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Seric profile predictive for recurrence in patients undergone curative hepatic resection for metastasis from Colo-Rectal Carcinoma

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ABBREVIATIONS

ALU Arithmetic Logic Unit

CEA Carcinoembryonic Antigen

CRC ColoRectal Cancer

CRS Clinical Risk Score

DFS Disease Free Survival

II Integrity Index

K-W Kruskal-Wallis

MTS Metastasis

MSI Microsatellite Instability

OS Overall Survival

Tf0 Pre-operative blood sample

Tf1 Blood sample 30 days after surgery

ABSTRACT

Introduction: Liver metastatic disease is the first cause of death in Colorectal Cancer. Specifically, 20-25 % of patients has metastatic disease at the time of diagnosis, while 25-30 % of individuals will develop liver metastases during the course of disease.

At diagnosis only 10-20% of patients is resectable remaining surgical resection the only potentially curative treatment.

However, two-third of patients who received curative surgery will experience recurrence of disease, and 75% will relapse within the first two years after hepatectomy. Several combinations of clinical-pathological parameters have been proposed to analyze the prognosis of patients with potentially resectable colorectal liver metastases, in particular various molecular markers have been considered, but any of these has not been validated for clinical use. Recently, some trials have proposed the detection of tumoral circulating DNA to be a prognostic marker in solid neoplasms.

Aim of the Study: Aim of the study is to determine if peri-operative tumoral circulating DNA detected in blood of patients with colorectal liver metastases can be a prognostic marker for recurrences.

Materials and Methods: Between March 2009 and March 2011 we analyzed 26 patients who underwent surgical resection for colorectal liver metastases. 19 patients were male, 7 patients were female. Mean age was 63.7 years (45-79). We collected a sample of venous blood before surgical procedure (Tf0) and after 30 days (Tf1). In these two samples we applied qRT- PCR to quantify total circulating DNA (ALU83) and tumoral circulating DNA (ALU244) in serum.

Results: Median follow-up was 15 months (range 3-26); median DFS was 19 months. Median ALU244/ALU83 ratio was 0.28 (range 0.0652-0.763). Patients with ALU244/ALU83 ratio > 0.28 had worst recurrence-free survival than patients with ALU244/ALU83 ratio ≤0.28. (Hazard Ratio 8.07; P-value: 0.0205).

Conclusions: In our Study the value of circulating DNA ALU244/ALU83 ratio in patients with colorectal liver metastases who underwent curative hepatic resection has a prognostic value for detecting recurrences. It is necessary to enforce the case-study by increasing the number of patients and extending follow-up for patients already included.

RIASSUNTO

Introduzione: Nella storia naturale del cancro del colon–retto le metastasi epatiche costituiscono la causa maggiore di decesso. Il 20-25% dei pazienti presenta metastasi epatiche al momento della diagnosi del tumore primitivo mentre il 25-30% dei casi le svilupperà nel corso dell'evoluzione della malattia.
Al momento della diagnosi solo il 10-20% di pazienti risulta candidato ad una resezione curativa del fegato pur rimanendo l'intervento chirurgico di resezione epatica l'unico trattamento potenzialmente curativo.
Circa due terzi dei pazienti trattati con intento curativo va incontro a recidiva e il 75% di queste recidive si manifesta nei primi 2 anni dall'intervento chirurgico.
Allo scopo di analizzare più accuratamente l'evoluzione clinica di questi pazienti sono stati considerati diversi fattori prognostici, in particolare è stato studiato l'andamento di molteplici marcatori molecolari, ma nessuno di questi è stato validato nella pratica clinica. Recentemente alcuni ricercatori hanno proposto lo studio del DNA circolante di origine tumorale come fattore prognostico in alcuni tumori solidi.

Scopo dello Studio: Lo scopo dello studio è stato di verificare se la quantità di DNA circolante di origine tumorale misurata nel sangue dei pazienti prima e a 30 giorni dalla resezione epatica possa essere considerato un fattore prognostico di rischio di recidiva.

Materiali e Metodi: Sono stati analizzati 26 pazienti sottoposti a resezione epatica per metastasi da cancro del colon-retto nel periodo compreso tra Marzo 2009 e Marzo 2011. 19 erano di sesso maschile e 7 di sesso femminile; l'età media è risultata di 63.7 anni (range 45-79). E' stato effettuato un prelievo di sangue venoso prima dell'intervento (Tf0) e dopo 30 giorni dallo stesso (Tf1). In queste due serie di campioni è stata quantificata nel siero la quota di DNA circolante totale (ALU83) e la quota di DNA circolante di origine tumorale

(ALU244).La quantificazione del DNA circolante è stata effettuata mediante mediante la q RT-PCR.

Risultati: Il follow-up mediano di questi pazienti è risultato di 15 mesi (range 3-26); l'intervallo libero da malattia (DFS) mediano è risultato di 19 mesi
Il valore mediano di ALU244/ALU83 è risultato pari a 0,28 (range 0.0652-0.763)
Nei pazienti con rapporto ALU244/ALU83 > 0,28 l'hazard ratio di recidiva nei 12 mesi successivi all'intervento è risultato 8 volte superiore rispetto ai pazienti con rapporto ALU244/ALU83 ≤0.28. (P- value: 0.0205).

Conclusioni: Nel nostro studio il valore del rapporto ALU244/ALU83 del DNA circolante tumorale nei pazienti affetti da metastasi epatiche da cancro del colon-retto e sottoposti a resezione curativa del fegato è correlato con un elevato rischio di recidiva. Sarà indispensabile aumentare il follow-up di questo gruppo di pazienti ed implementare la casistica allo scopo di confermare i dati ottenuti.

INTRODUCTION

The colorectal carcinoma is the third most frequent carcinoma in the Western countries, and 50% of the cases involve a liver metastasis, which is the major cause of death in those patients. [1; 2]

Liver resection is still today the only curative treatment, it accounts for up to a five-year survival rate of 40-58%. [3-9]

Unfortunately the recurrence rate of colorectal liver metastasis after curative resection is high: the overall rate varies between 60 and 70% according to different cases [5;8;10;11-14]; in particular, 75% of the patients have a recurrence in the first two years after resection.

These data show how important it is to try and identify patients at high recurrence risk.

Literature reports different “Clinical Risk Score” of disease recurrence after treating colorectal carcinoma taking into account different parameters: patient’s age, tumour grading, primitive tumour staging, diameter of neoplasia, number of liver metastasis, location of liver metastasis, resection margin on the liver, CEA level, extrahepatic disease, and disease-free interval between diagnosis of primitive tumour and metastasis. However those parameters are not applicable in all cases and therefore the use of this "Clinical Risk Score" has not been validated in clinical practice. [15-19].

Trying to accurately define the prognosis at the moment of diagnosis and to early identify recurrence, various studies have proposed to search the circulating DNA of tumor origin in patients suffering from various types of solid tumors [20-26].

It has been demonstrated that DNA of tumour origin can be found in the blood of patients suffering from the most frequent solid malignant neoplasms: tumor necrosis releases a significant quantity of genomic DNA fragments, which are wider than those released by the apoptotic process commonly present in healthy people. [22;24;27] Hence it becomes clear how tumoral circulating DNA can be a valid prognosis marker and it can be useful to diagnose the recurrence in some types of solid tumours.

1. LIVER METASTASES OF COLORECTAL CARCINOMA

1.1 Epidemiology of colorectal carcinoma

The colorectal carcinoma is the third most frequent carcinoma in the Western countries. [28]. There are about 1.2 million new cases every year, 413,000 new cases in Europe and 150,000 cases in the USA [29]

In Italy, colorectal carcinoma is the third most common cancer in males and the second among women. Cases in the Veneto region are about 3,350 yearly (55% men, 45% women). [30].

