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LIVER AND PANCREATIC CANCERS: THE ROLE OF METABOLIC SYNDROME AND NOVEL AVENUES FOR TREATMENT

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INTRODUCTION TO THE PhD THESIS

Cancer is a major public health problem worldwide being the second leading cause of death in Western Countries.

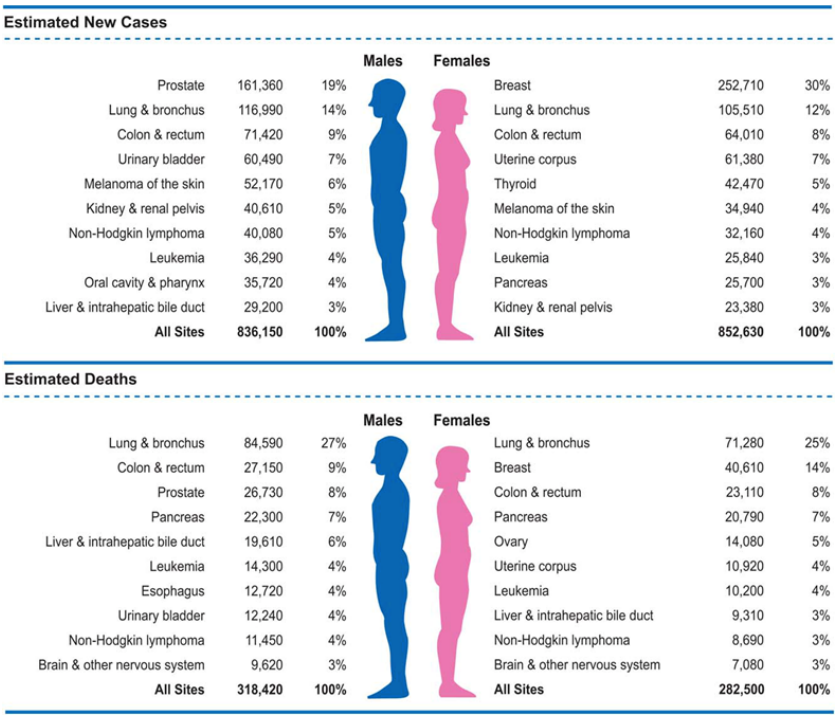
In the last decades, the identification of predisposing cancerogenic factors, both genetic and environmental, has significantly changed the incidence of several cancers.

Important examples are lung and colon cancer. In fact, as it is well reported in literature, lung cancer is strictly related to tobacco habit. The incidence of lung tumors from 1975 to 2013 showed a slight decrease in males while showed an increased incidence in females. This trend is the direct consequence of the decreasing and increasing of the amount of smokers in males and females respectively. At the same time, the knowledge of certain forms of precancerosis has enabled the development and the implementation of large-scale screening programs that have led to a decrease in incidence of several cancers. Examples are represented by the introduction of endoscopic screening programs for colon cancer in both sex and by the institution of Pap test for cervical cancer in women. Moreover, in the last decades important successes have been achieved in terms of mortality outcome for several tumors. Such improvements are due to the progress of both chemotherapy treatment and surgical technique.

Unfortunately, this promising and cheering trend has not been achieved for all tumors.

For example, in the last 25 years there has been a gradual increase in the

incidence of liver and pancreatic cancers. Currently, liver and pancreatic cancers are on the list of the 10 leading cancers with an incidence around 29.200 and 25.700 new cases respectively. (Fig. 1)



