still within dietary factors, consumption of garlic, milk, calcium, and high-in-fiber foods are regarded as protective; limited data suggest that there may also be a protective effect of vitamin D via antitumor immunity.

As for non-diet-related risk factors, the main ones consist of cigarette smoking, chronic use of NSAIDs and certain conditions such as chronic IBDs, genetic predisposition, and the presence of metabolic syndrome.
In the United States, one in five cases of (colo)rectal cancer is potentially attributable to smoking, particularly in those where BRAF gene mutations or microsatellite instability are detectable (10): exposure to constituents of tobacco smoke may be an initiating factor for colorectal carcinogenesis.
Studies to determine the role of NSAIDs in the prevention or regression of adenomas and colorectal cancers have shown a role of aspirin in significantly reducing the occurrence of sporadic adenomatous polyps.
Chronic inflammatory bowel diseases (*especially ulcerative colitis*) increase the risk of developing CCR. In the natural course of UC, the risk of malignant degeneration is a nonnegligible event (7-15%) and it is associated with both, the

extent of disease (*greater if pancolitis, where chronic inflammation is extended from the rectum up to the ileum*) and its duration (*increased risk up to 19 times in a long-term history of UC* (11)).

The metabolic syndrome (≥3 of the following components: high blood pressure, increased waist circumference, hypertriglyceridemia, low levels of HDL-cholesterol, or diabetes/hyperglycemia) has a modest, positive association with colorectal cancer incidence and there appears to be a dose response according to the number of components present (12).

Last but not least, the likelihood of developing CCR is significantly higher in individuals older than 50 years, especially of the male gender, underscoring how both age and gender can be considered important non-modifiable risk factors. This finding is probably due to protective effects of estrogen hormones, although the mechanisms have not yet been clarified.

Among the hereditary forms (~5%) it is important to mention the two most common:

1) Familial adenomatous polyposis (FAP), associated with autosomal dominant mutations in the APC onco-suppressor gene. It presents with the finding of numerous adenomas in the rectum and colon (>100), usually during the second decade of life. Patients with this mutation have a 100% risk of going on to develop a CRC if not detected and treated (12).
2) Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome): a form caused by loss-of-function mutations of mismatch-repair genes (MMR), such as MLH1, MSH2, MSH6 (12).

1.3. PATHOGENESIS

Rectal cancer usually begins with the non-cancerous proliferation of mucosal epithelial cells. Most of them develop from benign lesions known as “polyps”, visible macroscopically as protrusions on the intestinal mucosa, that can grow gradually for 10–20 years before becoming cancerous; indeed, three main phases can be distinguished in the process of (colo)rectal carcinogenesis: an "initiation" phase in which only a few epidermal cells, after a single exposure to a genotoxic carcinogen, can undergo mutations in a single critical gene, a "promotion" phase in which there is proliferation of the former altered cells into a large number of

daughter cells containing the mutation (from normal epithelium to adenoma) and a "progression" phase, in which the adenoma evolves into carcinoma.
Only about 10% of all adenomas progress to invasive cancer, although the risk of cancer increases as the polyp grows larger. Invasive cancer arising from such polyps is known as adenocarcinoma and accounts for 96% of all CRCs (3).
Certain dietary and lifestyle choices can promote intestinal inflammation and modify the microenvironment to promote an immune response, both of which can facilitate polyp growth and conversion to cancer. Likewise, hereditary or spontaneous mutations in oncogenes and tumor-suppressor genes can provide certain mucosal cells with a selective advantage and encourage hyper-proliferation and ultimately carcinogenesis.
Multiple genetic events are required for (colo)rectal carcinogenesis and genomic instability is now recognized as an essential cellular feature that accompanies the acquisition of these mutations. In colon cancer (13), at least 3 distinct pathways of genomic instability have been described, all of them being characterized by an early event, i.e. the alteration of the WNT (APC-β-catenin) system, which causes a disturbance in cell cycle regulation (13):

- the chromosomal instability pathway (CIN), observed in 65%–70% of sporadic colorectal cancers and FAP (13). Here, the accelerated rate of gains (or losses) of whole/large portions of chromosomes results in karyotypic variability between cells, with accumulation of characteristic set of mutations in specific tumor-suppressor genes and oncogenes. Germline mutations (in FAP) or somatic mutations of the APC gene result in failure to degrade (*and accumulation*) of β-catenin; subsequently, other mutations (KRAS, p53, SMAD4, PIK3CA...) contribute to adenoma evolution and progression (13)
- the microsatellite instability pathway (MSI), is characterized by mutations or hypermethylation in MMR (*mismatch repair*) genes, assigned to repair errors in DNA replication. Mutations in these genes (inherited in Lynch syndrome or sporadic) cause accumulation of mutations at microsatellites (*highly repetitive sequences found in the coding sequences of tumor-suppressor genes*) in other genes, going on to promote cancerous transformation (13).
- finally, the CpG Island Methylator Phenotype (CIMP) pathway, characterized by presence of hypermethylation (*and thus silencing*) of the promoters of several genes, usually tumor-suppressors. Genes most frequently affected by

mutations through this pathway include B-RAF, a kinase of the Ras pathway, with severe consequences particularly on cell apoptosis (13).

1.4. CLINICAL FEATURES

Rectal cancer may be diagnosed when a patient presents with clinical signs and symptoms or as the result of a screening program (9).
Unfortunately, early (colo)rectal cancer produces no symptoms and/or many of the actually present symptoms are non-specific (change in bowel habits, general abdominal discomfort, weight loss with no apparent cause, constant tiredness); when more specific signs appear (*rectal bleeding, anemia, obstruction, or perforation*), most patients are already in the advanced stage where cancers are aggressive and metastatic. Diagnosis at advanced stages (*which unfortunately occurs in ~ 25% of all CRC at diagnosis* (3)) is one of the determinants of the disparity in survival and large number of CRC deaths worldwide. Thus, population-based screening programs have been widely implemented in some highly developed countries, with the aim of shifting CRC distribution to early stages and improving therapy outcomes (14).

Rectal neoplasms are predominantly vegetative and ulcerated, easily bleeding lesions (15); different clinical pictures can be distinguished, depending on the specific location of the rectal lesion, that can be classified as: supra-ampullary, ampullary, or infra-ampullary (14). The first are characterized by the same clinical features of left colon cancers (*change in bowel habits, presence of blood in the stool and intermittent abdominal pain*). Ampullary rectal cancers, on the other hand, are specifically characterized by two main signs and symptoms: tenesmus, the unpleasant sensation of incomplete rectal emptying, and rectorrhagia, the emission of bright red blood during/and after defecation or independently from it. Infra-ampullary lesions typically progress rapidly to stenosis and are followed by perianal and perineal pain, accentuated during defecation, evacuation of ribbon-like stools, mixed with blood and mucus and important tenesmus (15).

Figure 2 shows the possible macroscopic presentation of a rectal cancer, seen on the surgical specimen.

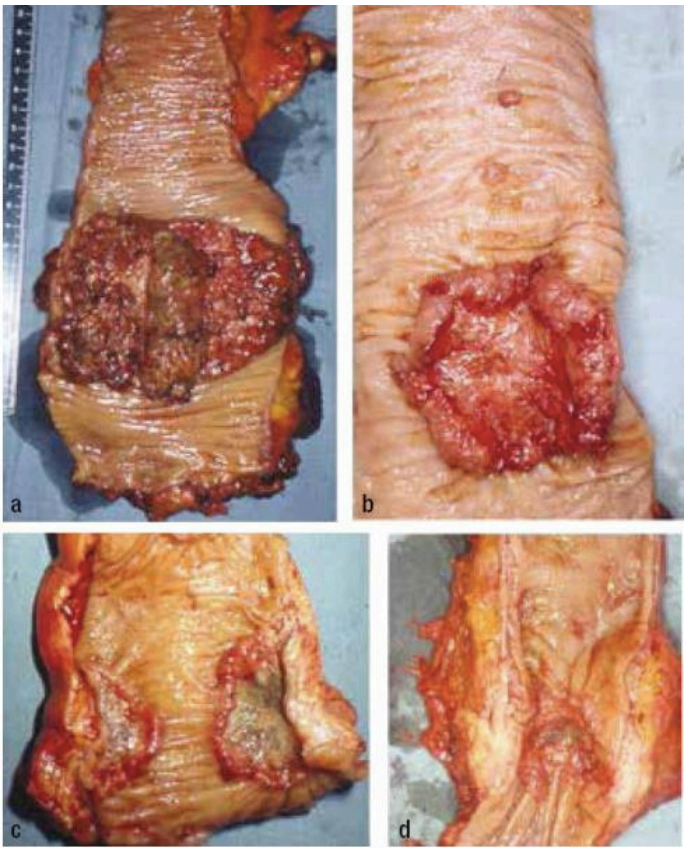


Figure 2. Macroscopic appearances of rectal cancer. a) Vegetating form. b) Ulcerative form. c) Infiltrating form. d) Stenosing-annular form. (From Renzo Dionigi, *Chirurgia generale (11)*).

1.5. DIAGNOSIS AND STAGING OF RECTAL CANCER

Diagnosis of rectal cancer is based on a digital rectal examination (DRE) and rigid sigmoidoscopy with biopsy for histopathological examination (16). Tumors with distal extension to ≤15 cm from the anal margin (as measured by rigid sigmoidoscopy) are classified as rectal, more proximal tumors as colonic.
Complete history and physical examination, complete blood count, liver and renal function tests, CEA measurements, chest X-ray (or alternatively chest-CT scan) and CT or magnetic resonance imaging (MRI) or ultrasound of liver and abdomen should be carried out.
Endoscopic rectal ultrasound (ERUS) for the earliest tumors (cT1-T2) or rectal MRI for all tumors, including the earliest ones, is required to select patients for

preoperative treatment and evaluate the extent of surgery (16). Preoperative complete colonoscopy is always required to exclude further manifestations. Nodal staging is very unreliable even using both ERUS and MRI. In addition to large size (which is not particularly accurate), roundness, irregular border, and hypoechoic nature/ heterogeneous signal on ERUS provide additional information.

Histopathological examination should include: surgical specimen with proximal, distal, and circumferential margins (the pathohistological circumferential resection margin (crm +/-) status is very important), and regional lymph nodes (*it is recommended to examine at least 12 nodes* (16)).

Figure 3 briefly depicts the diagnostic work-up in primary rectal cancer.

Parameter	Method of choice
Location (distance from anal verge)	Palpation Rigid sigmoidoscopy (flexible endoscopy)
Morphological verification	Biopsy
T stage	
Early	ERUS MRI
Intermediate/advanced	MRI (ERUS)
Sphincter infiltration	MRI (ERUS, palpation)
N stage	MRI (CT, ERUS)
M stage	CT, MRI (or US) of the liver/abdomen CT/chest X-ray of the thorax
Evaluation	MDT conference

Figure 3. Diagnostic work-up in primary rectal cancer. (From ESMO guidelines for Rectal Cancer *(16)*).

1.5.1 Staging

TNM staging system should be used for rectal cancer and it includes a clinical (*pre-operative*) classification (cTNM), and a pathologic (histopathologic *post-operative*) classification (pTNM). The two are distinct, the former being more focused on categorizing the disease for treatment choice, and the latter being more important for prognostic evaluation (16).

The prognosis and treatment of rectal cancer are strictly dependent on the stage of the disease, according to the American Joint Committee on Cancer (AJCC/UICC) TNM staging system (17); nevertheless, reliable prognostic criteria, which can

help predict tumor behavior and aggressiveness, thus guiding therapeutic choices, are still lacking.

Figures 4 and 5 report the actual TNM classification (*version 7* (16)) with sub-classifications and the actual group staging for rectal cancer, accordingly to ESMO (European Society for Medical Oncology) guidelines (16) and AJCC (American Joint Committee on Cancer) (18).

TNM	Extension to
Tis	Carcinoma *in situ*: intraepithelial or invasion of lamina propria
T1	Submucosa
T2	Muscularis propria
T3	Subserosa/perirectal tissue
	T3a[a] <1 mm
	T3b 1–5 mm
	T3c 5–15 mm
	T3d 15+ mm
T4	Perforation into visceral peritoneum (a) or invasion to other organs (b)[b]
N1	1–3 regional nodes involved
N1a	1 lymph node
N1b	2–3 lymph nodes
N1c	Small deposits in the fat
N2	4 or more regional nodes involved
N2a	4–6 lymph nodes
N2b	7 or more lymph nodes
M1	Distant metastases
M1a	One distant organ or set of lymph nodes
M1b	More than one organ or to the peritoneum

Figure 4. TNM classification (version 7) of rectal cancer. (From ESMO guidelines for Rectal Cancer *(16) (18)*)

I	T1-2, N0, M0
IIA	T3, N0, M0
IIB	T4a, N0, M0
IIC	T4b, N0, M0
IIIA	T1-2, N1/N1c, M0
	T1, N2a, M0
IIIB	T3-T4a, N1/N1c, M0
	T2-T3, N2a, M0
	T1-2, N2b, M0
IIIC	T4a, N2a, M0
	T3-4a, N2b, M0
	T4b, N1-2, M0
IVA	T1-4, N1-2, M1a
IVB	T1-4, N1-2, M1b

Figure 5. Group staging for rectal cancer. (From ESMO guidelines for Rectal Cancer *(16) (18)*).

1.6 TREATMENT PRINCIPLES OF RECTAL CANCER

Depending on the stage of the disease, different treatment options are available.

1.6.1 Management of local/locoregional disease

From a practical point of view, local/locally advanced rectal cancers (*i.e. non metastatic- M0*) can be divided into four groups (16):

a) very early (some cT1),

b) early (cT1-2, some cT3),

c) intermediate (cT3- some cT4a)

d) locally advanced (cT3crm +, some cT4a, all cT4b).

It is sometimes not possible to give a precise definition of which T and substages these tumors belong to. Furthermore, it's important to consider the "extramural vascular invasion (EMVI)", that can be identified on MRI. Presence of EMVI (EMVI+) is a poor prognostic signal for development of distant metastases, and possibly also local failure (16).

Total mesorectal excision (TME)

The standard of care today in rectal cancer surgery is TME, a surgical procedure in which the entire mesorectal compartment - including the rectum, surrounding mesorectal fat and perirectal lymph nodes - is completely removed along the mesorectal fascia (19); all mesorectal lymph nodes, should be excised (16).

TME is actually an umbrella term used to describe different surgical techniques all of which include a total resection of the mesorectum along the mesorectal fascia (19): this can be performed as either a low anterior resection (LAR) or an abdominoperineal resection (APR) (19).

- Low anterior resection (LAR): the anal canal is left in situ *(anal canal sparing)* and an anastomosis is made between the rectum and sigmoid colon. LAR is therefore typically applied in middle and upper rectal tumors where there is sufficient margin between the lower tumor border and the anal canal to make an anastomosis, most commonly using a "side-to-end" anastomosis (19);
- Abdominoperineal resection (APR, also known as or Miles Operation): the rectum and anal canal are resected *en bloc* and the patient receives a permanent colostomy. APR is indicated for low rectal tumors with a close

margin or involvement of the anal canal. Perineal repair techniques after APR include primary surgical closure, filling of the pelvis using omentoplasty, or plastic reconstructive techniques including vertical or oblique rectus abdominis myocutaneous flaps (19).

Possible variations of the standard APR are the "intersphincteric APR", where the external sphincter is spared and the "extra-levator APR", a more extensive procedure that includes the *levator ani muscles* and is indicated for tumors that invade the pelvic floor (19).

Local resection

In rare situations *(for very early and early rectal cancers)* minimally invasive techniques to excise rectal tumors endoscopically, through the anus, can be used (19).

- Endoscopic mucosal resection (EMR) and endoscopic submucosal resection (ESD) are superficial excision techniques used for non-cancerous polyps and T1a and T1b tumors (19).
- Transanal minimally invasive surgery (TAMIS) (or transanal endoscopic microsurgery TEM, *a very similar but older technique*) is a full thickness endoscopic resection of all layers of the bowel wall, that can be applied for T1 (and some small T2) tumors (19).

Figure 6 presents the different techniques used in rectal cancer surgery described above.