Prognosis is particularly influenced by the stage of neoplasia at the diagnosis. The presence of liver metastases is the most significant death cause in those patients: they are synchronous (within 6 months since the diagnosis of primitive tumour) in 20-25% of cases and metachronous in 25-30% of cases. [1;2]

1.2.Metastatic diffusion

The diffusion of colorectal carcinoma occurs in four ways:

- *local invasion*: from the mucosa the tumour spreads into the muscularis mucosae, then involving the submucosa, tunica submucosa and serosa, eventually invading the surrounding organs;
- *lymphatic dissemination*: neoplastic cells spread along the lymphatic vessels involving epicolic, paracolic, intermediate and principal lymph nodes;
- *peritoneal implantation*: it occurs by exfoliation of tumour cells from the serosa into the peritoneal cavity, thus allowing the clinical condition of the peritoneal carcinosis [31].
- *hematogenous dissemination*: the diffusion at distance occurs when the tumour cells enter the blood circle, and through the portal flow they affect the liver parenchyma. In those cases liver is considered to be the first site of metastasis dissemination, and it is the first and unique site of disease in 30-50% of cases [32]. 70% of cases are non-resectable because there is a metastatic extrahepatic disease or because hepatic lesions involve the main vessels. [33].

1.3. Signs and symptoms of liver metastases

In most cases liver metastases are asymptomatic and are diagnosed during the staging of the primitive tumour or the follow-up. [31]

Only 10-15% of patients with liver metastases have lesions big enough to determine the occurrence of non specific systemic symptoms (asthenia, unexplainable loss of weight, fever, sweating, loss of appetite), those symptoms are due to a significant involvement of the liver (hepatomegaly, palpable mass, burden and pain in the right hypochondrium or in the epigastrium, abdominal tension, signs of hepatic deficiency such as rapidly worsening jaundice, hypoalbuminemia and ascites, coagulopathy with bleeding of skin or mucosa even in case of weak trauma) and symptoms due to the compression of nearby organs (particularly of bile ducts with jaundice, inferior vena cava with edema of lower extremities). Ascites can occur later both for hepatic deficiency and other causes, such as thrombosis of the portal vein or peritoneal dissemination of the disease.

The objective examination of abdomen is generally negative: hepatomegaly is a very unfavourable prognostic sign showing that the disease is in an advanced stage. [33]

1.4.Liver metastases diagnosis

Laboratory tests

Laboratory tests show early but non specific cholestasis index in over 90% of patients, particularly in Alkaline Phosphatase (ALP) and in Gamma-Glutamyl-Transpeptidase (GGT). When bile ducts are obstructed, there is an increase in bilirubinemia.

When liver is massively involved in metastasis, there can be hypoalbuminemia, reduction in a1-antitrypsin, alteration of the electrophoretic trace of plasma proteins, reduction in blood urea nitrogen, hyperammonemia, aminoaciduria, hypoglycemia, fibrogen deficiency, and vitamin K-dependent clotting factors (thrombin or FII, FVII, FIX, FX), longer prothrombin time (PT).

Any increase in the plasma concentrations of tumour markers can help in the diagnosis and in the follow-up of patients with liver metastases. The most used neoplastic markers are: CEA (carcinoembryonic antigen) and CA 19.9. [33]

Imaging

The radiological analysis is fundamental both at preoperative staging and to decide whether or what type of operation to perform.

The **ultrasonography** is limited by the performer's experience; it has 70% sensitivity and 85% specificity. It is useful in differential diagnosis particularly with contrast medium. Metastases look like solid nodules with varying sonic characteristics: hypoecoic, isoecoic, hyperecoic, or they can have the so called "target pattern" because of a central hypoecoic area, due to necrosis and a peripheral surrounding hyperecoic area [34].

The Gold Standard in staging and follow-up of patients with liver metastases is **Computed Tomography** (CT) using a contrast medium: it does not only show a complete image, but it also enables to perform dynamic analyses on the portal and arterial vasculature of liver lesions. Total body CT is fundamental to dismiss an extrahepatic disease and to evaluate the resectability of liver metastases especially as far as the anatomic relation to the hepatic hilum and the suprahepatic veins is concerned. Metastases look like hypodense areas because they are hypervascularized, not well defined by the surrounding parenchyma and better visible during the portal phase. [33]

Magnetic Resonance (MRI) gives more specific information about vasculature of the lesion and on its relation to the adjacent vascular and biliary structures or other hepatic masses. The use of contrast media as gadolinium (non specific for the liver) or supermagnetic oxide (liver-specific) allows to enhance the method. Liver metastases are typically hypointense in T1-weighted images and hyperintense in T2-weighted images; necrosis, hemorrhage or fibrous tissue can alter the signal. [33]. MRI is not generally used as a routine test, but for differential diagnosis and in case of doubts.

PET (Positron Emission Tomography) uses the 2-deoxi-2-fluoro-D-glucose (FDG) produced in tissues with malignant cells caused by the increased glycolysis

compared to healthy tissues. It enables to examine the metabolic activity in relation to anatomic structures. In patients undergoing chemotherapy, PET should be used four weeks after the conclusion of the treatment since the drugs cause metabolic inhibition that interferes with the correct functioning of the test increasing the number of false negatives and compromising the results [35;36]. FDG-PET can identify a hidden extrahepatic disease or additional hepatic lesions in ¼ of the cases; for this reason it has become part of the staging protocol for this pathology.

Recently the use of **PET-TAC** method is increasing thanks to its combination of both tests, thus enabling a more accurate staging of the disease and the metastases. [33]

1.5. Treatment of liver metastases

Surgical Anatomy of the Liver

According to what Coinaud wrote in 1957 liver can be divided into eight segments according to the subdivision of the hepatic portal tree and the division of suprahepatic veins.

The right and middle/left hepatic veins divide the two lobes, right and left, in sectors and segments with autonomous vasculature support and separate biliary drainage.

The left lobe is composed of segments I,II,III and IV which is further divided into IVa and IVb; the right lobe is composed of segments V,VI,VII, VIII. [31]

The surgical treatment

Only 10-20% of patients at the diagnosis are considered resectable according to criteria that have undergone much revision in the last 10 years.

In the past, characteristics such as the number of metastases (3-4), the size of tumour lesions and disease-free surgical margin of at least 1 cm were considered fundamental to define resectability. At present only two criteria must be met to define a curative operation:

1. Disease-free surgical margins (resection R0) on all lesions notwithstanding the distance: 1 cm margin is not necessary any more;
2. Disease-free area of liver >20% of the initial liver volume or >30% if the patient underwent chemotherapy.

To evaluate the resectability and the real number of liver metastases an intraoperative ultrasonography is used (with or without contrast medium). This method enables to identify even small metastases (3-4 mm of diameter) enabling an upstaging/downstaging in 15-20% of cases during the operation.[37]

On the basis of the subdivision in segments described above, liver surgical operations are divided into typical and atypical resections.