Estimated New Cases

Males			Females		
Prostate	161,360	19%	Breast	252,710	30%
Lung & bronchus	116,990	14%	Lung & bronchus	105,510	12%
Colon & rectum	71,420	9%	Colon & rectum	64,010	8%
Urinary bladder	60,490	7%	Uterine corpus	61,380	7%
Melanoma of the skin	52,170	6%	Thyroid	42,470	5%
Kidney & renal pelvis	40,610	5%	Melanoma of the skin	34,940	4%
Non-Hodgkin lymphoma	40,080	5%	Non-Hodgkin lymphoma	32,160	4%
Leukemia	36,290	4%	Leukemia	25,840	3%
Oral cavity & pharynx	35,720	4%	Pancreas	25,700	3%
Liver & intrahepatic bile duct	29,200	3%	Kidney & renal pelvis	23,380	3%
All Sites	**836,150**	**100%**	**All Sites**	**852,630**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	84,590	27%	Lung & bronchus	71,280	25%
Colon & rectum	27,150	9%	Breast	40,610	14%
Prostate	26,730	8%	Colon & rectum	23,110	8%
Pancreas	22,300	7%	Pancreas	20,790	7%
Liver & intrahepatic bile duct	19,610	6%	Ovary	14,080	5%
Leukemia	14,300	4%	Uterine corpus	10,920	4%
Esophagus	12,720	4%	Leukemia	10,200	4%
Urinary bladder	12,240	4%	Liver & intrahepatic bile duct	9,310	3%
Non-Hodgkin lymphoma	11,450	4%	Non-Hodgkin lymphoma	8,690	3%
Brain & other nervous system	9,620	3%	Brain & other nervous system	7,080	3%
All Sites	**318,420**	**100%**	**All Sites**	**282,500**	**100%**

Figure 1. Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2017.

Moreover the incidence rates continue to increase very fast for liver cancer, by around 3% per year in females and 4% per year in males, although rates have begun to decline in adults aged younger than 50 years. (1)

Also from a survival point of view, in contrast to the constant increase in survival for most cancers, advances have been very poor for pancreatic cancer, for which the 5-year survival is currently 8%, (Fig. 2).

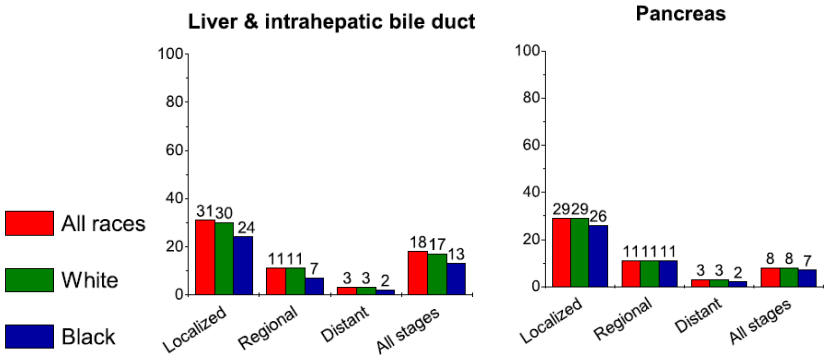


Figure 2. Five-Year Relative Survival Rates by Stage at Diagnosis and Race, United States, 2006 to 2012. The standard error of the survival rate is between 5 and 10 percentage points.

These low rates are in part because more than one-half of cases are detected at advanced stages (Fig. 3).

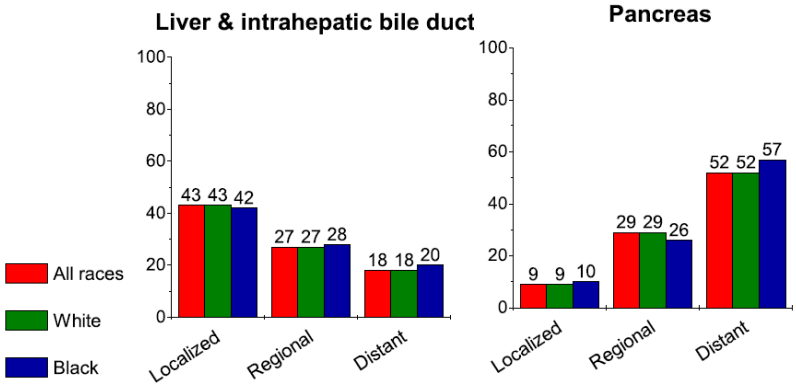


Figure 3. Stage Distribution by Race, United States, 2006 to 2012.