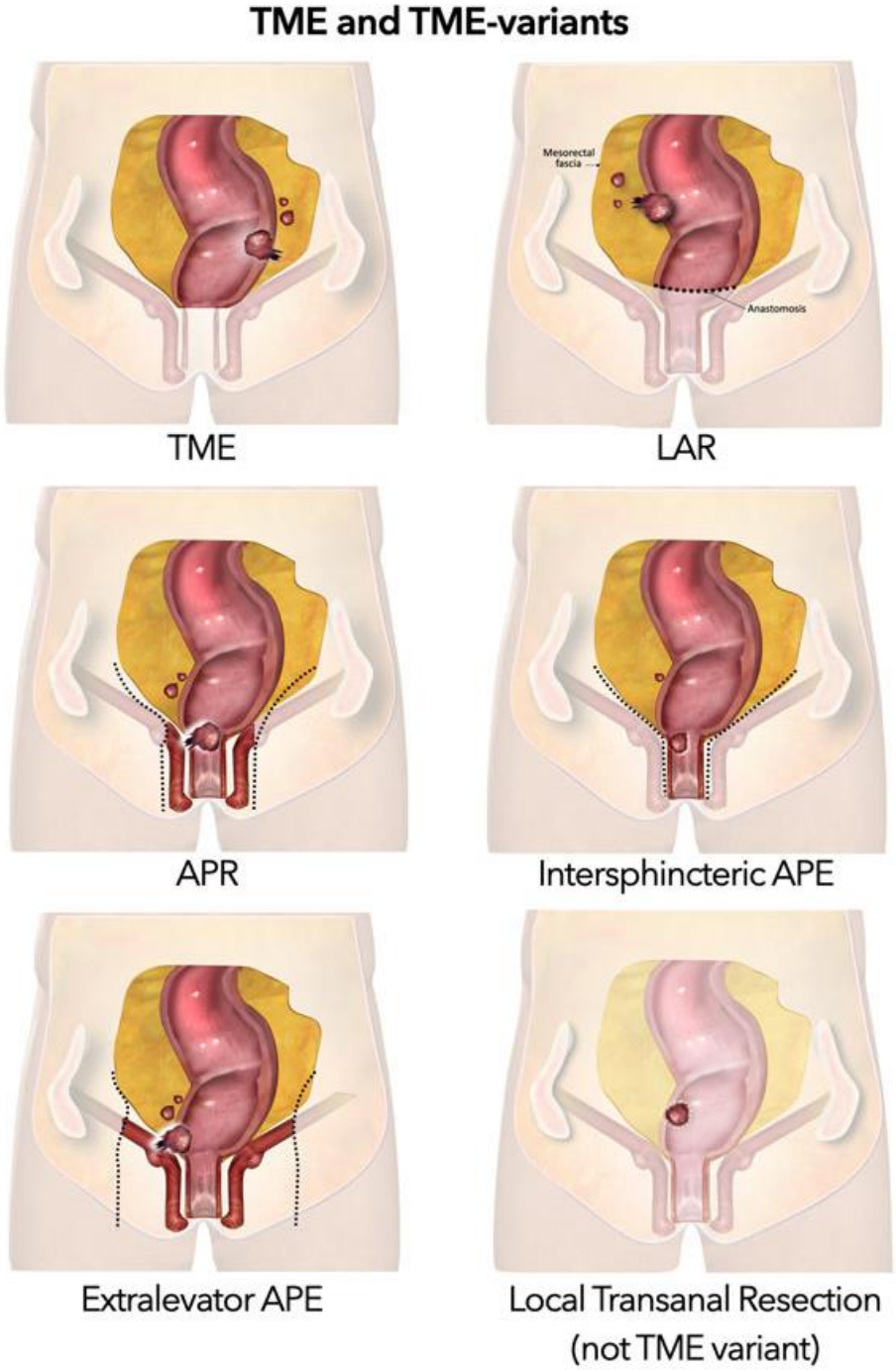


Figure 6. Rectal cancer surgery techniques. TME = Total Mesorectal Excision. LAR = Low Anterior Resection. APR/E = Abdominoperineal resection/Excision. (From *(19)*).

Stage-tailored treatment

Endoscopic resection is sufficient for hyperplastic or adenomatous polyps and intramucosal (pTis) adenocarcinomas of both colon and rectum (20) (21).

For very early rectal cancer (T1N0) with no adverse features, guidelines suggest a local excision of the tumor with trans-anal endoscopic microsurgery TAMIS (or TEM) (20). Local radiotherapy (RT) may be used as an alternative to local surgery (22).

More advanced rectal tumors up to and including cT2c/T3a/b should be treated by total mesorectal excision (TME). All mesorectal fat and including all LNs should be meticulously excised (23).

For patients with intermediate or locally advance rectal cancer (LARC), treatment decisions regarding neoadjuvant therapy with chemoradiotherapy (CRT) or short-course preoperative radiotherapy (SCPRT) should be based on preoperative, MRI-predicted circumferential resection margin (crm) (<1 mm), extramural vascular invasion (EMVI) and more advanced T3 substages (T3c/T3d), which define the risk of both local recurrence and/or synchronous and subsequent metastatic disease (24).

Finally preoperative CRT followed by surgery or preoperative SCPRT plus FOLFOX and delay to surgery is recommended for advance tumor (T3 with any mesorectal fascia (MRF) involved, any T4a/b, lateral node+) (20). As a matter of fact, in Europe, the addition of preoperative (C)RT is considered superior (*higher efficacy and/or less morbidity*) to surgical lateral node dissection, although this has not been subject to a randomized trial (16).

Postoperative chemoradiotherapy is no longer recommended but could be used in N+ patients or with positive crm+, perforation in the tumor area, defects in the mesorectum, or in other cases with high risk of local recurrence if preoperative radiotherapy has not been given (25).

A specialized and dedicated multidisciplinary team (MDT) should attend regular meetings and discuss all patients (26). MDT play a crucial role to define the initial diagnostic workup and then the treatment focus for patients with metastatic rectal cancer (M+) (27).

The details of the specific choice of treatment according to the risk category for primary non-metastatic (M0) rectal cancer is shown in Figure 7 (16)

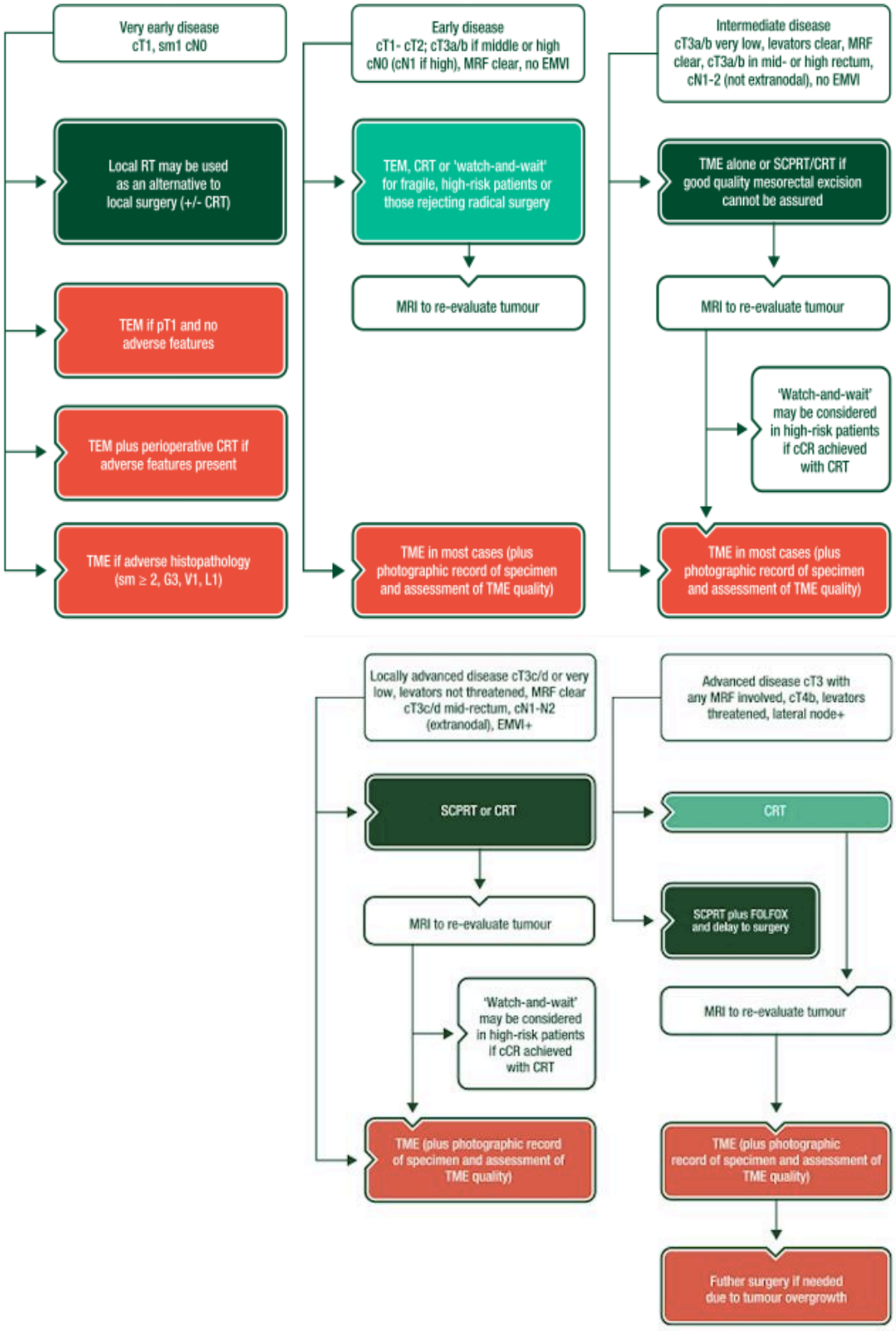


Figure 7. Choice of treatment according to risk category for primary rectal cancer without distant metastases. (From *ESMO Clinical practice guidelines for rectal cancer* (16)).

1.6.2 Management of metastatic disease

Generally, oligometastatic disease (OMD) may be characterized by the existence of metastases at up to 2-3 sites and visceral and/or five lymph nodal lesions. For patients with OMD systemic therapy should be considered as the initial part of every treatment strategy (27) . Exceptions are made for patients with single/few liver or lung resectable lesions for whom a complete ablation of all tumor masses, using surgical R0 resection and/or localized interventions (LAT) is recommended (27).

For non resectable M+ rectal cancer the definition of a (potential) treatment target and strategy is important for both the upfront integration of a multimodal treatment approach and for the choice of a systemic treatment strategy (first-line and later- line) as part of a 'continuum of care' (27). For these patients, knowledge of the RAS and BRAF mutational status of their disease is used to further refine treatment strategies (28). Targeted agents are indicated in the first-line treatment of most patients unless contraindicated. To date, there is no unequivocal evidence for the superiority of one class of biological over another (anti-VEGF versus anti-EGFR therapies). Each one of these antibodies should be used in combination with other agents including FOLFOX, CAPOX, FOLFIRI. (27) (26). Subsequent lines of therapy depend on the characteristics of the patient, the organ function, and the characteristics of the first-line therapy choice (27).

2. IMMUNE MICROENVIRONMENT IN COLORECTAL CANCER

Most solid tumors induce an immune response in the host, confirmed by histopathological studies: antigens derived from neoplastic processes have been reported as responsible for triggering immune responses. In this sense, tissue affected by (colo)rectal cancer is invaded by immune cells from the host, suggesting that the amount of lymphocytes may play a prognostic role with a potential impact upon patient's survival (29). According to literature, if colorectal neoplasia invades through the muscularis mucosa into the submucosa, local host reactions take place in cancer tissue and proinflammatory cells accumulate along the margins of the tumor and in the peritumoral healthy mucosa (the latter referring to the concept of "*field of cancerization*"), creating an immune microenvironment and triggering an immune response targeted towards the tumor (29).

2.1 Tumor Immune Microenvironment: Immune-Surveillance and Tumor-Infiltrating Lymphocytes (TILs)

In normal conditions, the immune system is an effective barrier against cancer. Antitumor activity of the immune system is initially mediated by innate immunity, mainly with effector cells such as Natural Killer (NK) cells, neutrophils, and macrophages. Subsequently, adaptive immunity mechanisms are activated. Cancer development can be explained, at least in part, by the success of the immunosuppressive escape mechanisms displayed by the tumor against the host's immune response: in this sense, cancer cells may escape the innate and immune host responses mainly by two mechanisms: selection of non-immunogenic tumor cell variants (*immunoselection*) or by active suppression of the immune response (*immunosuppression*) (30).

Tumor-infiltrating lymphocytes (TILs) are located in the inflammatory infiltrates in tumor islets and in the peritumoral stroma of solid tumors, as abovementioned (29). TILs include cytotoxic T-lymphocytes (CD8), NK cells, and helper T-lymphocytes (CD4). Among the latter, there is a subpopulation of cells known as regulatory T-cells (Tregs), main actors in suppressing and controlling the immune

response. The relationship between CD8+/NK and CD4+/Treg cells in the tumor-peritumor microenvironment offers an explanation to the final effect of a triggered immune response with an effective response or an immunosuppressive effect, based on the amount of CD8+/NK cells, as strongly supported by up-to-date literature (29) and discussed in detail further ahead in the chapter.

2.2 The Immune synapses: role of CD80, CD86 and CTLA-4

One of the key control points in the immune response relies in the HLA-antigen complex recognition by T-cell receptors (TCR). This interaction is very complex and involves a series of ligands present on APC (*antigen presenting cells*), which trigger immune responses, such as CD80 and CD86; when these interact with CD28 on T-cells (*immune synapses*), T- cells are activated. However, interactions with antigen 4 associated to cytotoxic T lymphocytes (CTLA-4) on T-cells lead to a status of anergy or immune tolerance (29). The blockade of CTLA-4 interaction with its ligands can result in an augmentation of antigen specific T-cell responses, and several studies (31) (32) have demonstrated that CTLA- 4 blockade can enhance immunity to tumors.

2.3 Prognostic value of Tumor-Infiltrating Lymphocytes (TILs) and their subtypes in colorectal cancer

A study published in 1987 by Jass et al. (33) reported the possibility that lymphocytes infiltrate of the invasive margins of rectal cancer could be an independent prognostic factor for survival, advocating for a new prognostic tool to calculate the risk of this disease.

In most colorectal tumors, tumoral tissue is infiltrated by a scarce number of lymphocytes and only along the margins of the tumor the highest density of lymphocytes and other inflammatory cells is observed. Proinflammatory cells such as neutrophils and macrophages usually appear with lymphocytes, which are usually CD4+ or CD8+ T-cells (29). The specific TILs composition has a crucial role in clinical evolution of colorectal cancer. Many research groups have focused their effort on analyzing the eventual relation between T effector cells and regulatory T-cells infiltrates and clinical outcomes. Intraepithelial lymphocytes are mainly CD8+ and their number is consistently correlated with higher disease-

free survival rates, as proven in several studies (34) (35). On the contrary, studies that analyze T-regs infiltration report conflicting results (29).

- **Regulatory T-cells (T-reg)**

  T-reg population represents roughly the 10% of CD4 T-cells and specifically expresses the forkhead-box-P3 transcription factor (FoxP3) which confers them suppressive properties upon effector T-cells (29). Increased numbers of FOXP3-infiltrating tumor cell nests have been demonstrated in several neoplasms, and this event is generally associated with unfavorable clinical outcomes: in fact, in the case of malignant neoplasia their presence seems related more to immunosuppressive mechanisms preventing immune-mediated tumor destruction (29). For colorectal cancer, stromal FoxP3+ cell density was strongly associated with tumor regression grade (36): a low stromal FOXP3+ density was observed in 84% of patients that had a pathologic complete response (pCR) after rectal surgery for rectal cancer, compared to 41% of patients who did not. Low stromal FOXP3+ cell density was also associated with improved recurrence-free survival; furthermore T-reg cells in the tumor microenvironment may inhibit response to neoadjuvant CRT and may represent a therapeutic target in rectal cancer (36).

- **Cytotoxic T CD8+ cells**

  In relation to T-regs, results regarding CD8+ infiltration in colorectal cancer are more robust and concordant suggesting strong antitumoral effects and a positive effect on patient survival (29). Diederichsen et al. (37) showed throughout flow cytometry that a high CD8+/CD4+ ratio is an independent prognostic factor for a better survival, proponing the hypothesis that the presence of CD8+ T-cells in tumor and peritumoral tissue could trigger an immunosurveillance status in the organism, avoiding the development of advance and metastatic CRC.