Typical resections are carried out based on criteria of functional anatomical subdivision of the liver as described above and are called hepatectomy (right or left) or segmentectomy. The “larger” resections (> 3 segments) are the right hepatectomy involving liver parenchyma on the right side of the main portal scissura (segments V, VI, VII and VIII), the left hepatectomy involving liver parenchyma on the left side of the main portal scissura (segments II, III, IV) and the trisegmentectomy characterised by resection of three segments (IV-V-VI or VI-VII-VIII). [31]

Atypical resections are carried out without considering the liver segmental anatomy; they are usually resections of small peripheral or superficial liver lesions which are not close to large vascular or biliary structures. Hepatectomies involving the removal of a higher number of segments compared to a major hepatectomy are called “enlarged”: right hepatectomy enlarged to segment IV, left hepatectomy enlarged to segment V and/or segment VI, and left hepatectomy superenlarged to segments I, V and VIII. [31]

The surgical treatment is curative when all liver lesions are removed. However in some cases despite removing all metastases, histological analysis show that resection margins have neoplastic cells: in that case they speak about microscopic residual disease (R1). The five-year survival rate is 40% after a radical surgical treatment. [38].

Two-stage hepatectomy is a surgical procedure used when there are resectable bilateral liver metastases but involving a percentage > 80% of the liver or >70% of the parenchyma in case the patient underwent a neoadjuvant chemotherapy. This operation consists of the resection of the liver metastases in two phases. In a first operation, atipycal resections or segmentectomies of one of the two sectors (right or left) are performed, and after an interval during which the residual liver regenerates, the remaining lesions in the contralateral lobe are removed. Two-stage hepatectomy is often associated with portal vein embolisation of the treated lobe in order to cause the hypertrophy of the contralateral lobe during 4 weeks thus increasing the parenchymal volume to about 20-40%. A chemotherapy cycle is carried out to control the tumour growth between the two operations. However the neoadjuvant chemotherapy can reduce efficacy and efficiency of the portal vein embolisation; moreover it can cause a steatosis or a non-alcoholic steatohepatatis (NASH) in a large number of patients.[9]

Port vein embolisation (PVE) is being increasingly used in the preoperative treatment of patients selected for a larger liver resection (>3 segments). It causes a selective hypertrophy of the healthy portion of liver in patients suffering from hepatic neoplasia making people, who previously were not suitable because of their too little remnant healthy liver, possible candidates for operation.

Contraindications to this procedure are: diffuse extrahepatic metastases or periportal lymphadenopathy, diffuse intrahepatic metastatization, severe coagulopathy, tumour invasion of the portal vein, biliary dilatation, portal hypertension and kidney failure. [39]

Mortality related to liver resections on non-cirrhotic livers is calculated around 1%. [8] The most frequent complications are: pleural effusion (occurring in 30% of the cases), hepatic or perihepatic abscess (25%), temporary hepatic deficiency (19%), ascites (10%), hemoperitoneum (10%) and biliary fistula (6%). [40]. Other less frequent complications are paralytic ileus and infection of the laparotomy. [41].

Chemotherapy

Chemotherapy (CT) can be used as a neoadjuvant or adjuvant treatment.

Neoadjuvant chemotherapy is used before a possible surgical treatment to make liver metastases, which were initially deemed inoperable, resectable and to have a higher success rate in terms of surgical curative operations; moreover it tests the chemoresponsiveness of the tumour in view of other chemotherapy curative treatments, it eliminates the micrometastatic disease and it enables to perform more limited resections in case of a complete response: [42] in case of a positive response to chemotherapy there is a change to resectability in 15/20% of cases. [43-46] In patients with disease progression during the neoadjuvant treatment the 5-year survival rate (8%) is worse than in patients with objective response (37%).

However neoadjuvant chemotherapy also has a negative side, among other things: a likely liver damage and consequently risk of affecting the surgical treatment; presence of metastatic sites that are not visible with imaging thus making it not possible to operate patients that were initially considered resectable because of visible metastases. [45;47]

Adjuvant chemotherapy is a successive treatment following a surgical treatment considered to be radical. It should theoretically work on the occult or dormant tumour cells that are still in the liver after resection, thus increasing the survival rate of patients suffering from colorectal liver metastases. [46] In a retrospective study, Parks et al. stated that adjuvant chemotherapy should be used in all cases of curative liver resection. [48]

New therapy protocols provide for the use of chemotherapy agents such as oxaliplatin and irinotecan that may be combined with biological agents.

Irinotecal inhibits topoisomerase I and can be given together with 5-FU and leucovorin under the name of FOLFIRI.

Oxaliplatin is a platinum-based agent combined with 5-FU and leucovorin: this triple therapy is called FOLFOX.

These two therapies have enabled to increase the survival rate by 20/30% if compared to the old protocols which used only leucovorin and 5-FU.

Combining neoadjuvant and adjuvant chemotherapy based on FOLFOX4 (perioperative treatment) for a limited time (3 months + 3 months) has enabled to

increase the disease-free survival reducing the risk of disease progression to ¼. [46; 49-51]

Recently traditional chemotherapy protocols have been increasingly matched with monoclonal antibodies (Cetuximab, Bevacizumab, Panituzumab) which competitively inhibit growth factors or their receptors. Those combinations enable to reach a survival rate of 45% (24 months) instead of 35% only considering therapy with FOLFIRI [52-54], and enable to increase the disease-free survival thus decreasing the number of recurrence. [55] Side effects of this therapy may include the risk of perforating the organ, bleeding and decreased cicatrisation of the wounds. [54]

Loco-regional treatments

The use of a catheter in the hepatic artery connected to a subcutaneous reservoir has made it possible to perform a loco-regional therapy. Floxuridine (FUDR), derived from 5-fluorouracil and composed of 5-FU and F-deoxiribonucleoside, has shown a significant improvement in the recurrence-free interval because it is easy to use and it has pharmacokinetic advantages (high doses – low side effects), but not in the survival rate.[50; 56] Together with surgical resection it has enabled to have a 4-year disease-free survival of 46% compared to 25% obtained by only using a surgical operation, and the survival rate has increased from 50 to 68 months. The use of an approach combining loco-regional therapy and systemic chemotherapy is justified by the higher control also on the extrahepatic disease and by the efficacy in making metastases, which were previously considered non-resectable, into resectable. [49;51]

Transarterial Chemoembolisation (**TACE**) is characterised by administration of chemotherapy together with biodegradable embolizing substances inside the hepatic artery: chemotherapy is taken directly to the metastasis through the arterial vase combined with an embolizing substance stimulating the necrosis of the metastasis. The most widely accepted TACE therapy includes the use of irinotecan in water-in-oil emulsion together with gelatin sponge. [57] Martin et al. report that three months after the administration of microspheres associated to irinotecan (DEBIRI) the response rate is 75%, and after 6 months is 66%. [58]

Side effects of this procedure are: nausea, vomiting, pain in the right hypochondrium reaching the shoulder, alopecia, asthenia without significant blood toxicity. [59]

RFA is relatively easy to use as a thermoablative treatment: it uses alternating electric current inside the tumoural tissue taking advantage of high temperature (>50° C), leading to a coagulative necrosis of the ill parenchyma.

Anatomic localization of metastases is a limit to the use of RFA. The survival rate one year after the treatment is 92.3%, after two years it is 46.2%, recurrence rate after one year is 55%.[60]

Complications connected with this method are fever, abdominal pain, leukocytosis, and serous increase of transaminase. [13]

Microwaves (**MW**) are a type of electromagnetic radiation at high frequency (900 MHz- 2.45 GHz) that produces heat by exciting water molecules. The consequent thermal insult leads to the coagulative necrosis and the tumour ablation. This technique is mainly used for the ablation of single liver metastases of colorectal origin in patients that cannot be selected for surgical operation or it is performed intraoperatively instead of radiofrequency.[61] Tanaka et al. showed that microwaves together with adjuvant chemotherapy lead to a survival rate of 56% after one year and 39% after 3 years. [62;63]

Isolated hepatic perfusion (**IHP**) is a loco-regional treatment which isolates the blood vessels supplying the liver in order to separate completely the hepatic circulation from the rest of the body. For this reason two extracorporeal circulations must be prepared, one to guarantee the integrity of the systemic circulation, and one to enable the perfusion of the liver, they are connected to a centrifugal pump (guaranteeing the systemic circulation) and to a peristaltic pump (enabling the liver perfusion). The following drugs are used: Melphalan (alkyliting) together with TNF, which increases the quantity of Melphalan going inside the cell. The perfusion lasts from 40 minutes to one hour. The temperature of the perfusate is between 41-41.5°C.