Stage categories do not sum to 100% because sufficient information is not available to stage all cases

Pancreatic cancer death rates continued to increase (by 0.3% per year) in males but have settled down in females and the 5-year survival is 4% and 3%, respectively. Similar data are reported also for liver cancer.

Therefore, both liver and pancreatic cancers are diseases for which risk factors and pathogenesis still remain not fully understood being areas of oncology warranting further investigation into their epidemiology. In addition, also the available therapeutic strategies are not enough to improve

survival rate in patients affected by liver and pancreatic cancers.

From an epidemiological point of view in the last years, metabolic syndrome has started to be considered one of the risk factors for the developing of both liver and pancreatic tumors.

Along this thesis, the role of metabolic syndrome in liver and pancreas tumors will be discussed.

This thesis is divided in four different sections.

The first section is the description the current knowledge regarding liver and pancreatic cancers their relation with the metabolic syndrome.

The second section describes the experimental activity carried out at Careggi University in Florence, Italy, under the supervision of Professor A. Arcangeli. During this experience, a highest rate of growth in HCC in diabetic rats was showed by fluorescence technique.

The third section describes a novel technique and type of central vein catheter. The advantages of this system are described in a paper currently under review. Some extracts of this paper are reported in this section.

In the fourth section, research data regarding the use of the laparoscopic approach in pancreatic cancer are reported. Laparoscopy as a less invasive technique has shown some advantages especially in patients with metabolic syndrome. These data were published in collaboration with the Mayo Clinic of Jacksonville (FL, United States) under the supervision of Prof. H.J. Asbun.

SECTION 1

Introduction to Section 1

Over the past 20 years, there has been a dramatic increase in the prevalence of obesity in most Western and some developing countries. In fact, the proportion of obese adults is now 34.9% in the US population and 14.5% in the French population. Several genes have been identified to be associated with the development of obesity in various animal models; in addition abnormal neural pathways have been proposed that may impact the regulation of energy balance, as well as innate and acquired immune activation in adipose tissue. These mechanisms do not account for the entirety of the obesity epidemic and clearly lifestyle choices including increased caloric intake, especially in fat, and low physical activity contribute to the increase in obesity. In addition, obesity has been noted to have adverse health implications such as a reduction in sleep duration, disruption of circadian rhythm, and an increased risk of diabetes. Obesity associated type 2 diabetes mellitus not only increases the risk of cardiovascular complications, but also the risk of cancer and cancer-related mortality, especially hepatobiliary cancers.

The so-called metabolic syndrome involves a subgroup of obese patients. This condition is associated with a high risk of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic fatty pancreas disease (NAFPD). Both this conditions can lead to HCC and pancreatic adenocarcinoma respectively.

OBESITY AND METABOLIC SYNDROME

The prevalence of obesity is increasing worldwide resulting in a major health problem. In fact obesity can cause an increased risk for several diseases such as cardiovascular diseases, diabetes, and cancers especially hepatobiliary cancer. (2)

According to Keaver et al. overweight and obesity are supposed to reach levels of 89% and 85% in men and women, respectively by 2030. As a consequence there will be also an increase in the obesity-related prevalence of coronary heart disease by 97%, cancers by 61% and type 2 diabetes by 21%. Obesity is usually classified by Body Max Index (BMI). It is calculated as body weight in kilograms divided by the height in meters squared (kg/m2). However, BMI does not give an estimation about the proportion of body weight which consists of fat or the distribution of fat; these factors of the body composition are those that mostly affects the health risks in obese patients.

According to BMI; individuals are allocated to five different categories as:

- - 18.5– 24.9 kg/m^2 : normal range,
- 25.0–29.9 kg/m^2 : overweight,
- 30.0–34.9 kg/m^2 : class 1-obesity,
- 35.0–39.9 kg/m^2 : class 2-obesity,
- equal or greater 40 kg/m^2 : class 3-obesity.