  In 2006, Galon et al. published in Science (38) a very relevant study with clinical-pathological transcendence. Genomic analyses were conducted on 75 cases of colorectal carcinoma in stages I to III, observing that tumors with lower rates of recurrence had higher density of immune cells (TCD3, TCD8, memory- TCD45RO, and granzyme B) in the analyzed regions in comparison to recurrent tumors. This study shows that adaptive immunity,

expressed by Th1, is inversely proportional to tumor recurrence; thus, patients with increased Th1 gene expression present a better prognosis (38).

Preliminary results of the IMMUNOREACT 1 and 2 studies form our research group (39) (40) suggested the role of immunosurveillance in rectal cancer outcome. In early rectal cancer, CD8+/CD4+ ratio in healthy mucosa of patients with lymphovascular invasion was significantly lower than in patients without, hence the CD8+/CD4+ ratio resulted to be a significant predictor of lymphovascular invasion (39). Furthermore, in healthy mucosa of patients who had previously received neoadjuvant therapy, CD8+ rate tended to be higher in patients who had complete response respect to those without and it was a significant predictor of complete response (40).

- **Antigen Presenting Cells (APCs).**

  Along with TILs, antigen presenting cells (APCs) are other components of adaptive immune system worthy of consideration, and among them dendritic cells (DC) are retained as the most potent antigen presenting cells. In colorectal cancer, dendritic cells are found along the invasive margins of the tumor once they have developed completely in lymphoid follicles. The prognostic value of these cells is very important (29): DCs concourse seems essential to trigger antitumor responses, with the ability to generate effector and memory T-cells (29).

3. OBESITY

3.1 DEFINITION

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (41). Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2).

For adults, WHO defines overweight, and obesity as follows (41):

- overweight is a BMI ≥ to 25.
- obesity is a BMI ≥ to 30.

BMI provides the most useful population-level measure of overweight and obesity as it is the same for both sexes and for all ages of adults. However, it should be considered a rough guide because it may not correspond to the same degree of fatness in different individuals. Figure 8 shows WHO BMI classification (41). BMI classifications are based upon risk of cardiovascular disease; figure 8 presents the classification adopted by the NIH and WHO for White, Hispanic, and Black individuals (41). Because the reported cutoffs underestimate risk in the Asian population, WHO and NIH guidelines for Asian individuals define overweight as a BMI ≥ 23 and obesity as a BMI ≥ 25 kg/m^2 (42).

BMI < 18.5:	Below normal weight
BMI >= 18.5 and < 25:	Normal weight
BMI >= 25 and < 30:	Overweight
BMI >= 30 and < 35:	Class I Obesity
BMI >= 35 and < 40:	Class II Obesity
BMI >= 40:	Class III Obesity

Figure 8. BMI classification. (From WHO reports, *June 2021 (41)*)

3.2 EPIDEMIOLOGY

According to recent WHO global estimations (41), in 2016 more than 1.9 billion adults aged 18/+ were overweight; of these, over 650 million adults were obese. Roughly ~ 39% of adults aged 18 years and over were overweight (39% of men

and 40% of women), while about 13% of the world's adult population (11% of men and 15% of women) were obese.
The worldwide prevalence of obesity nearly tripled between 1975 and 2016 (41); In 2019, an estimated 38.2 million children under the age of 5 years were overweight or obese. Once considered a high-income country problem, overweight and obesity are now on the rise in low- and middle-income countries, particularly in urban settings (41). Overweight and obesity are linked to more deaths worldwide than underweight. Globally there are more people who are obese than underweight – this occurring in every region except parts of sub-Saharan Africa and some parts of Asia.

3.3 HEALTH CONSEQUENCES OF OBESITY

Overweight or obesity increase the risk of a range of diseases (*Figure 9 presents some of the most frequent*), many of which are found to occur more frequently with increasing BMI (43). Excess of adiposity is also linked to increased mortality, with those living with obesity found to have a life expectancy five years shorter than those with a "healthy" weight status (BMI 18.5–24.9 kg/m2) (44). Recent estimates suggest that overweight and obesity cause more than 1.2 million deaths across the WHO European Region every year (43); this represents more than 13% of total deaths, ranking fourth behind high blood pressure, dietary risks and tobacco use. Overweight and obesity are also the leading risk factor for disability, accounting for 7% of total YLDs ("*years of life lost due to disability*") in European region (43).

These health issues arise in people living with obesity in part because adipose tissue is a metabolically active endocrine organ, with fat cells releasing and receiving hormones: adipocytes release substances called *adipocytokines*; these are associated with a range of systemic or local actions including: glucose and lipid metabolism, cell development, inflammation, and oxidative stress, which can lead to several health problems. Recent evidence from 2022 (43), argues that the negative health effects of obesity stem not simply from an excess of fat, but from the decline in its ability to respond to changes - *its plasticity*: as fat declines in plasticity due to aging and obesity, it loses its ability to respond to bodily cues,

leading to metabolic issues, such as insulin resistance, inflammation, and cell death.

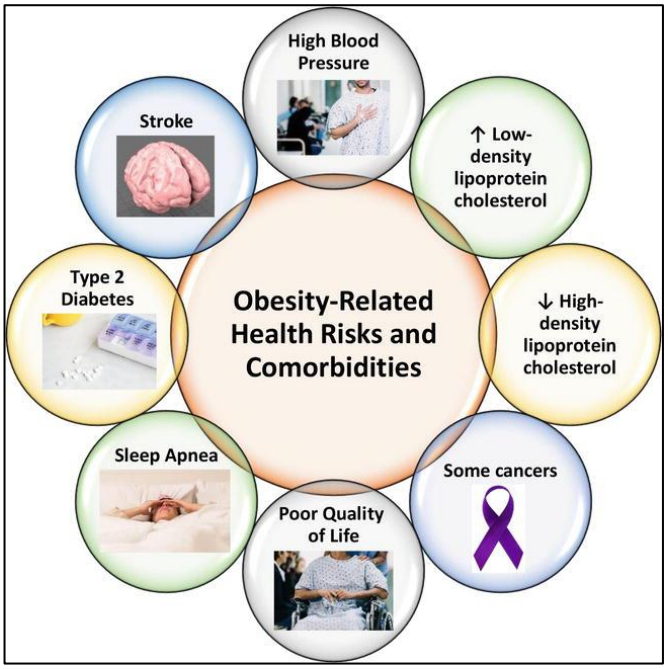


Figure 9. Some of health risk and comorbidities mostly related to obesity. (From *(99)*)

- Obesity is linked with clusters of diseases that greatly increase CVD risk (43): these include atherosclerosis, hypertension, dyslipidemia, insulin resistance and overt diabetes, coagulability, endothelial dysfunction and inflammation. Consequently, those living with obesity are at elevated risk of a range of CVDs (45): in particular, obesity increases the risk for stroke and coronary heart disease (45), the two most common forms of CVD mortality.

- Similarly, excess body weight increases the risk of many different neoplastic diseases (43), including not only colorectal cancer, but also endometrial, breast (*particularly postmenopausal*), gallbladder, pancreatic, liver, kidney, gastric and esophageal, thyroid, ovarian and blood cancers. At the same time, WHO reports how obesity is linked with the development of more severe forms of cancer, such as metastatic, as well as an increased risk of dying from it (43) (45).

- In addition to these two prominent chronic diseases, obesity may lead to a greater risk of many others, including metabolic diseases, such NAFLD,

T2DM, musculoskeletal, respiratory, and reproductive issues, and various psychological and mental health problems. Recent concern has focused on the link between obesity and COVID-19, with people living with overweight and obesity found to be at increased risk of severe COVID-19 outcomes (43).

4. OBESITY AND (COLO)RECTAL CANCER

4.1 GENERALITIES

The association between BMI and colorectal cancer incidence has been evaluated in several epidemiological studies, and it has been shown that the risk and incidence of colorectal cancer increase with BMI (46). A meta-analysis of 30 prospective studies found an increased risk of colorectal cancer in obese men and women, though the association was higher in men (46).

While the relative risk of colorectal cancer with obesity is moderate, due to the high prevalence of obesity it is estimated that ~35.4% of colorectal cancer cases in men and ~20.8% in women in the U.S. are attributable to obesity (47).

The relationship between body mass and colorectal cancer development has also been studied in animal models, where both APC^{min} mice (*a model for spontaneous colorectal cancer*) and mice treated with azoxymethane (*carcinogen used to induce colorectal cancer*) fed a high-fat diet had increased colon polyp formation (48).

4.2 OBESITY AND (COLO)RECTAL CARCINOGENESIS: TUMOR MICROENVIRONMENT AND INFLAMMATION

The importance of the tumor microenvironment in the development, growth, and progression of cancer is now well recognized; research on the tumoral microenvironment is leading to the identification of new therapeutic targets. The tumor microenvironment consists of a complex network of different cell types, including infiltrating immune cells, such as lymphocytes (T and B), antigen-presenting cells (*i.e.* macrophages and dendritic cells), granulocytes, cancer-associated fibroblasts, endothelial cells, but also extracellular matrix (ECM), and other stromal components (49); their dynamic interaction - within tumoral but also surrounding peritumoral healthy tissue - activates thousands of immune-signaling cascades, potentially favoring or controlling tumor progression (49).

A state of superimposed chronic inflammation influences negatively on the ongoing immune perturbation within the (peri)tumoral microenvironment, and has been associated with the development and progression of several types of cancers, including colorectal (49).
Obesity is now a well-known cause of chronic subclinical inflammation; in this chapter we will focus on discussing the mechanisms through which adipose tissue inflammation fosters a tumor-supportive microenvironment through local (4.2.1, 4.2.2, 4.2.3) and systemic (4.2.4) effects, with reference to colorectal cancer.

4.2.1 White adipose tissue (WAT) inflammation

Adipose tissue inflammation may be the key process by which obesity promotes cancer. Locally, white adipose tissue (WAT) in obese patients is infiltrated by immune cells, including macrophages and lymphocytes. In this manner, the obese fat pad resembles chronically injured tissue and can be a rich source of proinflammatory mediators, potentially fostering tumor growth (49).
Several are the underlying mechanisms that promote inflammation within adipose tissue in overweight/obese patients and their understanding is based on the physiological function of adipocytes, endocrine cells that secrete a large range of cytokines, hormones, and growth factors (collectively referred to as *adipokines*) and specialize in the storage of energy as triglycerides in cytoplasmic lipid droplets.

- Chronic excess nutrients leads *per se* to the upregulation of metabolic signaling pathways such as c-Jun N-terminal kinase (JNK), nuclear factor κ B (NFκB), and protein kinase R (50), which results in chronic production of low levels of inflammatory cytokines triggering and maintaining a low-grade inflammatory response (34). NFκB is a well-known transcription factor that has been linked to both inflammation and the development and progression of tumors (33).
- Excess nutrients and obesity also produce hyperplasia and hypertrophy of WAT adipocytes, followed by an increased production of adipokines. Over time, as adipose tissue outgrows its blood supply leading to hypoxia, further adipocyte stress and death may occur, with change in the adipokine pattern, additional upregulation of the abovementioned inflammatory pathways and free fatty acids (FFA) liberation (33). This is paralleled by the increased

production of MCP-1/CCL2 (Monocyte Chemoattractant Protein-1), a chemokine involved the recruitment and proliferation of macrophages within adipose tissue. The recruited and activated macrophages tend to form an envelope around dead or dying adipocytes in a configuration termed crown-like structures (CLS), a histologic biomarker of inflammation (33).
The macrophages that form CLS engage in phagocytosis of a dead or dying adipocyte and become lipid loaded, forming foam cells. Free fatty acids (FFAs) released from the foam cells and from the necrotic adipocytes activate macrophage plasma membrane TLR4, increasing NFκB-dependent expression of pro-inflammatory genes, *including TNF-α, IL-1β, and COX-2*, in a vicious cycle that sustains WAT inflammation and promotes carcinogenesis (33).

Figure 10 shows the complex adipocyte-macrophage cross-talk in obesity (51)

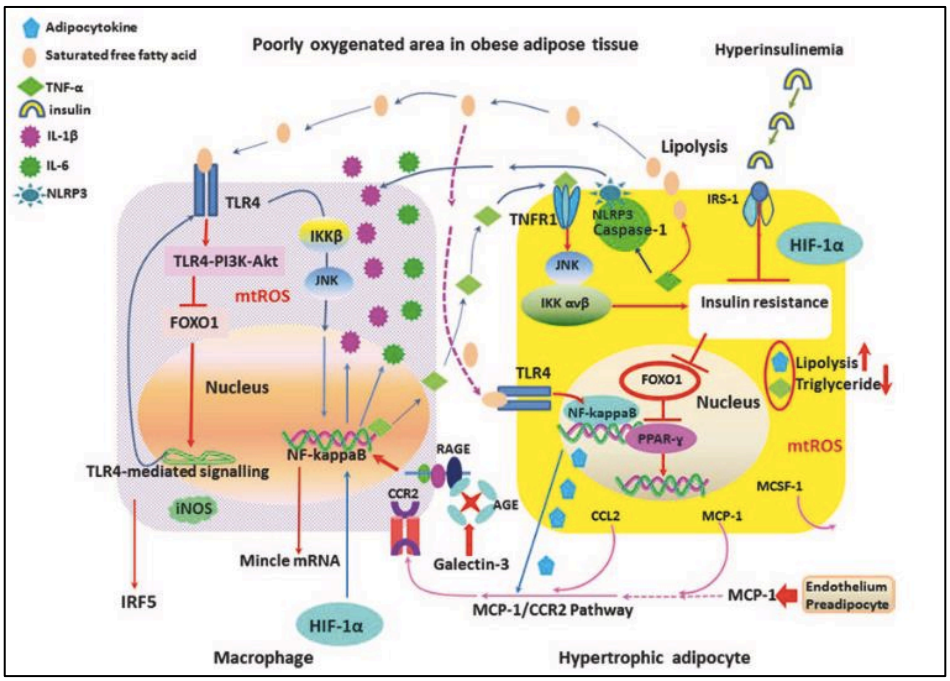


Figure 10. Macrophage-elicited metabolic inflammation and adipocyte-macrophage interaction has a primary importance in obesity. Large amounts of macrophages are accumulated by different mechanisms in obese adipose tissue. (From (35))

- The impact of obesity on immunity is not limited to macrophages; within WAT we see increasing Th1 cell and CD8 cytotoxic T-cell responses, augmentation of natural killer (NK) cells and decrease of regulatory T-reg (34): CD8 T cells have been shown to be the early event showing up in

adipose tissue of diet-induced obesity, which plays an important role in further recruiting M1 macrophages thus sustaining inflammation (50). It is important to notice how, paradoxically, in obese tumor tissues, CD8 T-cells and M1-polarized macrophages are reduced in number: one potential explanation could be that changes in the microenvironment during tumor development change adipose tissue macrophages from a more M1-like phenotype, to a more tumor-promoting M2-like stage under obesity (50). It is likely that there are significant differences between the microenvironments from obese adipose tissues and obese tumor tissues (50).

4.2.2 Discussed role of adipokine imbalance

Various studies, up to now carried-out on colorectal cancer (and *not specifically* on rectal cancer), have demonstrated an imbalance of obesity-related adipokines, *i.e.* the change in leptin and adiponectin ratio. According to the up-to-date literature, obese white adipose tissue tends to have an increase in the production of leptin, which is pro-inflammatory, pro-angiogenic, and pro-proliferative, and a decrease in adiponectin, which is anti-inflammatory, anti-angiogenic, and anti-proliferative (34).

A few epidemiological studies have examined the relationship between levels of circulating leptin and colorectal cancer risk, finding that high levels of leptin are associated with an increased risk of colon cancer (*but not rectal cancer*) in men with no association in women (52). Leptin has also been shown to promote proliferation, survival and invasiveness of colon cancer cells through activation of MAPKs, PI3K, NF-κB and STAT3 signaling (53), also stimulating the production of inflammatory carcinogenic cytokines IL-6 and TNF-α. Adiponectin can inhibit colorectal cancer cell growth through activation of AMPK, and loss of adiponectin enhances development of both colitis-associated colorectal cancer and in APC^{min} mice (54). However, the extent of the impact of adiponectin in sporadic-CRC development is still controversial: some epidemiological studies have found that circulating levels of adiponectin is inversely correlated with colorectal cancer risk, while others declared no significant correlation (50).