This method enables to use high concentration of drugs (about 70-80 times) without resulting into the systemic toxicity that would be found if the same doses

were given intravenously or intra-arterially. Together with hyperthermia it enhances the efficacy of the drug in the neoplastic tissue.

However this technique has its complications: systemic or hepatic toxicity (to doses higher than 1.5 mg/kg), post-operative hemorrhage and mortality risk (6% vs 2% systemic chemotherapy). [64]

In our Institute the results of this method are promising: the response was complete or partial in 60% of the patients suffering from liver metastases with colorectal origin. [65].

The **SIRT** (Selective Internal Radiotherapy Therapy) technique uses radioactive spheres that are introduced into the circulation supplying the hepatic parenchyma. Before starting the treatment an angiography must be performed to guarantee that the arterial tree is adequate for the procedure, then Technetium^{99} macroaggregated albumin is injected into the hepatic artery to determine the shunt grade between liver and lungs or the gastrointestinal track. An arterial shunt $\geq$ 20 % is a contraindication to the procedure because of the risk of radiation pneumonia or gastroduodenal ulcer. Other complications are: abdominal pain, fever, lethargy, fatigue and increase of transaminase.

Combining selective internal radiotherapy with chemotherapy (fluorouracil and leucovorin) has enabled to downstage the disease increasing the average survival rates to 11.5 months (from 4.6), with a two-year survival rate of 50%, 6% after five years, and an objective response rate of 37% (response to the sole use of CT 14%). [66]

1.7. Clinical and biomolecular markers of recurrence

Clinical Risk Score

According to data found in literature, at least two thirds of the patients has a recurrence, in particular at hepatic and pulmonary level. [67] The recurrence rate after resection is 60-90% [68]: 75% of recurrence occurs within the first 2 years after the surgical resection of the liver, and approximately 90% occurs within the third year. Prognostic factors that may indicate recurrence are: involvement of lymph nodes into the primitive tumour, CEA level, disease-free survival (DFS),

number of liver metastases and their maximum diameter, having an extrahepatic disease or not, evaluation of disease-free margins. A resection margin involved in the disease is associated with a worse survival rate, and a disease-free survival is a good measure for the biological aggressiveness of the tumour and a predictive factor of the outcome after the liver resection. [43]

Aiming to predict the prognosis of patients suffering from liver metastases with colorectal origin when it is possible to perform surgery, and to categorize patients according to risk, various (at least 5) systems have been developed by different authors selecting prognostic factors for recurrence risk (Clinical Risk Scores), but none of these has been validated because the parameters applied are not applicable in all cases. [15-19] An ideal scoring system has not been developed yet.

Biomolecular markers

The need to find a metastatic disease early in patients suffering from colorectal carcinoma has led to look for adequate markers that are potentially predictive for the disease in the clinical practice. They are divided into biological markers and molecular markers.

Among the biological markers there is the carcinoembryonic antigen **CEA**, isolated in 1965 for the first time from gastrointestinal carcinoma. At present, it is used in the follow-up of patients suffering from colorectal carcinoma who underwent a surgical treatment. Various studies have shown that high preoperative CEA levels are independently associated with a higher probability of recurrence and worse prognosis. Recently ASCO guidelines recommend to measure CEA every 3 months together with a thoracic-abdominal CAT scan and in case a pelvic CAT every year in the first 3 years for patients at high risk of having metastatic disease [69]. In particular, CEA should be first measured at the beginning of a possible chemotherapy treatment and then every 1-3 months. The increase of figures seems to suggest that the disease is progressing even when the imaging does not show it. However an increase of the marker could be caused by the administration of drugs (oxaliplatin). Other non-neoplastic causes of an increase of CEA values are gastropathies, peptic ulcer, diverticulitis, liver pathologies, chronic obstructive pulmonary disease, diabetes and acute and

chronic inflammations. The College of American Pathologists stated that preoperative CEA levels > 5ng/ml could indicate a worse prognosis. However only 37.5% of patients with high preoperative CEA level develop recurrence, and 75% of patients with normal values have had the disease again, with a positive predictive value of 22% [12; 70]. As far as the postoperative stage is concerned, various studies have shown that continuously high levels of CEA are very powerful indicators of future disease recurrence. In particular, high levels of CEA after the operation have a 50% sensitivity and 90% specificity in predicting recurrence, 70% positive predictive value. More specifically, some studies have shown that abnormal CEA levels in the bile and in the portal circulatory system have a higher sensitivity in identifying patients at high risk of recurrence compared to the levels found in the serum. It is however important to underline that even though high levels of the antigen are associated with a higher risk for the patient, they still have a limited role in predicting exactly who is going to develop a metastatic disease in the liver or elsewhere.

Perioperative values of alkaline phosphatase and lactate dehydrogenase can also be taken into consideration: their increase compared to the basal value is considered to be among the most significant negative prognostic factors, especially if associated with the high levels of serum bilirubin or low levels of albumin.

As far as molecular markers are concerned, mainly microsatellite instabilities are taken into consideration; mutations of BRAF and KRAF, expression levels of VEGF and EGFR, methylated DNA, the mutation of mitochondrial DNA and circulating cells.

Microsatellite instability **MSI** is associated with Lynch syndrome (HNPCC) and patients with high instability (MSI-H) have a fenotype characterised by primitive tumour on the right colon and diagnosis is performed at a relatively early stage [29]. Alterations of microsatellites including loss of heterozygosis (LOH) were found both in plasma and serum samples, and they can be considered prognostic factors both singularly and in combination with gene mutations or hypermethylation of DNA.

KRAS is a proto oncogene involved in the down regulation of different cellular processes.
Mutations of that gene have been found in 40% of the cases of colorectal carcinoma and for this reason they are the most frequent in this kind of carcinoma. Recently, it has been underlined how fundamental the mutation of KRAS is in the tumour response to Cetuximab (EGFR inhibitor), and how it is at the basis of an early development of hepatic recurrence and of a worse survival rate.[29]

The **BRAF** gene codifies protein serine/threonine kinase in the pathway RAS-MEK-ERK. Mutations of BRAF and KRAS are considered mutually exclusive in the development of tumours, and different studies have suggested that if mutated BRAF is involved in the development of liver metastases, it is associated with a lower risk of developing them. [29]

VEGF is a protein that is involved in angiogenesis, and it plays a fundamental role in the tumour growth and in the development of metastases. [29] Expression levels of VEGF can be useful in predicting which tumours are more likely to develop into liver metastases: Takeda et al. demonstrated that serum levels of VEGF in patients suffering from CRC indicate the real development of liver metastases. [71]

EGFR receptor: protein c-erbB-2 (Her/neu), a receptor of the EGFR class, has been taken into consideration, it has been shown that primitive tumours with liver metastases have a high expression of that protein. Moreover some additional studies suggest that it could play a role in predicting liver metastases in patients with negative lymph nodes who could therefore wrongly be considered patients at low risk. [29]

TGF-α, IGF-II and matrix **metalloprotease** are significantly increased in patients with liver metastases and their attendant overexpression predicts the risk of disease with a percentage of 99.5%.

Even the low expression of **Smad4** and the **Ki-67**-positivity are associated with an increased likelihood to develop the metastatic disease; more specifically, the normal expression of Smad4 and the Ki-67-negativity have a negative predictive value of 100%. [29]
The combination of the expressions of **disaderin, matricillin and E-cadherin** is highly associated with the development of liver metastases with 85.7% sensitivity and 58.9% specificity, thus accounting for a positive prognostic value of 25% and a negative prognostic value of 96%. [29]

Several studies have reported to have found **methylated DNA** in the serum/plasma and other body fluids in patients with different neoplastic types and not to have found it in the healthy controls. [72]. This characteristic makes it possible to develop new tests that can categorize risk in neoplastic patients. First of all, the hypermethylated genes in the primitive tumour must be identified, and only then they can be looked for in the serum or plasma samples. Consequently, if mutilation changes occur (yes/no), we will be able to evaluate their validity in term of predictivity and prognosis.
In particular, a study has shown a correlation with the methilation of p16 Dukes C patients, suggesting that methilation of that gene may be a possible prognostic marker. [73]

Mutated mitochondrial DNA: every cell contains several hundreds of mitochondrial DNA copies that codify the sub-unities of the respiratory chain, for tRNA and rRNA.
Several mutations of mitochondrial DNA (mtDNA) have been described in patients suffering from colorectal carcinoma, bladder carcinoma, lung carcinoma, and recently even in patients with prostate carcinoma and hepatocarcinoma. Therefore this suggests that mtDNA may be highly diluted in the plasma of neoplastic patients. [73]
Circulating tumour cells: looking for tumour cells circulating in the blood to find micrometastases may predict which patients may develop metastatic disease. Characterizing circulating tumour cell at molecular level is called “real-time

tumour biopsy". If this technique were really applicable, there would be a reduction in the influence of the toxic effects of useless therapies. The presence of circulating cells of possible tumour origin has a predictive value of 66% associated also with other parameters, such as the depth of the invasion and the lymphovascular involvement, but it is not useful to predict possible metachronous liver metastases. [70]

Most of the prognostic markers listed so far have not been validated in clinical trials, however some of them are often used in clinical practice to influence the choice of therapy. [29]

2. CIRCULATING DNA

2.1. Tumour markers and circulating DNA

In the last few years, a lot of detailed research on tumour markers that can actively correlate with the neoplastic disease activity has been increasing, and several studies of molecular biology have shown that is possible to use circulating DNA as an efficient prognostic marker for cancer.

Free circulating tumour-associated DNA has been found in serum and plasma of patients suffering from different types of cancer, such as of the pancreas, colorectal, of the head and neck, esophageal, of the lungs, the kidneys, the liver, the breast, and melanoma[21], and it is a promising biomarker for cancer diagnosis and prognosis. The final proof that tumour DNA is released in the circulation was given by Sorenson et al. and by Vasioukhin et al. who demonstrated that there is circulating DNA of neoplastic origin in patients suffering from cancer of the pancreas and in patients suffering from myelodysplastic syndrome and acute myelogenous leukemia respectively. It has also been shown that in healthy subjects the median concentration of circulating plasma DNA is about 14-18 ng/ml, whereas in patients with different types of neoplasm the concentration increases to 180-318 ng/ml. [74]

In healthy subjects the circulating DNA is mainly produced by apoptic processes that release fragments of the nucleic acid of uniform length from 185 to 200 bp,

as the result of programmed cleavage. There can be several other sources of circulating DNA, such as inflammatory processes, pregnancy, fracture, attendant pathologies, previous chemotherapy, trauma, autoimmune diseases as SLE and DM, etc… [75] How circulating DNA in healthy subjects enter the circulation and how precisely it originates has not been completely explained, but in literature two possible sources of circulating endogenous DNA have been discussed: cells that are facing death, both for apoptosis or necrosis, and the DNA that is actively secreted by circulating cells.

On the contrary, the DNA released from malignant cells varies in size because the pathological cell death is the result of various processes that can be traced back to apoptosis, necrosis, autophagia, mitotic catastrophe, or the presence of viral genes [64], in all these situations the fragments are not uniformly chunked, but they are longer. [23; 25] Tumour necrosis is a frequent event in solid malignant neoplasm, it generates a range of DNA fragments of different lengths because of the random and incomplete digestion of genomic DNA by a wide variety of deoxyribonuclease. In order to validate this hypothesis, Wang et al. performed a study where the integrity of the DNA strand was tested by using a real-time PCR on a total of 126 samples of plasma from neoplastic and non-neoplastic patients, showing that the increased value of the integrity index in the plasma is associated with cancer, and measuring that value could be an easy and cheap way to test whether there is cancer or not.[24]

Which is the source of all the circulating DNA is still an unsolved enigma. There are several theories about the possible origin of those fragments: in the healthy controls, for example, they may derive from lymphocytes or other nucleated cells; in a neoplastic patient, on the contrary, it is now widely accepted that tumour cells are the main source of a great portion of circulating DNA. [22]

The most popular theory, according to which tumour-specific circulating DNA derives from the lysis of neoplastic cells or from the micrometastases, has revealed to be wrong, because there are not enough circulating cells to justify the quantity of DNA found in the blood flow.[74]

On the contrary, it would seem that it is released from the tumour necrosis or through active release mechanisms.

An alternative hypothesis claims that the altered circulating DNA itself can cause the development de novo of tumour cells in organs that usually host metastases. It is supported by the fact that experiments on animals have shown that a horizontal transfer of circulating DNA in tissues has a malignant transformation power. This mechanism has been called *genometastasis.* [74]

As far as the best source of nucleic acid is concerned, Umetani et al. state that serum must be considered a better resource of circulating DNA than plasma, because it is less likely contaminated by foreign DNA released by leukocyte, for example. [76]

On the contrary, Wang and Lecomte et al. used plasma samples as starting point to extract circulating DNA in association with colorectal carcinoma. [21;24] As far as the quantification of circulating DNA is concerned, Wang suggests to use the ratio between longer fragments of DNA (of tumour origin) and shorter fragments of DNA (total DNA), defined integrity index. Increase of that value indicates the presence of neoplastic cells. In particular, if the ratio between the circulating DNA of tumour origin and the total tends to 0, DNA tends to be mainly of apoptic origin, on the contrary, if it tends to 1, then it is probably of non apoptic origin (tumour origin) [24].

2.2. Circulating DNA and colorectal carcinoma

Lecomte et al. demonstrated that circulating DNA of tumour origin in patients suffering from colorectal carcinoma is an indicator of bad prognosis with a significant reduction of the overall survival rate and of the disease-free survival, whereas there was an increase of the survival rate in patients without circulating tumour DNA in the plasma before the operation. [21] Circulating DNA can therefore have a prognostic value in patients suffering from colorectal carcinoma, and at stages I,II,III, it is associated with a lower survival rate and with a likely disease recurrence. [21] In a univariate analysis, Yamada et al. [77] showed how circulating DNA of tumour origin correlates with disease-free survival; moreover based on follow-up data, they have correlated the neoplastic response with the negativisation of the abnormal quantity of circulating DNA, and the disease progression with the persistence of plasma alterations.

Somatic, genetic or epigenetic alterations in tumours are potential targets of the molecular research on plasma. The most commonly used target has been K-RAS2, and in the study of Lecomte 45% of the patients which showed that alteration in the primitive tumour had the same alteration in the plasmatic DNA. However to be more clinically relevant it is necessary to look for other genetic alterations to increase the number of neoplastic types that can be screened with the method of circulating DNA. [21]

Three alterations could become interesting in the study of colorectal carcinoma: p53 mutation, alteration of microsatellites (loss of heterozygosis or instability), epigenetic modifications as p16 hypermetilation.