4.2.3 Gut dysbiosis and endotoxemia

A growing amount of evidence has linked CRC to intestinal microbiota and dysfunction of the intestinal barrier. Studies have also shown that altered gut microbiota is present in patients with adenomas of the colon, suggesting a role for dysbiosis in the early stages of colorectal cancer development (55). The development of colorectal cancer in animal models has been shown to be linked to leakage of the intestinal barrier and activation of pro-inflammatory tumor promoting IL-23/IL-17 signaling (56). A previous study also supported a role for intestinal barrier dysfunction and subsequent endotoxemia in the development and progression of colorectal cancer in APC^{min} mice (50). Obesity is also associated with changes in the gut microbiome and dysfunction of intestinal barrier (50). Pfalzer A.C. et al reported that genetically obese or diet-induced obese (HFD-mice) APC^{1638N} colon tumor-bearing mice had altered gut microbiome compared to non-obese mice (57). HFD-fed mice have also been demonstrated to have increased levels of circulating lipopolysaccharide (LPS), a marker of intestinal barrier dysfunction and endotoxemia, which has been shown to promote colorectal cancer development (57).

These studies indicate that inflammation due to obesity-mediated gut dysbiosis and intestinal barrier dysfunction, along with changes in the balance between leptin and adiponectin, plays a role in the increased risk of colorectal cancer in obese individuals.

Figure 11 sums up the mechanisms previously described as involved in the pathogenesis of (colo)rectal cancer in obese patients. Such mechanisms involve the interplay between different signaling events; however, the underlying theme is the obesity-associated inflammation, which is known to promote progression of several types of cancers.

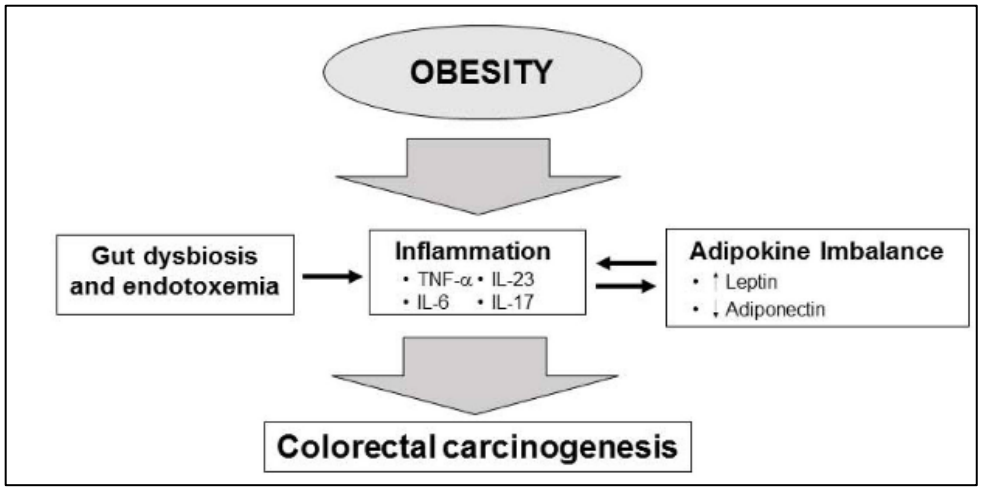


Figure 11. Obesity and colorectal carcinogenesis. Obesity is associated with changes in the intestinal microbiome and dysfunction of the intestinal barrier. Gut dysbiosis and endotoxemia promotes inflammation through the upregulation inflammatory cytokines, particularly IL-6, TNF-α, IL-17 and IL-23, which promote CRC carcinogenesis. Obesity-associated changes in leptin and adiponectin also leads to increased inflammation and CRC carcinogenesis. (From *(50)*)

4.2.4 Insulin resistance and IGF-1

Obesity is associated with an increase in insulin release and a decrease in insulin sensitivity, mediated by the decreased expression of insulin-receptor levels and reduced intracellular insulin signaling in response to insulin receptor binding (58). This results in hyperinsulinemia and insulin resistance, with an associated increase in IGF levels: IGF is involved in the control of normal growth, maintenance of tissue homeostasis and a differentiated phenotype, alterations in the balance of proliferation and apoptosis, angiogenesis, cell adhesion, migration, and wound healing.

There is strong evidence from animal and human studies that cancer development is promoted by high concentrations of insulin and IGFs acting through the insulin/IGF axis. Stimulation of the IGF-1 receptor (IGF-1R), a tyrosine kinase, activates two main signaling pathways, PI3K-AKT and RAS-Raf-MAPK, which have multiple effects on gene regulation and protein expression, activation and translocation (58) Another important pathway, the c-Jun N-terminal kinase (JNK), appears to play a crucial role in obesity and insulin resistance and in colorectal carcinogenesis (58).

Experimental data have shown that a HFD might increase insulin levels, inactivate AKT and increase JNK activity (59). Interestingly, in this study, a HFD was associated with an increase in the number of aberrant crypt foci and the proliferation of colon epithelial cells. Both these effects were prevented by the use of a JNK inhibitor (59).

Figure 12 summarizes the whole set of potential factors that are believed to relate obesity and colorectal cancer. Blue arrows indicate the metabolic consequences of obesity; black arrows are for some of the suspected consequences of a dysbiotic microbiota; purple arrows are for the cellular events induced by obesity-related metabolic changes; red arrows locate these cellular events in the carcinogenic process; green arrows suggest the stage of the normal epithelium-to-carcinoma sequence when the different biological factors might start to act; orange lines suggest some of the potentially beneficial effects of bariatric surgery (59).

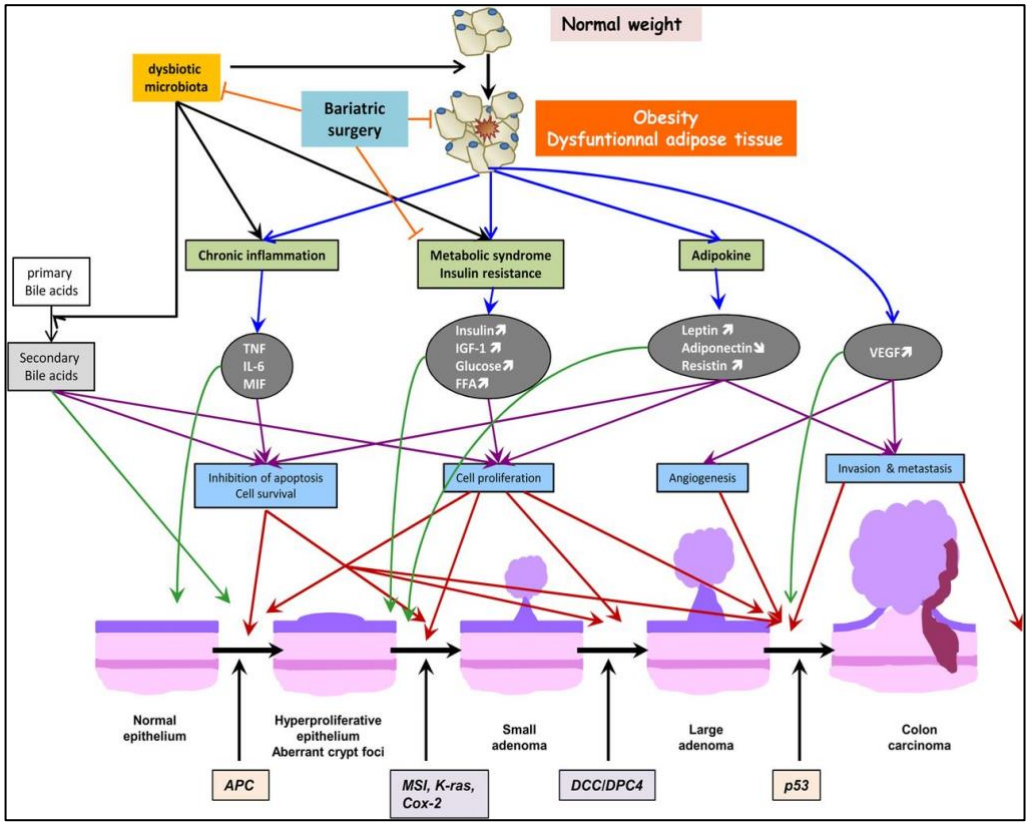


Figure 12. Summary of potential factors that are believed to relate obesity and colorectal cancer. (From *(59)*).

AIM OF THE STUDY

Hypothesis

The main hypothesis underlying this research is that the aggressiveness of rectal cancer is determined by the complex interactions between the malignant cells and their immune microenvironment. The second hypothesis is that relevant trace of this cross talk between tumor cells and immune microenvironment can be detected in the normal "healthy" mucosa surrounding the cancer, according to the concept of field of cancerization, and sampling of this mucosa may provide useful information about rectal cancer behavior. The third hypothesis, which is the effective part of the bigger and still *in fieri* "IMMUNOREACT 10 project" ("*IMMUNOlogical Microenvironment in REctal Adenocarcinoma Treatment*" ClinicalTrials.gov Identifier: NCT04915326) (60) is that the immune microenvironment may be influenced by body weight and thus BMI was chosen in this study as indicator of body fatness. The signaling governed by NF-κB and triggered by pro-inflammatory cytokines and adipokines has emerged as pivotal in orchestrating host defense against cancer and this pathway displays different activation between normoweight and overweight/obese rectal cancer patients.

Specific aim

The aim of this study was to analyze the healthy rectal mucosa surrounding the cancer on rectal cancer operated patients ("Azienda Ospedale Università of Padova" cohort), by using state of the art flow cytometry (FACS analysis) and immunohistochemistry (IHC) markers, and bio-statistical analysis to stratify results according to the BMI, in order to:

- evaluate the **impairment in immune surveillance** of healthy rectal mucosa of overweight/obese patients with rectal cancer;
- find the **differences** between healthy rectal mucosa of overweight/obese patients with rectal cancer and of normal weight patients with rectal cancer.

Possible clinical-translational application

The results, might be useful for evaluating a future tailored screening and/or treatment for obese patients with rectal cancer diagnosis.

MATERIALS AND METHODS

1. STUDY DESIGN

As a first research outline, clinical and pathological records of a series of consecutive rectal cancer patients operated on in three different surgical wards of the "Azienda Ospedale Università of Padova": 1- ex General Surgery Unit, 2- ex Surgery Clinic 1st Unit, 3- actual General Surgery 3 Unit were retrieved for this observational study.
This study is an offshoot of the IMMUNOREACT 1 and 2 trials (ClinicalTrials.gov Identifier: NCT04915326) (60): here all patients enrolled in the two pivotal trials are included, and eligibility criteria are expanded *(see below)*. The IMMUNOREACT project started in 2019 and is currently ongoing in 9 Italian centers (secondary or tertiary center in North-Central Eastern Italy).
The present observational study involves two groups/cohorts of patients: the retrospective and the prospective cohort; a total of 213 patients with rectal cancer, whose data of body mass index were available, were analyzed. The retrospective cohort includes 103 patients with surgically treated non-metastatic rectal cancer (data collected from January 2011 to March 2018), while the prospective cohort comprises of 110 non-metastatic rectal cancer operated patients (data collected from April 2016 up to August 2022).

In this study, tissue samples were obtained from cancer tissue (*when possible*) and from normal rectal mucosa adjacent to the cancer at surgery. Complete medical record and follow-up were collected for each patient. The analysis were centralized at "Azienda Ospedale Università di Padova".

The retrospective cohort study was conducted on formalin-fixed paraffin-embedded (FFPE) slides retrieved from the pathology archives, testing a panel of molecular markers exploring the immune reaction to cancer (i.e. antigen-presenting cells and T lymphocytes activation). Such panel of immune markers was retrospectively investigated at immunohistochemistry: CD3, CD4, CD8, CD8beta, Tbet, FoxP3, PD-L1, MSH6, and PMS2, and CD80.

The prospective cohort analyses were conducted on fresh tissue samples obtained from normal rectal mucosa proximal (3-15 cm) to cancer or to the site of previous cancer (*in case of complete response to CRT*) at the time of surgery: fluorescence-activated cell sorting (FACS) was used to determine the proportion of epithelial cells expressing CD80, CD86, CD40, HLA ABC, or HLA DR and the proportion of activated CD8+ T cells, CD4+ Th1 cell, and T reg.
Immune-markers of healthy rectal mucosa were compared between normal-weighted patients and overweight/obese (i.e. BMI $<$ 25 and $\geq$ 25 respectively), between infiltrated margins or not, and between complete response or not.

1.1. Tissue sampling

Mucosal samples were obtained from the healthy rectal mucosa proximal (3-15 cm) to the rectal cancer or to the site of the previous rectal cancer at the time of the surgery. A pilot study was formerly carried on (*as part of the first steps of IMMUNOREACT 1 and 2 projects* (39) (40)) to demonstrate the homogeneity of the expression of the immunological markers within the range of 3-15 cm from the rectal cancer and was available for consultation.

1.2. Histopathology

Sections (3 µm) from formalin-fixed and paraffin-embedded (FFPE) specimens were stained with hematoxylin-eosin. Pathological staging, though not of crucial importance for the present study, was performed using AJCC classification (8th edition) (61).

1.3. Immunohistochemistry (IHC)

Using a tissue microarray instrument (Beecher Instruments, Alphelys, Plaisir, France), two different and representative areas of the tumor and healthy mucosa were selected. The center of the tumor (CT) and the invasive margin (IM) were punched (0.6 mm and 1 mm-diameter, respectively) from paraffin-embedded tissue-blocks. Tissue microarrays were constructed and cut into 5-µm sections for immunohistochemical staining (IHC).
IHC analyses were performed using a monoclonal anti-CD80, anti-PD1, anti-CD8beta, anti- PDL1, anti-Tbet, anti-FoxP3, anti-MSH2, anti-MLH1, anti-MSH6 and anti-PMS2 antibodies. Immunocomplexes were detected using an avidin-

biotin-peroxidase conjugate and 3-3' di- aminobenzidinetetrahydrochloride chromogen as a substrate (ABC Kit, Vector Laboratories, Burlingame, CA, USA; and DAB kit Dako, Glostrup, Denmark) and the resulting sections wereevaluated by a single pathologist in a blinded fashion.

1.4. Flow cytometry (FACS)

Rectal biopsies from healthy and cancer tissue, freed of mucus by a 30-min were washed in HBSS containing 10 mM DTT and digested to obtain single-cell suspensions. Freshly isolated cells (10^5) were then stained in PBS/2% FBS with appropriate combinations of FITC- and PE-conjugated antibodies. Single-cell suspensions were subjected to flow cytometry to determine the proportion of epithelial cells (Cytokeratin 20, Cyt-20+) acting as antigen-presenting cells (expressing CD80, CD86, CD40, HLA ABC or HLA DR) and the proportion of activated CD8+ T cells (positive for CD28, CTLA-4, CD38 or CD69), of activated CD4+ Th1 cell (T-bet, TNF-beta, IFNgamma, IL17) and of activated T reg (FoxP3, CD25, IL-35)

2. PATIENTS AND ELIGIBILITY CRITERIA

The IMMUNOREACT protocol was approved by the Ethical Committee of the coordinating center (CESC code 4448/AO/20) and of each of the collaborating centers. The two arms of the pivotal trial (i.e. IMMUNOREACT 1 (39) and IMMUNOREACT 2 (40)) are registered at ClinicalTrials.gov (NCT04915326 and NCT04917263, respectively) (60). All the consecutively enrolled patients gave their written informed consent to be enrolled in the study. The study was conducted according to Helsinki's declaration principles (62).

The enrolled patients had to respond to the following criteria:

- Ages eligible for study: 18 years and older (adult, older adult)
- Sexes eligible for study: all
- Acceptance of healthy volunteers: no
- Patients operated for NON-METASTATIC (M0) rectal cancer at the Azienda Ospedale Università of Padova (either standard *anterior*

resection or *Miles abdominal-perineal amputation* or *trans-anal micro dissection*)

- Full availability of clinical records and at least 1 year of follow up.

3. DESCRIPTION OF DATA COLLECTION FORM AND REDCap® DATABASE

The retrospective cohort (103 patients) and the prospective cohort (110 patients) data were collected using the platform REDCap® (Research Electronic Data Capture), a web application for building and managing online surveys and databases (63).

The form used comprises several fields and it was the same for the two cohorts, though analyzed separately. Each record, corresponding to a single patient, had its single identification code (*patient ID*) and all data remained anonymous, in full respect of privacy. The forms were filled using information gathered, under permission, from the AOPD "Galileo" management application software, by consulting medical history forms and discharge letters regarding the single patient at the time of the rectal cancer surgery.