Unfortunately, at the moment there are too few available data to be able to clearly determine the prognostic value of circulating plasma DNA of patients suffering from neoplasia and to be able to confirm the preliminary results achieved so far.

2.3.Methods of extraction and quantification of circulating DNA

As far as the best source of nucleic acid is concerned, Umetani et al. maintain that serum must be considered a better resource of circulating DNA than plasma because it is less likely contaminated by foreign DNA released by leukocytes, for example. [26]

On the contrary, Wang and Lecomte et al. used plasma samples as starting point to extract circulating DNA in association with colorectal carcinoma. [21;25] In order to quantify circulating DNA, Wang suggests to use the ratio between longer fragments of DNA (of tumour origin) and shorter fragments of DNA (total DNA), defined integrity index. The increase in that value of the plasma indicates that there is a carcinoma being the index of death of neoplastic cells. [24]

In order to measure the integrity of free DNA circulating in serum, Umetani et al. developed a highly sensitive technique without the need to purify DNA using qRT- PCR, taking advantage of ALU repetitions interspersed in genome and using an ad hoc probe to detect the fluorescence. [26]

ALUs (Arithmetic Logic Unit) are among the most frequent sequences repeated inside the human genome, with a number of copies of about 1,4 x 10 ^6, they

typically consist of 300 nucleotides and account for more than 10% of the genome. [25]

On the basis of this peculiar characteristic, investigating them aims to quantify circulating DNA in plasma and to discriminate DNA of tumour origin from the total DNA of the sample. Since in healthy subjects circulating DNA is mainly produced by apoptic processes releasing fragments of uniform length between 185 and 200 bp, in order to identify DNA of tumour origin you only need to choose ALU that are longer than 200 bp, so that you can be completely sure to have a sequence of neoplastic origin. [24;25]

qRT-PCR

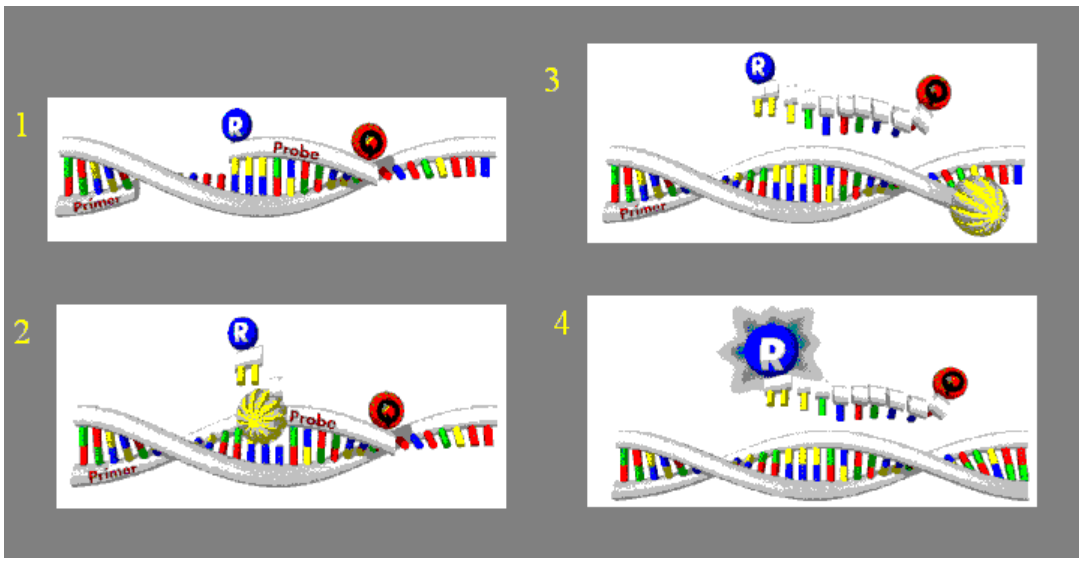
The quantitative polymerase chain reaction in real-time (qRT-PCR) monitors the amount of DNA products using a group of new fluorescent reagents [78-80] that bind the amplification product. The fluorescence intensity produced during the process shows the concentration of amplicon in real time.

The repetition of the denaturation step (at 95°C for 5'), strand dissociation (at 95°C for 15') annealing and extension (at 62°C for 1') for a *n* of cycles produce a DNA quantity that grows exponentially every cycle.

The TaqMan probe consists of two types of fluorophores: the quencher (Q) fluorophore at the 3' end of the probe and reporter (R) fluorophore at the 5'end. When the probe is attached to the template DNA and before the polymerase acts, the quencher fluorophore reduces the fluorescence from the reporter fluorophore.

After DNA denaturation, the TaqMan probe binds its specific piece of the template DNA and the primers anneal to the DNA. When *Taq* polymerase removes the probe from the template DNA there is a separation of the quencher and the reporter, and the reporter begins to give off its energy which is then quantified using a computer [81]. (figure 1)**.**

Figure 1. TaqMan probe[81]



5' REPORTER (R): high energy fluorochrome emitting fluorescence
3' QUENCHER (Q): low energy fluorochrome quenching fluorescence

AIM OF THE STUDY

This study aims to verify whether the quantity of the circulating tumor DNA measured in the blood of patients before and/or after the liver resection can be a prognostic tool to quantify the risk of liver recurrence.

MATERIALS AND METHODS

Patients

For our study 26 patients, who underwent a liver resection because of colorectal metastasis, were analyzed between March 2009 and March 2011. Each patient provided written informed consent. For each patient age, timing of metastasis, number and diameter of liver metastases, affected percentage of liver, chemotherapy, type of surgery, resection margins, and ALU244, ALU83, ALU244/ALU83, CEA, VES, PCR values were measured before and after the surgical operation.

All patients underwent a staging with thoracic-abdominal CT scan, PET/CT before the surgical operation, and intraoperative ultrasonography. Free resection margins were obtained in 21 patients.

Out of 26 patients 7 were women (26.9%) and 19 were men (3.1%): the average age was 63.7 (range 47-79).

Liver metastases were synchronous in the primitive tumour in 12 patients (46.2%) and metachronous in the remnant 14 patients (53.8%). The average lesion diameter was 3.2 cm (range:1.5- 8 cm). In 12 patients (46.2%) metastases were bilobar, in the remnant 14 (53.4%) they were only limited to one lobe. 10 patients (38.5%) only had one metastasis, in 16 patients (61.5%) nodules were multiple (up to a maximum of 4).

18 patients (69.3%) underwent neoadjuvant chemotherapy according to the schedules FOLFIRI + Avastin, FOLFIRI, FOLFOX; the treatment finished at least one month before surgery. 3 patients (11.5%) underwent only adjuvant therapy, 2 patients (7.7%) underwent neoadjuvant and adjuvant therapy; 3 patients (11.5%) did not undergo any chemotherapy treatment (table I).

In our study we evaluate:

1. The prognostic role of preoperative ratio ALU244/ALU83 to identify patients at risk of liver recurrence. Patients included were 26.
2. The correlation between circulating tumor DNA pre- and postoperatively and the surgical radicality (R0-R2): patients were divided into 3 groups considering a disease free survival of 12 months and the surgical

radicality. Group 0 includes patients who had recurrence within 12 months from the curative operation R0 (5 patients), group 1 includes patients without recurrence or with liver recurrence > 12 months after radical surgery (10 patients), group 3 includes patients who underwent non-radical surgery R2 (5 patients).

6 patients were excluded from this analysis because their follow-up was too short (< 6 months).

Table I. Clinical-pathological features

	Number	**%**
Gender		
M	19	73.1%
F	7	26.9%
Timing		
Synchronous	12	46.2%
Metachronous	14	53.8%
Nodule		
Single	10	38.5%
Multiple	16	61.5%
Neoadjuvant CT		
Yes	18	69.2%
No	8	30.8%

Sample analysis

Blood samples were collected at regular and pre-defined intervals: the first sample on the day of surgery (Tf0), the second (Tf1) 30 days after surgery in the ward or during the visit. CEA and CA19.19, as well as VES and PCR were also measured. Within 1 hour, blood was centrifuged at 2800 X g for 15', the supernatant (serum) was aspirated with a Pasteur pipette at room temperature. At least 1 ml of serum

was distributed in 4 vials each, then they were frozen at -80°C. DNA extraction from serum was performed with the protocol *QIAamp UltraSens Virus kit* ®.
In order to have a higher sensitivity for the DNA quantification, this study used two types of ALU primers: **ALU83** identifying total DNA and **ALU 244** identifying tumour DNA. A TaqMan-like probe, marked Fam, was built; TAMRA was used as quencher (Table II).

Table II. DNA sequence ALU 83; ALU244; SONDA.

	DNA sequence
ALU83	CTGAGGTCAGGAGTTCGAGACC AAAATTAGCCGGGCGTGG
ALU244	GCGGTGGCTCACGCCTGTAA AGATCGCGCCACTGCACTCC
SONDA	CCTGGCCAACATGGTGAAACCCC

Amplification of samples (95°C for 5 minutes followed by 40 cycles with 2 steps: 95°C for 15 seconds and 62° for 1 minute) was performed with q RT- PCR *7300/7500 Real Time PCR Systems.* A software obtains a numeric value from the mass spectrum of each sample.

Statistical analysis

In order to find differences between preoperative and postoperative median values of ALU83, ALU244, ALU244/ALU83, CEA, the non-parametric test Kruskal-Wallis was used.
The following variables were taken into considerations in a univariate analysis: sex, age, ALU83 and ALU244 pre- and postoperatively, CEA pre- and postoperatively, ratio ALU244/ALU83 pre- and postoperatively. For the categorical variables the log rank test was used, whereas for the continuous variables the Cox regression was used. The values of the ratio ALU244/ALU83

were dichotomized according to the median for obtaining 2 balanced groups in terms of sample size.

The Kaplan- Meier method was used to draw the DFS and OS curves.

An alpha error lower than 5% was considered significant.

The software used was STATA/SE 11.1 (StataCorp LP. College Station, Texas, USA).

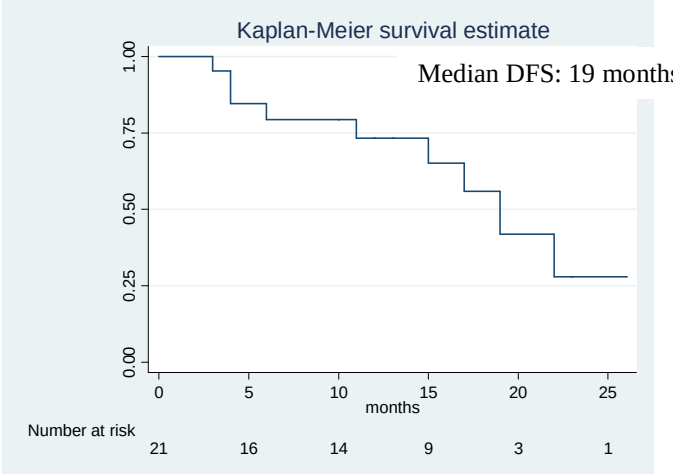
RESULTS

Survival analysis

In 21 patients had free disease margins (R0). In 5 cases the surgical treatment was not radical (R2).

The median disease-free survival was 19 months (Fig 2). The median follow up was 15 months (range 3-26 months).

Fig 2. Median DFS



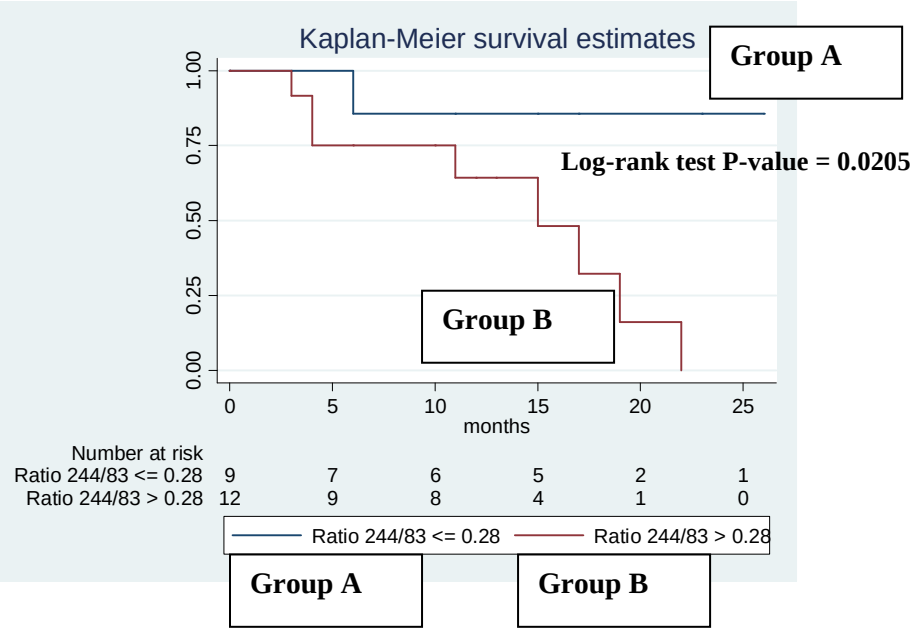
First we analyzed the relationship between the ratio ALU244/ALU83 in the patients before surgical treatment and the disease-free survival. Median ratio ALU244/ALU83 was 0.28 (0.0652-0.763); on the basis of that value we dichotomised patients into two groups:

Group A = ALU244/ALU83 ≤ 0,28

Group B =ALU244/ALU83 > 0,28.

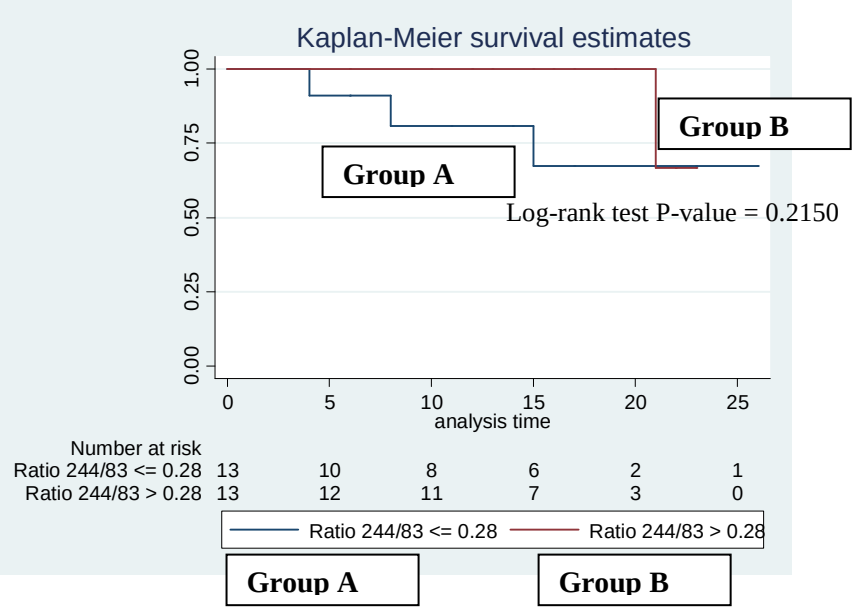
The risk to develop recurrence after surgery was 8 times more in Group B (ALU244/ALU83 >0,28) rathen then Group A patients (ALU244/ALU83 ≤ 0,28). (Hazard Ratio= 8.07, P-value= 0.0205). (Fig 3)

Fig 3. Disease-free survival according to the ratio ALU244/ALU83 before surgery



There was no correlation between survival and ratio ALU244/ALU83 before surgery. (P-value = 0.2150). (Fig 4)

Fig 4. Overall Suvival according to the ratio ALU244/ALU83 before surgery



On the other hand, when considering the 3 subgroups of patients according to R0 and disease-free survival (group 0= DFS < 12 months and R0; group 1= DFS > 12 months and R0; group 2= DFS 0 and R2) there were not significant differences in the median levels of circulating DNA (P- value: 0.3253).

the values of median, average and standard deviation for each analyzed parameter in the three different subgroups of patients are shown in tables III-VIII.