The fields used in the form do not evaluate only the anthropometric parameters of the patients (height (m) and weight (Kg), *main interest in our study*), but also other variables, in order to further strengthen the validity of data (*reported in the below* Table 0)

▪ **Height (m)**	
▪ **Weight (Kg)**	
▪ **BMI (Kg/m^2)**	
▪ **BMI categorization**	☐ <= 18.5, Underweight ☐ Range between 18.5 and 25, Normal ☐ Range between 25 and 30, Overweight ☐ >= 30, Obese
▪ **Comorbidities**	☐ Absent ☐ Hypertension ☐ Ischemic heart disease ☐ Diabetes ☐ Stroke/TIA ☐ Hepatic steatosis ☐ Eating disorders

	☐ Endocrinopathies
▪ **Smoking habit**	Yes/no
▪ **Potus**	Yes/no
▪ **Corticosteroids (systemically, at least 1 month in the last year)** ▪ **Type of corticosteroid (*if used*)**	Yes/no ☐ Prednisone ☐ Prednisolone ☐ Beclomethasone ☐ Budesonide
▪ **Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))**	
▪ **Neutrophile/lymphocyte ratio (NLR)**	
▪ **Creatinine (*μ*mol/L)**	
▪ **eGFR**	
▪ **eGFR categorization**	☐ >= 90 Normal renal function ☐ Range between 60 and 90, Mild renal failure ☐ Range between 30 and 60, Moderate renal failure ☐ <= 30 Severe renal failure

Table 0: sample of the online REDCap® Database form form filled for each patient of both the retrospective and prospective cohort.

- **BMI categorization (Kg/m^2)** is defined accordingly to the WHO definition (64), *as previously discussed in Chapter 3.* It needs to be specified that BMI was calculated with height and weight of the patient at the time of the surgical intervention; however, it is proven that UWL (*unintentional weight loss*, *defined as loss of >5% of body weight within the last 6-12 months*) is a common symptom of rectal malignant disease (65) and typically indicates an advanced stage disease (66). Indeed, many patients reported a +/- relevant weight loss in the last 3-6-12 months, and BMI results as such may be biased: previously obese/overweight patients, at time of cancer development, in many cases resulted to have a normal BMI in consequence of neoplastic cachexia or important weight loss. This must be stressed as possible limit of the present study.

- **Comorbidities** (in particular, the most frequently reported were chronic diseases, such as: arterial hypertension, diabetes and, among endocrinopathies, hypercholesterolemia and thyroid disorders) were further investigated to corroborate the results and their association with overweight and obesity. It led to analyze the patient's present therapy, that could bias the results concerning the immunological microenvironment *(e.g., immunosuppressors).*
- **Smoking habit** was defined accordingly to the "Behavioral Risk Factor Surveillance System (BRFSS)" definitions (67):
    - Non-smoker is a person who states that he/she has smoked less than 100 cigarettes (5 packs of 20) in his/her life and is not currently a smoker (67). For these patients "**NO**" was filled in the form under the field "smoking habit"
    - Smoker (current) is a person who states that he or she has smoked at least 100 cigarettes (5 packs of 20) in his or her life and that he or she is a smoker at the time of the interview or has stopped smoking for less than six months. (67)
    - Ex-smoker is a person who states that he/she has smoked at least 100 cigarettes (5 packs of 20) in his/her life, that he/she was NOT a smoker at the time of the interview and that he/she quit smoking more than six months ago. (67)

  Current smokers and ex-smokers were signed as "**YES"** at the smoking habit field.

  It is known that cigarette smoking is another important risk factor for colon and rectal cancer. The incidence of colorectal cancer was proven to be significantly higher in current and former smokers compared with lifelong nonsmokers (68), and as such be an independent confounding variable.
- **Potus (alcohol consumption)** was defined accordingly to the "ISS Passi" indicators (69). Passi *(Progressi delle Aziende Sanitarie per la Salute in Italia)* is a surveillance program started in 2006 that estimates the average consumption of alcoholic accordingly to frequency of intake (expressed in days/month) and the number of UA taken on average, on the days of consumption. The set threshold levels based on the number of alcoholic units consumed on average per day are stratified accordingly to sex and age:

- moderate consumption threshold for adult men = 2-3 alcohol units (AU) on average per day. Older male adults (65+) have lower normal thresholds of 1-2 UA/die. (69)
- moderate consumption threshold for adult women = 1-2 alcoholic unit per day on average. Older women (65+) threshold is of 1 UA/die. (69)

Consumption levels above these thresholds are classified as high habitual consumption, and were ticked as "**YES"**, at the alcohol consumption field. As for cigarette smoking, also alcohol consumption is a well-established risk factor for colon and rectal cancer and some epidemiologic studies suggest that even moderate drinking increases the CRC risk and can also represent a confounding variable in our study (70).

- **Use (and type) of corticosteroids,** systemically at least 1 month in the last year. Few patients used such medications that are known to compromise the immune response in the patients, and as such influence the immunological environment analyzed in the present study, biasing the results. Systemic glucocorticoids are potent immunosuppressants, potentially facilitating colorectal carcinogenesis (71). Studies examining glucocorticoids and colorectal cancer risk are few.
- **Albumin, lymphocyte count and neutrophilic count** of patients were collected and used to determine
  - the Onodera prognostic nutritional index (OPNI), an indicator calculated from serum albumin and total lymphocyte counts in peripheral blood, which can be utilized to evaluate patients' preoperative nutritional and immune status (72). Its potential prognostic value in colorectal cancer having recently been postulated in Chinese study (73) (*there still is no data on the predictive value of in a Western population*), where a low pre-treatment OPNI (<40) has an independent, unfavorable predictive value on survival of patients with resected colorectal cancer. (73)
  - the Neutrophil-to-Lymphocyte Ratio (NLR) in peripheral blood reflects the balance between systemic inflammation and immunity and is emerging as a prognostic biomarker in many diseases (74), including rectal cancer. It is overall a marker of systemic inflammatory response. In a systematic review including over 10,000 patients with LARC

rectal cancer, an elevated pretherapeutic NLR has been found to correlate with poor cancer-specific and overall survival (75).

- **Renal function** was assessed by evaluating the patients' creatinine level (expressed in μmol/L or mg/L) and estimating the glomerular filtration ratio (eGFR) by using MDCALC 2021 CKD-EPI equation, which is now the recommended standard (76). We then stratified the patients according to their renal function (see Table I for eGFR categorization, accordingly to *KDIGO 2012 Clinical Practice Guideline* (77)). Chronic kidney disease (CKD) is a relatively common comorbidity among cancer patients affecting the available therapy and outcomes (78): patients with CKD are more likely to be older and anemic with higher serum CRP, which reflects a general inflammatory state and a poorer prognosis (78).

4. STATISTICAL METHODS

Descriptive statistics were reported as mean (standard error, SE) for continuous variables and percentages (absolute numbers) for categorical variables and for these, in hypothesis analysis, Pearson's Chi-squared test and Fisher's exact test have been computed. Instead, Mann-Whitney U rank sum exact test was used to compare for continuous variables.

Set the standardized effect size at 0.80, a probability of type I error (α) at 0.05 and a probability of type II (β) error at 0.20 the minimal sample size was 25 subjects per group. Univariable and multivariable Cox proportional hazard models were used to assess the correlation between BMI and the expression of immunological gatekeepers in healthy rectal mucosa and a p-value less than 0.05 was considered statistically significant and the test considered were two-sided. All statistical analyses were conducted using the open-source R software (version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria (79) available from https://www.R-project.org) with the package Gtsummary (available from https://cran.r-project.org/web/packages/gtsummary/index.html).

RESULTS

1. CLINICAL FINDINGS (Descriptive statistics)

A total of 213 patients with rectal cancer, whose data of body mass index were available, were analyzed: 103 in the retrospective cohort and 110 in the prospective cohort. In our study group, 88 patients were normal weight (BMI <25) while 125 were overweight or obese (BMI≥25).

Tables I and II report descriptive statistics related to tumor grade (G), TNM stage and neoadjuvant treatment (YES/NO), stratified by BMI, respectively in the overall retrospective (Table I) and overall prospective cohort (Table II).

Table I. Descriptive statistics of tumor grade, TNM stage and neoadjuvant therapy stratified by BMI of overall retrospective cohort.

Characteristic	**N**	**Overall, N = 103[1]**	**<25, N = 41[1]**	**>=25, N = 62[1]**	**p-value[2]**
Gender	103				0.217
Female		33 (32%)	16 (39%)	17 (27%)	
Male		70 (68%)	25 (61%)	45 (73%)	
Age	103	65 (54, 73)	63 (49, 71)	68 (56, 74)	0.050
Grading	76				0.268
G0		3 (3.9%)	3 (8.8%)	0 (0%)	
G1		16 (21%)	6 (18%)	10 (24%)	
G2		47 (62%)	20 (59%)	27 (64%)	
G3		10 (13%)	5 (15%)	5 (12%)	
T stage	103				0.481
T0		9 (8.7%)	5 (12%)	4 (6.5%)	
T1		42 (41%)	14 (34%)	28 (45%)	

T2		36 (35%)	14 (34%)	22 (35%)	
T3		15 (15%)	8 (20%)	7 (11%)	
T4		1 (1.0%)	0 (0%)	1 (1.6%)	
N	100				0.923
N+		23 (23%)	9 (22%)	14 (23%)	
N0		77 (77%)	31 (78%)	46 (77%)	
M	103				0.112
M0		96 (93%)	36 (88%)	60 (97%)	
M1		7 (6.8%)	5 (12%)	2 (3.2%)	
Neoadjuvant therapy	103				0.175
No		73 (71%)	26 (63%)	47 (76%)	
Yes		30 (29%)	15 (37%)	15 (24%)	

[1]n (%); Median (IQR)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Fisher's exact test

- T parameter was assessed in all 103 retrospective patients: T0 was found in 9 patients (8.7% *of which 5 (55.6%) had a BMI<25 and 4 (44.4%) a BMI ≥ 25*), T1 in 42 patients (41%, *of which 14 (33.3%) had a BMI<25 and 28 (66.6%) a BMI ≥ 25)*, T2 in 36 (35%, *of which 14 (38.9%) had a BMI<25 and 22 (61.1%) a BMI ≥ 25*), T3 in 15 patients (15%, *of which 8 (53.3%) had a BMI<25 and 7 (46.7%) a BMI ≥ 25*) and T4 in 1 patient (*who had a BMI ≥ 25*). N parameter was assessed in 100 patients: 23 (23%) were N+ (*of which 9 (39.1%) had a BMI<25 and 14 (60.1%) a BMI ≥ 25*), while 77 (77%) were N0 *(of which 31 (40.2%) had a BMI<25 and 46 (59.8%) a BMI ≥ 25*). M parameter was determined in all 103 patients; 96 (93%) were M0 *(of which 36 (37.5%) had a BMI<25 and 60 (62.5%) a BMI ≥ 25)* and 7 (6.8%) were M1 *(5 (71.4%) had BMI<25 and 2 (28.6%) a BMI≥ 25).*
- Furthermore, of the overall 103 retrospective patients, 73 (71%) were treated without neoadjuvant treatment, whereas 30 patients (29%) were treated with neoadjuvant treatment before surgery.

Table II. Descriptive statistics of tumor grade, TNM stage and neoadjuvant therapy stratified by BMI of overall prospective cohort.

Characteristic	**N**	**Overall, N = 110[1]**	**<25, N = 47[1]**	**>=25, N = 63[1]**	**p-value[2]**
Gender	110				0.359
Female		46 (42%)	22 (47%)	24 (38%)	
Male		64 (58%)	25 (53%)	39 (62%)	
Age	108	67 (56, 77)	67 (56, 76)	67 (56, 77)	0.764
Grading	82				0.159
G0		9 (11%)	1 (2.9%)	8 (17%)	
G1		20 (24%)	11 (31%)	9 (19%)	
G2		28 (34%)	12 (34%)	16 (34%)	
G3		23 (28%)	11 (31%)	12 (26%)	
G4		2 (2.4%)	0 (0%)	2 (4.3%)	
T stage	98				0.367
T0		18 (18%)	6 (14%)	12 (22%)	
T1		18 (18%)	7 (16%)	11 (20%)	
T2		18 (18%)	6 (14%)	12 (22%)	
T3		41 (42%)	23 (53%)	18 (33%)	
T4		3 (3.1%)	1 (2.3%)	2 (3.6%)	
N	83				0.716
N+		22 (27%)	10 (29%)	12 (25%)	
N0		61 (73%)	25 (71%)	36 (75%)	
M	97				>0.999
M0		92 (95%)	41 (95%)	51 (94%)	

M1		5 (5.2%)	2 (4.7%)	3 (5.6%)	
Neoadjuvant_therapy	109				0.830
No		43 (39%)	18 (38%)	25 (40%)	
Yes		66 (61%)	29 (62%)	37 (60%)	

[1]n (%); Median (IQR)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Fisher's exact test

- T parameter was assessed in 98 of the 110 prospective patients: T0 was found in 18 patients (18%, *of which 6 (33.3%) had a BMI<25 and 12 (66.6%) a BMI ≥ 25*), T1 in other 18 patients (18%, *of which 7 (38.9%) had a BMI<25 and 11 (61.1%) a BMI ≥ 25)*, T2 in other 18 patients (18%, *of which 6 (33.3%) had a BMI<25 and 12 (66.6%) a BMI ≥ 25*), T3 in 41 patients (42%, *of which 23 (56%) had a BMI<25 and 18 (44%) a BMI ≥ 25*) and T4 in 3 patients (3.1%, *1 of which (33.3%) had BMI<25 and 2 (66.6%) BMI ≥ 25*). N parameter was assessed in 83 prospective patients: 22 (27%) were N+ (*of which 10 (45.5%) had a BMI<25 and 12 (54.5%) a BMI ≥ 25*), while 61 (73%) were N0 *(of which 25 (41%) had a BMI<25 and 36 (59%) a BMI ≥ 25*). M parameter was determined in 97 prospective patients; 92 (95%) were M0 *(of which 41 (44.6%) had a BMI<25 and 51 (55.4%) a BMI ≥ 25)* and 5 (5.2%) were M1 *(2 (40%) had BMI<25 and 3 (60%) BMI≥ 25)*.
- Furthermore, of the overall 110 prospective patients, 43 (39%) were treated without neoadjuvant treatment, whereas 66 patients (61%) underwent neoadjuvant treatment prior to surgery.

Retrospective cohort

Tables III, IV, V report the descriptive statistics found among the retrospective patients stratified by BMI and gender. While table III shows the results of overall 103 patients, the following table IV and V report the results in those who have not and have undergone neoadjuvant treatment (pre-surgery) respectively.

- Of the 103 retrospective patients, 33 (32%) were females and 70 (68%) were males. Among the 33 females, 16 were normal-weight or underweight (BMI <

25), whilst 17 were overweight or obese (BMI ≥ 25); among the 70 males, 25 were normal-weight/underweight and 45 were overweight/obese. In conclusion, of the whole 103 patients, regardless of gender, 41 (39.8%) were found to be normal weight/underweight and 62 (60.2%) overweight/obese (See table III and supplementary table III in appendix for the whole set of results).

- Of the 103 retrospective patients, 73 were treated without neoadjuvant treatment, 25 (34%) were females and 48 (66%) were males; regardless of gender, 26 patients (35.6%) were normal weight/underweight (BMI < 25), whilst 47 (63.4%) were found to be overweight/obese (BMI ≥ 25) (See table IV and supplementary table IV in appendix for the whole set of results).
- Of the 103 retrospective patients, 30 were treated with neoadjuvant therapy, of which 8 (27%) were females and 22 (73%) were males. Overall and despite sex, 15 (50%) were normal weight/underweight (BMI < 25) and 15 (50%) were overweight/obese (BMI ≥ 25). (See table V and supplementary table V in appendix for the whole set of results).

  Supplementary tables III, IV, V are reported in appendix and show the whole set of immunohistochemistry (IHC) results on the healthy rectal mucosa of the surgically treated retrospective cohort patients.

Table III. Descriptive statistics of overall retrospective patients stratified by BMI.