Table III. ALU83 Tf0: Serum values of circulating DNA (ALU83) before surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	1100000	1518642	1189174
1	10	511144.9	2948548	5859351
2	5	1380000	1491923	594908.6

P value: 0.3253

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Table IV. ALU83 Tf1: Serum values of circulating DNA (ALU83) after surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	807321	2288329	2822088
1	10	1750000	7442643	1.54e+07
2	5	1430000	2404899	2640713

P value: 0.6618

Table V. ALU244 Tf0: Serum values of circulating DNA ALU244 before surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	364494	649956	661808.4
1	10	156737.5	972616	2063360
2	5	220940	206595.4	60630.95

P value: 0.0840

Table VI. ALU244 Tf 1 : Serum values of circulating DNA ALU244 after surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	448499	1052180	1267710
1	10	434283.5	3163355	7994214
2	5	137291	451981.4	573991.3

P value: 0.4994

Table VII. Ratio ALU244/ALU83 Tf0: Serum values of circulating DNA ratio ALU244/ALU83 before surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	0.39	0.412	0.131
1	10	0.295	0.42	0.37
2	5	0.15	0.166	0.100

P value: 0.0755

Table VIII. Ratio ALU244/ALU83 Tf1: Serum values of circulating DNA ratio ALU244/ALU83 after surgery

Group	No. Pt	Median	Average	St. Dev.
0	5	0.44	0.432	0.125
1	10	0.33	0.391	0.347
2	5	0.19	0.216	0.184

P value:0.1333

DISCUSSION

The surgical resection is still the gold standard treatment in patients with colorectal liver metastases, achieving a 5 year survival rate of 40-58% [3-9]. Unfortunately only 20-35% of patients are resectable at diagnosis. The recurrence rate after resection is up to 60-70%[5;8;10-14]. These data underline the importance of markers that can accurately predict the risk of recurrence.

There are at least 5 “Clinical Risk Scores” taking into account different clinical parameters as: patient’s age, Grading and TNM stage of the primary tumour, maximum diameter of neoplasia, number and diffusion (uni/bilobar) of liver metastases, CEA level, extrahepatic disease, DFS (time considered from the primitive tumour to the finding of liver metastases). Unfortunately none of those “Clinical Risk Score” has been validated in clinical practice. [15-19]

Among all the biomarkers proposed in literature, CEA is the only one that is currently used as parameter to evaluate the risk of recurrence in patients previously treated for a colorectal neoplasia.

Data from literature show that both high preoperative levels of CEA (CEA > 5 ng/ml) and an increase of this marker in the follow-up do not often correlate with the real state of the disease. [69] Therefore the need for a prognostic marker has increasingly grown, a marker that can correlate with the tumor recurrence: in the last few years the analysis of circulating tumour DNA in the peripheral blood origin has been emerged as a promising tool to measure the risk of tumour recurrence.

Recent studies have demonstrated that in the plasma or serum of patients with different types of solid tumours there are high levels of circulating tumour DNA, that can therefore become a prognostic biomarker for the disease recurrence[20-26]. Circulating DNA is mainly produced by apoptic processes releasing fragments of the nucleic acid of uniform size, from 185 to 200 bp, as result of planned cleavage processes.

However, has been found also in healthy subjects, with a concentration of about 14-18 ng/ml [74]: there are other sources of free circulating DNA such as inflammatory processes, pregnancy, attendant pathologies, previous chemotherapy, trauma, autoimmune diseases as SLE and DM. [24].

On the other hand, it has been demonstrated that solid tumours release DNA fragments (average concentration of 180-318 ng/ml) larger that the DNA that can be found in healthy subjects. Actually the pathological death is the result of several processes that can be traced back to necrosis, autophagia, mitotic catastrophe, or the presence of viral genes [24] and the fragments deriving from them are not uniformly chunked. [23;25] The lymphovascular invasion enable the entrance of neoplastic DNA into circulation because the blood or lymphatic flow makes it easier to disseminate the circulating cells in the blood flow through the tumour.

The circulating DNA could therefore be directly correlated with cancer progression and with cell turnover, as a marker of the biological aggressiveness of the neoplasia [21;23;24].

In order to quantify the circulating DNA in the blood, it is necessary to define the better substrate to use for the extraction. Umetani et al. [23] demonstrated that serum contains 6 times more DNA than plasma, and therefore stated that serum is the best way to quantify free circulating DNA. To discriminate the DNA of tumour origin from the total DNA of the sample, was proposed the analysis of the ALU sequences: they are the most numerous sequences repeated inside the human genome [25].

In our study we used ALU244 to identify DNA fragments of tumour origin and ALU83 to quantify the total circulating DNA. The ratio between ALU244/ALU83 gives a more precise estimate of the circulating DNA of tumor in the patient's serum. Our results have shown that patients whose preoperative circulating tumour DNA was higher than 0.28 had a significantly shorter disease-free survival than patients whose circulating tumour DNA was lower or equal than 0.28. Specially, the first group of patients had a risk of recurrence in the first year after surgery 8 time higher (Hazard Ratio = 8.07, p value = 0.0205).

Our results are similar to other authors that demonstrated worse survival rate and a significant reduction of the disease-free survival in patients with solid tumours that have a significant quantity of free circulating DNA of neoplastic origin.[20-25]

To better analyze the impact of the levels of circulating tumour DNA on survival, we have been focused on the possible correlation between ratio ALU244/ALU83, disease-free survival and surgical radicality: unfortunately we didn't find a statistically correlation. This may be due to the small sample size and the short period of follow-up.

CONCLUSIONS

Liver metastases of colorectal cancer are the main cause of death in those patients. Even after curative liver resection, the percentage of hepatic recurrence is still very high. At present there are no clinical markers or molecular biomarkers that can identify patients at high risk of recurrence. Recently there has been proposed the analysis of circulating tumour DNA in the early diagnosis of recurrence in several types of solid tumours.

Confirming the experimental studies in literature, the data of our study have demonstrated that the value of the preoperative ratio ALU244/ALU83 may play a significant prognostic role in predicting which patients are potentially at high risk of hepatic recurrence after curative resection for liver metastases of colorectal origin. The lack of statistically significant correlation between the amount of circulating neoplastic DNA, the duration of the disease-free survival and the radicality of the operation is due to the limited number of the analyzed sample and to the short follow up period.

It will therefore be necessary to implement and increase the case record both by recruiting new patients and by increasing the follow-up of patients already included in the study, to confirm the preliminary results achieved.

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