Characteristic	**N**	**Overall, N = 103[1]**	**<25, N = 41[1]**	**>=25, N = 62[1]**	**p-value[2]**
Gender	103				0.217
Female		33 (32%)	16 (39%)	17 (27%)	
Male		70 (68%)	25 (61%)	45 (73%)	
Albumin (g/dL):	71	3.83 (0.48)	3.92 (0.48)	3.78 (0.47)	0.267
Hemoglobin (g/dL):	95	13.60 (1.61)	13.36 (1.42)	13.74 (1.71)	0.162
Lymphocytes	99	57,724 (556,612)	1,755 (729)	92,590 (709,094)	0.627
Weight (Kg)	22	76 (16)	63 (8)	87 (14)	<0.001

Height (m)	22	1.72 (0.07)	1.70 (0.06)	1.74 (0.07)	0.112
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	16	48.5 (4.8)	46.2 (4.6)	50.2 (4.5)	0.210
Neutrophile/lymphocyte ratio (NLR)	21	2.97 (2.12)	2.84 (2.78)	3.07 (1.58)	0.554
eGFR	21	94 (15)	101 (13)	88 (15)	0.053
Creatinine (µmol/L)	21	74 (16)	65 (15)	83 (13)	0.026

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test; Fisher's exact test

Table IV. Descriptive statistics of retrospective patients treated WITHOUT neoadjuvant therapy, stratified by BMI.

Characteristic	**N**	**Overall, N = 73[1]**	**< 25, N = 26[1]**	**>= 25, N = 47[1]**	**p-value[2]**
Gender	73				0.111
Female		25 (34%)	12 (46%)	13 (28%)	
Male		48 (66%)	14 (54%)	34 (72%)	
Albumin (g/dL):	52	3.73 (0.48)	3.86 (0.51)	3.66 (0.46)	0.172
Hemoglobin (g/dL):	68	13.85 (1.55)	13.47 (1.32)	14.04 (1.64)	0.084
Lymphocytes	71	1,924 (572)	1,915 (631)	1,929 (544)	0.750
Weight (Kg)	10	72 (12)	63 (6)	81 (8)	0.008
Height (m)	10	1.69 (0.05)	1.66 (0.03)	1.72 (0.05)	0.094
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	7	49.9 (6.0)	46.7 (5.9)	54.1 (3.1)	0.114
Neutrophile/lymphocyte ratio (NLR)	9	1.98 (0.70)	2.08 (0.51)	1.91 (0.88)	0.286
eGFR	9	93 (21)	104 (13)	78 (21)	0.065

Creatinine (µmol/L)	9	75 (19)	65 (13)	87 (19)	0.085

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test; Fisher's exact test

Table V. Descriptive statistics of retrospective patients treated WITH neoadjuvant therapy, stratified by BMI

Characteristic	**N**	**Overall, N = 30[1]**	**< 25, N = 15[1]**	**>= 25, N = 15[1]**	**p-value[2]**
Gender	30				>0.999
Female		8 (27%)	4 (27%)	4 (27%)	
Male		22 (73%)	11 (73%)	11 (73%)	
Albumin (g/dL):	19	4.11 (0.32)	4.07 (0.39)	4.14 (0.29)	0.931
Hemoglobin (g/dL):	27	12.96 (1.60)	13.15 (1.62)	12.77 (1.63)	0.466
Lymphocytes	28	199,216 (1,046,696)	1,448 (828)	370,616 (1,430,067)	0.747
Weight (Kg)	12	79 (19)	63 (10)	91 (16)	0.007
Height (m)	12	1.74 (0.07)	1.73 (0.06)	1.76 (0.09)	0.368
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	9	47.4 (3.6)	45.7 (3.4)	48.2 (3.7)	0.714
Neutrophile/lymphocyte ratio (NLR)	12	3.71 (2.53)	3.44 (3.78)	3.90 (1.46)	0.268
eGFR	12	95 (11)	99 (13)	93 (8)	0.416
Creatinine (µmol/L)	12	74 (15)	65 (18)	80 (10)	0.219

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test; Fisher's exact test

Prospective cohort

Tables VI, VII, VIII report the descriptive statistics found among the prospective patients stratified by BMI and gender. Table VI shows the results of overall 110 patients, the following table VII and VIII report the results in those who have not and have undergone neoadjuvant treatment respectively.

- Of the 110 prospective patients, 46 (42%) were females and 64 (58%) were males. Among the 46 females, 22 were normal-weight or underweight (BMI $<$ 25), whilst 24 were overweight or obese (BMI $\geq$ 25); among the 64 males, 25 were normal-weight/underweight and 39 were overweight/obese. In conclusion, of the whole 103 patients, regardless of gender, 47 (42.7%) were found to be normal weight/underweight and 63 (57.3%) overweight/obese (See table VI and supplementary table VI in appendix for the whole set of results).
- Of the 110 prospective patients, 43 were treated without neoadjuvant treatment, 17 (40%) were females and 26 (60%) were males; regardless of gender, 18 patients (41.9%) were normal weight/underweight (BMI $<$ 25), whilst 25 (58.1%) were found to be overweight/obese (BMI $\geq$ 25) (See table VII and supplementary table VII in appendix for the whole set of results).
- Of the 110 prospective patients, 66 were treated with neoadjuvant therapy, of which 28 (42%) were females and 38 (58%) were males. Overall and despite sex, 29 (43.9%) were normal weight/underweight (BMI $<$ 25) and 37 (56.1%) were overweight/obese (BMI $\geq$ 25) (See table VIII and supplementary table VIII in appendix for the whole set of results).

  Supplementary tables VI, VII, VIII are reported in appendix and show the whole set of results of flow-cytometry (fluorescence-activated cell sorting - FACS) on the healthy rectal mucosa of the surgically treated prospective cohort patients.

Table VI. Descriptive statistics of overall prospective patients stratified by BMI

Characteristic	**N**	**Overall, N = 110[1]**	**< 25, N = 47[1]**	**>= 25, N = 63[1]**	**p-value[2]**
Gender	110				0.359

Female		46 (42%)	22 (47%)	24 (38%)	
Male		64 (58%)	25 (53%)	39 (62%)	
Albumin (g/dL):	88	6 (9)	5 (6)	7 (11)	0.264
Hemoglobin (g/dL):	110	16.97 (22.67)	12.57 (1.93)	20.25 (29.58)	0.156
Lymphocytes	99	3,927 (21,433)	2,157 (2,459)	5,286 (28,452)	0.172
Weight (Kg)	104	74 (14)	64 (11)	82 (11)	<0.001
Height (m)	100	1.69 (0.09)	1.69 (0.09)	1.69 (0.09)	0.607
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	86	84 (144)	60 (59)	104 (185)	0.828
Neutrophile/lymphocyte ratio (NLR)	97	3.10 (2.04)	3.10 (2.23)	3.09 (1.89)	0.658
eGFR	90	88 (20)	90 (22)	86 (17)	0.125
Creatinine (µmol/L)	88	81 (50)	84 (71)	78 (17)	0.058

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test

Table VII. Descriptive statistics of prospective patients treated WITHOUT neoadjuvant therapy stratified by BMI

Characteristic	**N**	**Overall, N = 43[1]**	**< 25, N = 18[1]**	**>= 25, N = 25[1]**	**p-value[2]**
Gender	43				0.068
Female		17 (40%)	10 (56%)	7 (28%)	
Male		26 (60%)	8 (44%)	18 (72%)	
Albumin (g/dL):	31	6.5 (9.4)	6.5 (9.7)	6.5 (9.4)	0.350
Hemoglobin (g/dL):	43	16.30 (22.86)	12.66 (2.20)	18.92 (29.91)	0.396
Lymphocytes	35	8,531 (35,851)	2,848 (3,083)	12,793 (47,418)	0.714

Weight (Kg)	38	74 (15)	63 (12)	84 (10)	<0.001
Height (m)	35	1.71 (0.10)	1.69 (0.10)	1.72 (0.09)	0.144
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	30	114 (212)	79 (97)	145 (276)	0.423
Neutrophile/lymphocyte ratio (NLR)	34	2.39 (1.71)	3.05 (2.32)	1.87 (0.74)	0.215
eGFR	31	83 (23)	89 (25)	77 (20)	0.114
Creatinine (µmol/L)	28	85 (46)	81 (61)	90 (21)	0.014

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test

Table VIII. Descriptive statistics of prospective patients treated WITH neoadjuvant therapy stratified by BMI

Characteristic	**N**	**Overall, N = 66[1]**	**< 25, N = 29[1]**	**>= 25, N = 37[1]**	**p-value[2]**
Gender	66				0.879
Female		28 (42%)	12 (41%)	16 (43%)	
Male		38 (58%)	17 (59%)	21 (57%)	
Albumin (g/dL):	56	6.1 (8.6)	4.0 (0.9)	7.7 (11.4)	0.487
Hemoglobin (g/dL):	66	17.51 (22.86)	12.51 (1.78)	21.43 (30.09)	0.237
Lymphocytes	63	1,422 (1,642)	1,787 (2,017)	1,130 (1,221)	0.088
Weight (Kg)	65	74 (14)	64 (10)	82 (12)	<0.001
Height (m)	64	1.69 (0.09)	1.69 (0.09)	1.68 (0.09)	0.665
Onodera index (formula: 10 × Albumin (g/dL) + 0.005 × total lymphocytes (mm3))	55	69 (87)	50 (12)	85 (116)	0.624
Neutrophile/lymphocyte ratio (NLR)	62	3.38 (1.98)	3.13 (2.22)	3.59 (1.76)	0.115
eGFR	58	90 (18)	90 (21)	91 (14)	0.580
Creatinine (µmol/L)	59	79 (52)	85 (77)	74 (13)	0.626

[1]n (%); Mean (SD)
[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test

2. FLOW-CYTOMETRY (fluorescence-activated cell sorting -FACS) FINDINGS

Figure 13 reports the boxplot of flow cytometric detection of HLA-ABC antibodies, stratified by BMI, in IMMUNOREACT prospective cohort (110 patients) as absolute values of MFI (mean fluorescence intensity). HLA-A, -B, and -C are major histocompatibility complex (MHC)-class I antigens; MHC molecules play a central role in the immune response and are expressed by antigen-presenting cells (APC): they are involved in the maturation of T cell repertoire and in the activation of T lymphocytes by presentation of xenogenic peptides or in the allogenic response. HLA-A, -B and -C are "classical" MHC Class I molecules and are expressed on the surface of most nucleated human cell types, including the here analyzed epithelial cells of rectal cancer patients' peritumoral healthy mucosa.

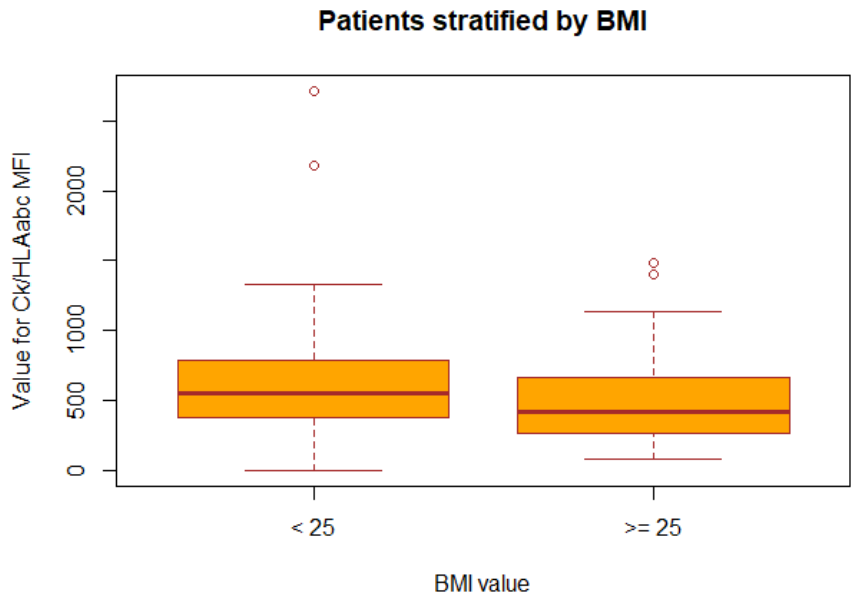


Figure 13. Boxplot of HLAabc, stratified by BMI, in IMMUNOREACT prospective cohort

These results suggest that overweight/obese (BMI ≥ 25) patients with rectal cancer tended to have a lower expression of HLA-ABC on the surface of their epithelial cells than those with BMI < 25 (CK+HLA-ABC+ MFI 549 (377, 792) vs 416 (261, 663), p= 0.069).

Moreover, in patients undergoing neoadjuvant therapy, overweight/obese ones had a lower frequency of high expression of HLA-ABC on epithelial cells than normal-weight patients, as shown in figure 14.

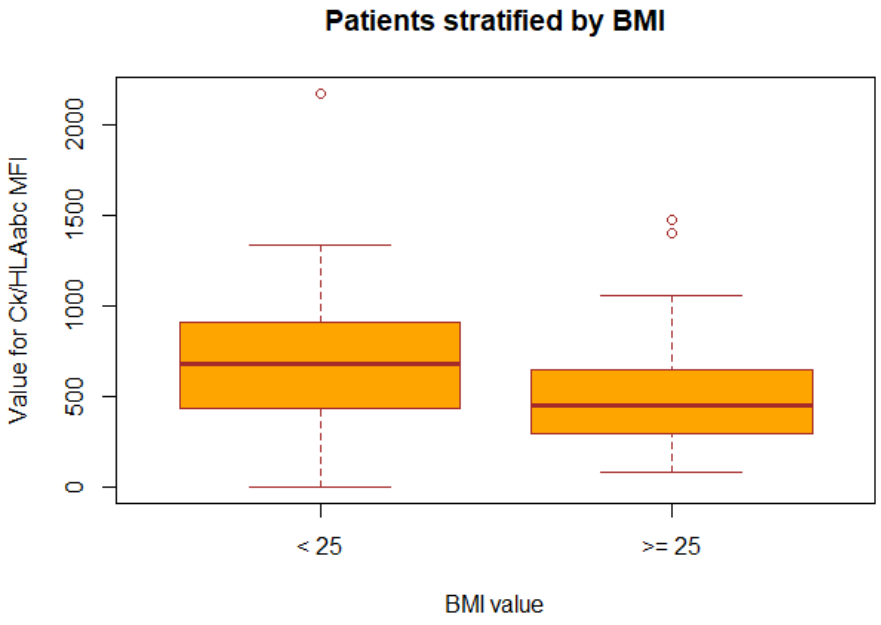


Figure 14. Boxplot of HLAabc, stratified by BMI, in IMMUNOREACT prospective cohort of neoadjuvantly treated patients.

3. IMMUNOHISTOCHEMISTRY (IHC) FINDINGS

A panel of immune markers was retrospectively investigated at immunohistochemistry (IHC): CD3, CD4, CD8, CD8beta, Tbet, FoxP3, PD-L1, MSH6, and PMS2 and CD80.

Whilst results showed no statistically significant difference in the number of CD4+ T-cells and expression of FoxP3 (well-established marker of T-reg cells) between normal weight/underweight patients (BMI < 25) and overweight/obese patients (BMI ≥ 25), a statistically significative difference among the two BMI stratified groups was found regarding the expression of CD8beta (and thus

number of CD8+ T-cells in peritumoral healthy mucosa of normal weight/underweight and overweight/obese patients).

In fact, as shown in the figure 15, overweight/obese patients have a lower infiltration of CD8beta+ T cells within the healthy mucosa surrounding the cancers than normal weight/underweight patients (CD8beta+ T cell /field: 87 (IQR: 60-106) in BMI<25 group, 16 (IQR: 5-68) in BMI ≥ 25 group, p=0.04).

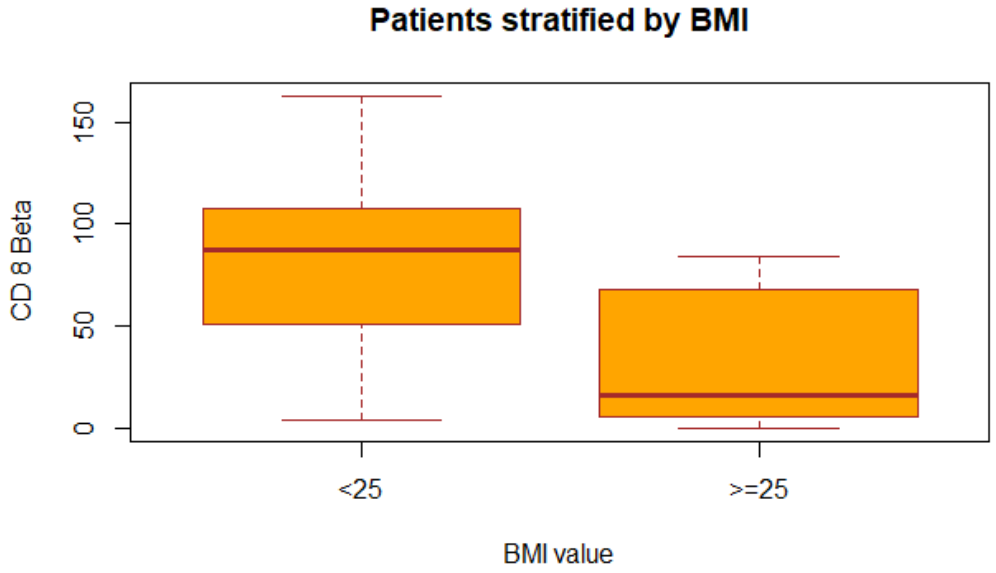


Figure 15. Boxplot of CD8beta expression stratified by BMI, in IMMUNOREACT retrospective cohort.

Finally, as presented in Figure 16 , the number of CD8 (cells/HPF [40x]) inversely correlated with BMI, (rho=-0.34, p=0.03).

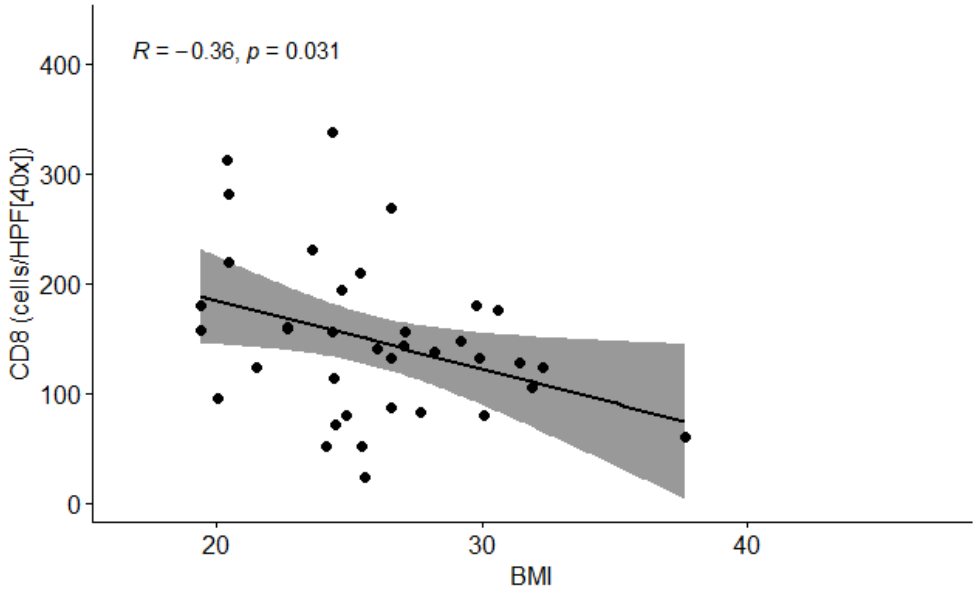


Figure 16. Regression of correlation between CD8 (cells/HPF [40x]) and BMI in IMMUNOREACT retrospective cohort.

Supplementary Figures 16.1,16.2,16.3, 16.4 can be found in appendix and present the regression of correlation between IHC analyzed CD8/CD3, CD8/CD4, CD8beta, CD8/FOX P3 and BMI. These correlations were not found to be statistically significative ($p > 0.05$).

DISCUSSION

Rectal cancer represents ~ 35% of the total CRC incidence, reflecting 15–25 cases/100.000 population per year (mean European incidence) (3) and is predicted to increase further in both genders. In fact, rectal cancer incidence has been steadily rising worldwide, especially in developing countries that are adopting the "western" way of life (3).

Although the aetiology of *sporadic* rectal cancer (which accounts for ≥70% of all cases) has not been fully understood, several risk factors have been proven to increase the likelihood of developing it, among which diet and obesity play a major role (8). Furthermore, the association between BMI and cancer incidence has been evaluated in several epidemiological studies, and it has been shown that the risk and incidence increase with BMI (46): it is in fact estimated that ~35.4% of rectal cancer cases in men and ~20.8% in women in the U.S. are attributable to obesity (47).

The importance of the tumor microenvironment in the development, growth, and progression of cancer is now well recognized: the peritumoral microenvironment is known to act to suppress the immune response against cancer cells, and tumor-infiltrating lymphocytes have a crucial role in immune surveillance (49).

Obesity leads to an imbalance in adipokines, gut dysbiosis, and endotoxemia, as well as IGF-1 activation pathways and free fatty acids release that can influence the immune microenvironment, as proven by different studies (49) (50) (51). The crosstalk between tumor cells and the immune microenvironment can be detected in the normal "healthy" mucosa surrounding cancer, according to the concept of the "field of cancerization" (30).

The aim of this study is to analyze the healthy rectal mucosa surrounding rectal cancer in overweight/obese patients who underwent surgery to evaluate the potential alteration of immune surveillance mechanisms. A detailed understanding of antitumoral immunity is essential for optimal cancer immune therapy.

In our study group, we enrolled 213 patients (103 in the retrospective cohort and 110 in the prospective cohort), 88 were normal weight or underweight (BMI <25), while 125 were overweight or obese (BMI≥25).

Flow-cytometry (FACS) analyses on peritumoral healthy mucosa of the prospective cohort showed that obese/overweight patients with rectal cancer have a statistically significative lower expression of HLA-ABC on the surface of their epithelial cells than the normal weight/underweight ones.
HLA-A, -B, and -C are major histocompatibility complex (MHC)-class I antigens, that are expressed on the surface of non-professional antigen presenting cells (APC) (*in our study rectal mucosa epithelial cells);* they play a central role in the immune response, as they are involved in the activation of T lymphocytes by presentation of immunogenic antigens. Although defective expression of HLA-ABC genes in peritumoral (colo)rectal mucosa have been reported in several studies (*using short-read sequencing data collected during large-scale cancer genome sequencing projects* as in (80) and (81)), the effects of these defects on tumor immunity have not been quantitatively evaluated yet. Kawazu M *et al,* (82) in a 2022 study, identified a subtype of CRC tumors in which lymphocyte infiltration was reduced, partly due to reduced expression of HLA-ABC and showed that survival time of such patients was shorter than in patients with normal antigen expression (82).
In our study, we found that overweight/obese patients had a lower expression of HLA-ABC antigens on the surface of peritumoral mucosa epithelial cells compared to normal/under-weight patients, accordingly to the still scarce literature. This result could be interpreted as a possible mechanism of immune evasion in overweight/obese people, with a possible effect on lymphocyte infiltration and long-term survival outcomes. Such difference of antigen expression among the two BMI groups may be explained by the chronic inflammatory state related to obesity and excessive fat accumulation, that has been proven to influence negatively on the ongoing immune perturbation within the (peri)tumoral microenvironment, potentially favoring tumor progression (49).

The results of the immunohistochemical (IHC) analyses on peritumoral healthy mucosa of the retrospective cohort of patients find greater supporting literature.

Our study found out that overweight/obese patients had a statistically significative lower infiltration of CD8beta+ T cells within the healthy mucosa surrounding the cancers than those with BMI under 25. CD8-β is a well-established marker of CD8+ T-cells (*thus reflecting their number in a tissue sample*): CD8 is, in fact, a transmembrane glycoprotein that serves as a co-receptor for the TCR and is predominantly expressed on the surface of cytotoxic T-cells. It can be an homomeric protein, but more often is heterodimeric, composed of a CD8-α and CD8-β chain. The expression of CD8beta is associated to cytotoxic T cell activation.

Furthermore, another important immunohistochemical finding was that of the inverse correlation between the number of CD8 (cells/HPF [40x]) in peritumoral healthy mucosa and BMI, as shown through the previous regression of correlation (Figure 16). This data suggest that the constitutive CD8 T cell infiltration of the healthy rectal mucosa, and thus the first line of defense against mutated epithelia cells, is lower the higher is the BMI.

Literature regarding the role of CD8+-T cell infiltration in rectal cancer is extensive and concordant studies suggest strong antitumoral effects and a positive prognostic significance (30): a high CD8+/CD4+ ratio is an independent prognostic factor for a better survival (37), advocating that the presence of CD8+ T-cells in tumor and peritumoral tissue could trigger an immunosurveillance status in the organism. Although CD8+ T cells infiltration have been shown to be an early event showing up in adipose tissue of diet-induced obesity (50), as part of the chronic inflammatory state triggered by excessive WAT (white adipose tissue), it is important to notice how - paradoxically - in obese tumoral and peritumoral tissues CD8+ T-cells are reduced in number.

Limitations of the study

Two are the main limitations of our study:

1) The first limitation of the study is that no measurements of <u>leptin, adiponectin and IGF-1 expression in adipose tissue samples</u> from peritumoral depots of patients with early rectal cancer have been taken. As reported in literature, one of the mechanisms proposed to explain the association between obesity and cancer risk is the potential action of adipokines (e.g., leptin, adiponectin and insulin growth factor-1 (IGF-1))

on tumor tissue (83). Adiponectin exerts pro-apoptotic, anti-proliferative and anti-inflammatory effects and expression of its receptor (AdipoR) in tumor cells is associated with a better prognosis in several GI-tract cancers, such as early esophageal cancer (83) and gastric cancer (84). IGF-1 has been suggested as another potential player in the association between visceral obesity and esophageal cancer (83) and its circulating levels are increased in obesity and other metabolic complications. Finally, leptin is known to exert a pro-inflammatory, mitogenic, anti-apoptotic and angiogenic functions on different cells and tissues; obesity and the involvement of leptin signaling have been associated with increased lymphangiogenesis and lymph node metastasis and increased leptin receptor (ObR) expression has been observed in several obese patients' tumor tissues, such as EAC (83) and breast cancer (85).

Collection of cultured biopsies of adipose tissue of peritumoral rectal mucosa and measurements of mRNA levels of leptin (ObR) and adiponectin receptors (AdipoR) is thus advised to further investigate the role of peritumoral adipose tissue in the interplay between obesity and cancer progression, by studying appropriately the local secretion of adipokines (*specifically leptin*) and their paracrine effect on tumor behavior.

2) The second limitation is linked to the relatively scarce number of obese patients analyzed in our study. The first aim was to investigate the influence of <u>obesity (BMI ≥30)</u> on the immunological peritumoral microenvironment; however, obese patients in our study series were too few to achieve a statistically significative sample size, thus the former purpose has been modified in the evaluation of the effect of <u>overweight and obesity (BMI ≥25)</u> on peritumoral healthy mucosa. As such, all analyzed parameters have been dichotomized in two different groups: that of normal weight and underweight patients (BMI <25) and that of overweight and obese patients (BMI ≥25).

   Willingly, future larger-scale studies will be designed to evaluate *more specifically* the effects of obesity in the immune microenvironment of rectal cancer peritumoral healthy mucosa.

CONCLUSIONS

In conclusion, our findings suggest that in patients with rectal cancer, those who are obese/overweight have a lower activation of epithelial cells as antigen-presenting cells (APC cells) and a lower activation of cytotoxic T-cells (CD8+ T-cells) in the healthy mucosa surrounding rectal cancer, compared to normal weight/underweight patients.

The difference in HLA-ABC antigen expression among the two BMI groups may be, in part, explained by the chronic inflammatory state related to obesity and excessive fat accumulation, that has been proven to influence negatively on the ongoing immune perturbation within the peritumoral microenvironment. We interpret such result as a newly discovered possible mechanism of immune evasion in overweight/obese rectal cancer patients, with a significant effect on lymphocyte infiltration and long-term survival outcomes. Surely, *as literature on the subject is quite scarce,* further studies need to be undertaken to identify causal mechanisms of BMI-associated influence on MHC class I expression upon antigen-presenting cells.

The second conclusion we can draw from the study is that of the lower level of cytotoxic (CD8+) T-cell infiltration in peritumoral healthy mucosa in overweight/obese patients compared to normal weight/underweight ones. *In accordance with the robust literature present in the matter*, the lower CD8+ T cell count could predict worse prognosis in overweight/obese rectal cancer treated patients, as a reflection of the diminished immune surveillance in peritumoral healthy mucosa. Future studies need to be done to explain the bases of the changes in the microenvironment that take place during tumor development, from overweight/obese adipose tissues (*rich in CD8+ T-cells and other inflammatory cells*) and overweight/obese tumoral and peritumoral tissues (*where the number of CD8+ T-cells is reduced*).

Possible clinical-translational application of the study

These data could be useful to plan a tailored approach to overweight/obese patients with a rectal cancer diagnosis. Moreover, being adipose inflammation a reversible process, it could represent a novel therapeutic target *(that surely warrants further study)* to break the obesity-cancer link. Collectively, these

findings and the existing literature, help to establish the rationale for interventions aimed at improving adipose tissue health as a novel opportunity to reduce the risk of both development of rectal cancer and improvement of rectal cancer treatment outcomes in overweight/obese patients.

APPENDIX

Supplementary Table III. Descriptive statistics of IHC findings on healthy mucosa of overall retrospective patients stratified by BMI

IHC findings on healthy mucosa					
CD4 (cells/hpf [40x]) healthy mucosa:	37	363 (398)	364 (397)	362 (410)	0.988
CD8 (cells/hpf [40x]) healthy mucosa	37	148 (72)	172 (83)	128 (57)	0.094
CD8/CD4 healthy mucosa	34	6 (19)	4 (13)	7 (24)	0.635
CD3 [/4 (40) hpf] healthy mucosa	37	398 (221)	421 (252)	379 (195)	0.784
CD8/CD3 healthy mucosa	35	0.47 (0.49)	0.60 (0.70)	0.36 (0.17)	0.766
CD4/CD3 healthy mucosa	35	0.90 (1.01)	0.87 (0.94)	0.93 (1.09)	0.840
PDL1 healthy mucosa	38	0.35 (0.94)	0.17 (0.52)	0.55 (1.24)	0.187
MSH6 healthy mucosa	35				0.443
1		8 (23%)	5 (29%)	3 (17%)	
2		27 (77%)	12 (71%)	15 (83%)	
PMS2 healthy mucosa	37				0.105
1		3 (8.1%)	3 (17%)	0 (0%)	
2		34 (92%)	15 (83%)	19 (100%)	
CD8β healthy mucosa	19	55 (47)	82 (49)	34 (35)	0.043
Tbet healthy mucosa	19	7.1 (7.0)	8.3 (8.3)	6.3 (6.1)	0.590
FOXP3 healthy mucosa	18	5.6 (5.3)	4.1 (1.8)	6.5 (6.6)	0.440
CD8/FOXP3 healthy mucosa	16	53 (86)	59 (40)	50 (106)	0.093

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test; Fisher's exact test

Supplementary Table IV. Descriptive statistics of IHC findings on healthy mucosa of retrospective patients treated WITHOUT neoadjuvant therapy, stratified by BMI

IHC findings on healthy mucosa					
CD4 (cells/hpf [40x]) healthy mucosa:	13	392 (480)	315 (529)	516 (411)	0.207
CD8 (cells/hpf [40x]) healthy mucosa	13	182 (89)	220 (92)	123 (42)	0.091
CD8/CD4 healthy mucosa	12	5 (15)	6 (18)	3 (6)	0.545
CD3 [/4 (40) hpf] healthy mucosa	12	379 (208)	379 (240)	378 (181)	0.755
CD8/CD3 healthy mucosa	11	0.74 (0.81)	0.98 (0.94)	0.31 (0.15)	0.130
CD4/CD3 healthy mucosa	12	1.07 (1.23)	0.62 (0.92)	1.69 (1.45)	0.141
PDL1 healthy mucosa	14	0.64 (1.33)	0.37 (0.75)	1.14 (2.04)	0.559
MSH6 healthy mucosa	13				>0.999
1		1 (7.7%)	1 (14%)	0 (0%)	
2		12 (92%)	6 (86%)	6 (100%)	
PMS2 healthy mucosa	14				
2		14 (100%)	8 (100%)	6 (100%)	
CD8β healthy mucosa	7	91 (48)	111 (33)	44 (57)	0.190
Tbet healthy mucosa	7	9 (9)	10 (10)	5 (1)	0.857
FOXP3 healthy mucosa	6	4.95 (3.37)	3.67 (2.05)	7.50 (4.95)	0.348
CD8/FOXP3 healthy mucosa	6	61 (37)	79 (32)	26 (14)	0.133

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test; Fisher's exact test

Supplementary Table V. Descriptive statistics of IHC findings on healthy mucosa of retrospective patients treated with neoadjuvant therapy stratified by BMI

IHC findings on healthy mucosa					
CD4 (cells/hpf [40x]) healthy mucosa:	24	347 (357)	404 (275)	307 (411)	0.182
CD8 (cells/hpf [40x]) healthy mucosa	24	130 (56)	130 (47)	130 (62)	0.698
CD8/CD4 healthy mucosa	22	5.7 (21.4)	2.1 (5.5)	7.7 (26.7)	0.298
CD3 [/4 (40) hpf] healthy mucosa	25	408 (230)	451 (269)	379 (205)	0.637
CD8/CD3 healthy mucosa	24	0.35 (0.15)	0.30 (0.09)	0.38 (0.17)	0.232
CD4/CD3 healthy mucosa	23	0.82 (0.89)	1.07 (0.97)	0.66 (0.84)	0.177
PDL1 healthy mucosa	24	0.18 (0.58)	0.00 (0.00)	0.32 (0.77)	0.107
MSH6 healthy mucosa	22				0.652
1		7 (32%)	4 (40%)	3 (25%)	
2		15 (68%)	6 (60%)	9 (75%)	
PMS2 healthy mucosa	23				0.068
1		3 (13%)	3 (30%)	0 (0%)	
2		20 (87%)	7 (70%)	13 (100%)	
CD8β healthy mucosa	12	33 (31)	35 (33)	32 (33)	>0.999
Tbet healthy mucosa	12	6.2 (6.0)	5.2 (4.1)	6.5 (6.7)	0.780
FOXP3 healthy mucosa	12	5.9 (6.2)	4.7 (1.6)	6.3 (7.1)	0.853
CD8/FOXP3 healthy mucosa	10	49 (107)	19 (8)	56 (120)	0.889

[1]n (%); Mean (SD)

[2]Fisher's exact test; Wilcoxon rank sum test; Wilcoxon rank sum exact test

Supplementary Table VI. Descriptive statistics of FACS on healthy mucosa of overall retrospective patients stratified by BMI

FACS on healthy mucosa					
Ck/CD80 % healthy mucosa	101	24 (20)	23 (19)	26 (21)	0.501
Ck/CD80 MFI healthy mucosa	101	151 (109)	139 (55)	159 (136)	0.778
Ck/CD86 % mucosa sana:	97	20 (16)	18 (15)	22 (16)	0.241
Ck/CD86 MFI healthy mucosa	97	152 (101)	145 (72)	158 (118)	0.621
Ck/HLAabc % healthy mucosa	100	48 (30)	42 (31)	52 (29)	0.140
Ck/HLAabc MFI healthy mucosa	100	567 (416)	657 (505)	498 (321)	0.069
CD8/CD28 % healthy mucosa	100	5.3 (7.3)	5.6 (7.5)	5.1 (7.2)	0.472
CD8/CD28 MFI healthy mucosa	100	52 (37)	57 (45)	49 (30)	0.646
CD8/CD38 % healthy mucosa	99	7 (8)	7 (8)	7 (9)	0.280
CD8/CD38 MFI healthy mucosa	99	51 (23)	54 (17)	50 (27)	0.460
CD4/CD25 % healthy mucosa	96	2.83 (5.35)	3.73 (7.06)	2.10 (3.30)	0.190
CD4/CD25 MFI healthy mucosa	96	26 (41)	31 (48)	21 (36)	0.115
CD25/FoxP3 % healthy mucosa	96	1.24 (2.25)	1.25 (1.85)	1.24 (2.54)	0.284
CD25/FoxP3 MFI healthy mucosa	96	218 (393)	318 (501)	137 (256)	0.079
CD3/CTLA4 % healthy mucosa	96	6.2 (7.8)	6.8 (8.2)	5.6 (7.4)	0.233
CD3/CTLA4 MFI healthy mucosa	96	49 (86)	53 (70)	46 (98)	0.123

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test

Supplementary Table VII. Descriptive statistics of FACS on healthy mucosa of retrospective patients treated without neoadjuvant therapy stratified by BMI

FACS healthy mucosa					
Ck/CD80 % healthy mucosa	42	26 (16)	22 (15)	28 (17)	0.291
Ck/CD80 MFI healthy mucosa	42	139 (54)	135 (43)	141 (62)	0.799
Ck/CD86 % mucosa sana:	39	21 (16)	17 (13)	25 (17)	0.097
Ck/CD86 MFI healthy mucosa	39	133 (40)	144 (45)	126 (35)	0.213
Ck/HLAabc % healthy mucosa	41	55 (32)	51 (35)	59 (29)	0.665
Ck/HLAabc MFI healthy mucosa	41	525 (426)	596 (562)	471 (281)	0.458
CD8/CD28 % healthy mucosa	41	6 (9)	5 (6)	7 (10)	0.752
CD8/CD28 MFI healthy mucosa	41	55 (47)	67 (61)	46 (30)	0.344
CD8/CD38 % healthy mucosa	40	9 (9)	8 (7)	9 (11)	0.796
CD8/CD38 MFI healthy mucosa	40	48 (21)	52 (19)	45 (23)	0.462
CD4/CD25 % healthy mucosa	37	3.4 (4.9)	4.1 (5.2)	2.8 (4.6)	0.220
CD4/CD25 MFI healthy mucosa	37	26 (42)	27 (23)	25 (55)	0.114
CD25/FoxP3 % healthy mucosa	37	1.15 (2.50)	0.91 (1.68)	1.37 (3.11)	0.809
CD25/FoxP3 MFI healthy mucosa	37	145 (313)	217 (406)	76 (175)	0.468
CD3/CTLA4 % healthy mucosa	37	7 (8)	7 (7)	8 (8)	0.939
CD3/CTLA4 MFI healthy mucosa	37	51 (117)	38 (38)	63 (160)	0.436

[1]n (%); Mean (SD)

[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test

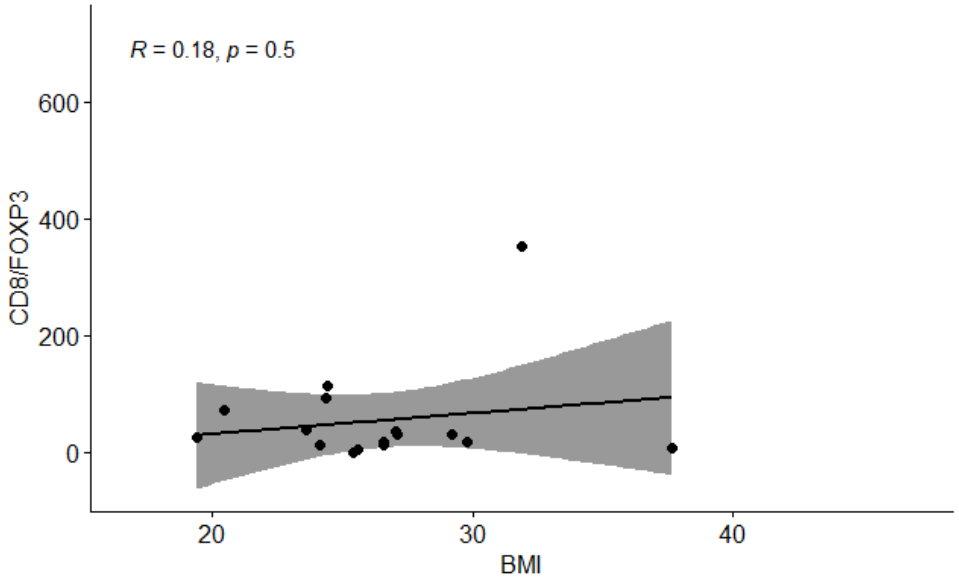
Supplementary Table VIII. Descriptive statistics of FACS on healthy mucosa of retrospective patients treated with neoadjuvant therapy stratified by BMI

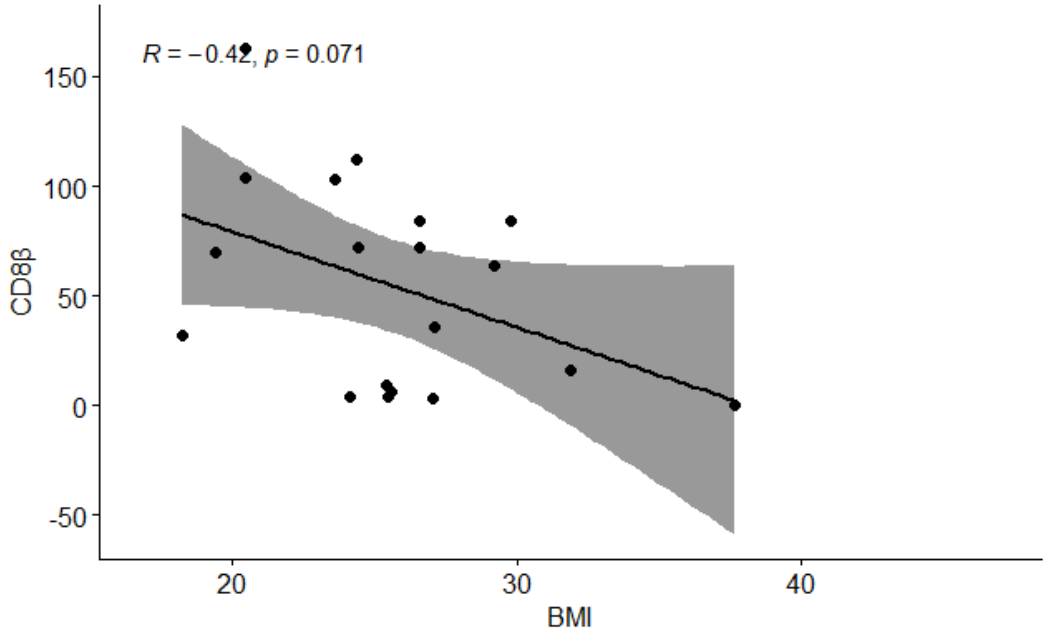
FACS healthy mucosa					
Ck/CD80 % healthy mucosa	58	23 (22)	23 (21)	24 (23)	0.962
Ck/CD80 MFI healthy mucosa	58	162 (135)	143 (63)	177 (171)	0.981
Ck/CD86 % mucosa sana:	57	19 (16)	19 (17)	20 (15)	0.860
Ck/CD86 MFI healthy mucosa	57	165 (126)	146 (86)	180 (149)	0.834
Ck/HLAabc % healthy mucosa	58	43 (28)	36 (26)	48 (28)	0.101
Ck/HLAabc MFI healthy mucosa	58	603 (409)	701 (467)	528 (347)	0.087
CD8/CD28 % healthy mucosa	58	4.7 (6.4)	5.7 (8.4)	4.0 (4.4)	0.483
CD8/CD28 MFI healthy mucosa	58	51 (30)	50 (29)	51 (31)	0.826
CD8/CD38 % healthy mucosa	58	6.0 (7.3)	6.8 (7.9)	5.4 (6.8)	0.300
CD8/CD38 MFI healthy mucosa	58	54 (25)	55 (16)	53 (30)	0.869
CD4/CD25 % healthy mucosa	58	2.48 (5.69)	3.48 (8.26)	1.72 (2.24)	0.625
CD4/CD25 MFI healthy mucosa	58	26 (42)	34 (60)	19 (19)	0.406
CD25/FoxP3 % healthy mucosa	58	1.33 (2.11)	1.49 (1.96)	1.21 (2.23)	0.249
CD25/FoxP3 MFI healthy mucosa	58	269 (435)	390 (556)	177 (291)	0.110
CD3/CTLA4 % healthy mucosa	58	5.6 (7.8)	6.9 (8.9)	4.6 (6.9)	0.404
CD3/CTLA4 MFI healthy mucosa	58	49 (61)	63 (85)	37 (29)	0.150

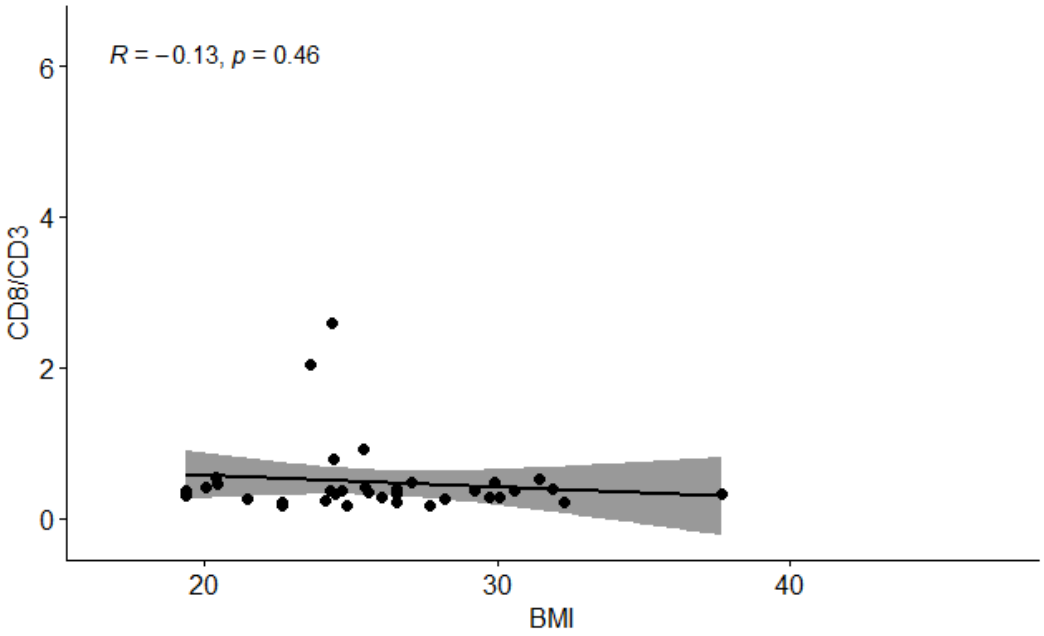
[1]n (%); Mean (SD)

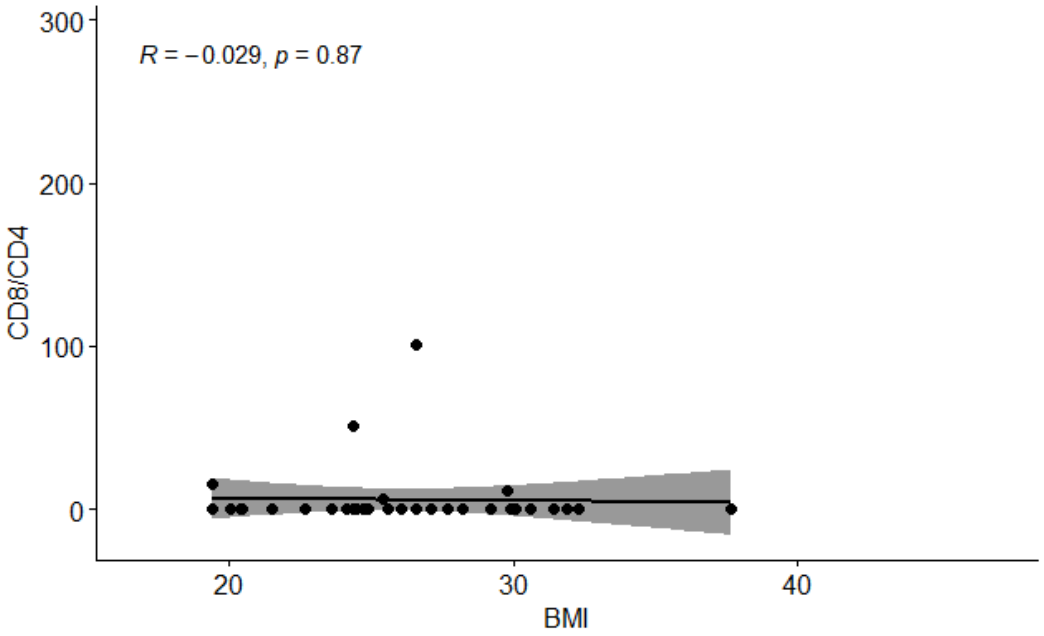
[2]Pearson's Chi-squared test; Wilcoxon rank sum test; Wilcoxon rank sum exact test

Supplementary figures 16.1,16.2,16.3,16.4. Regression of correlation between CD8/FOXP3,CD8beta, CD8/CD3, CD8/CD4 and BMI in IMMUNOREACT retrospective cohort.






R = −0.13, p = 0.46
CD8/CD3
6
4
2
0
20
30
40
BMI


R = −0.029, p = 0.87
CD8/CD4
300
200
100
0
20
30
40
BMI

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