Obesity is associated with significant decrease in life expectancy approximately by 3.3–18.7 years and also large increase in healthcare costs. (3) Grade obesity 2 and 3 are considered morbid obesity. (4)

Above BMI 25, every 5 kg/m^2 higher BMI is associated with around 30% higher overall mortality, which is mainly due to raised risk of cardiovascular death as 40%. While BMI at 30–35 kg/m^2 , median survival is reduced by 2–4 years; at 40–45 kg/m^2 , it is reduced by 8–10 years. (5) On the opposite side there is an interesting condition called the obesity paradox. (6) According to this condition, although the incidence of cardiovascular diseases, hypertension, dyslipidemia, type 2 diabetes mellitus and mortality are directly proportional with the BMI, obese individuals may have better outcomes compared to lean persons. However is important to emphasize that while the risk of cancer mortality decreases depending on the obesity status, it increases depending on the metabolic health status. Therefore mortality rate from cancer rises with the progress of metabolic dysfunction. (7) From an oncological point of view, epidemiological studies have proved that obesity is also linked with increased risk of several tumor types. Newly the protein kinase B/ phosphatidylinositol 3-kinase/mammalian target of rapamycin (Akt/PI3K/mTOR) cascade is an important pathway involved in the process of cancerogenesis in obese patient with cancer. Another mechanism involved is mediated by leptin protein, positively related with adipose stores, it induces cancer progression by activation of PI3K/Akt, mitogen-activated protein kinases (MAPK), mTOR and signal transducer and activator of transcription 3 (STAT3) pathways as a potential mediator of obesity-associated tumor (8).

Metabolic Syndrome is a condition that occurs between 20% and 45% in

Western Countries and its incidence is expected to increase to approximately 53% at 2035.

According to the American Heart Association/ National Heart, Lung, and Blood Institute (AHA/ NHLBI) criteria (9), to reach a diagnosis of metabolic syndrome three or more of the following risk factors should be present:

- abdominal obesity (Waist Circumference>102 cm in men, and >88 cm in women),
- hypertension ≥ 130/≥ 85 mmHg or specific medication,
- level of triglycerides ≥ 150 mg/dL (1.7 mmol/L) or specific medication,
- low HDL cholesterol: in men <40 mg/dL (1.03 mmol/L), and in women<50 mg/dL (1.29 mmol/L) or specific medication,and
- fasting plasma glucose ≥ 100 mg/dL(5.6 mmol/L) or history of diabetes mellitus or taking antidiabetic medications.

Abdominal obesity is the most frequently observed element of metabolic syndrome and

Waist Circumference and BMI are the most accurate representative indicators of visceral adiposity in young adults, and are good markers of Insulin Resistance (IR) and powerful predictors of the presence of hepatic steatosis.

According to the 2005 International Diabetes Federation criteria of metabolic syndrome, subsequently revised in 2009, abdominal obesity is identified as the Waist Circumference >94 cm in men, and >80 cm in women.

Abdominal adiposity and IR appear to have a key role in metabolic syndrome. In fact in a context of IR, non-esterified free fatty acids mobilization is accelerated from stored adipose tissue triglycerides. Consequently raised levels of glucose, triglyceride and very low-density lipoprotein are present. (10)

Data from several epidemiological studies showed that there is an association between obesity and metabolic syndrome with cancer risk. Therefore in countries where obesity prevalence has increased rapidly, such as the Western Countries, a significant proportion (~20%) of all new cancers may be related to obesity. (11) Specific examples of common cancers include breast, endometrial, colon, and prostate cancers. In a recent meta-analysis written by Esposito et al (12) a strong correlation is observed between metabolic syndrome and liver cancer in men and pancreatic cancer in women. Figure 4 shows the results of meta-analyses of RR (for presence of metabolic syndrome) in men and in women, respectively. (12)

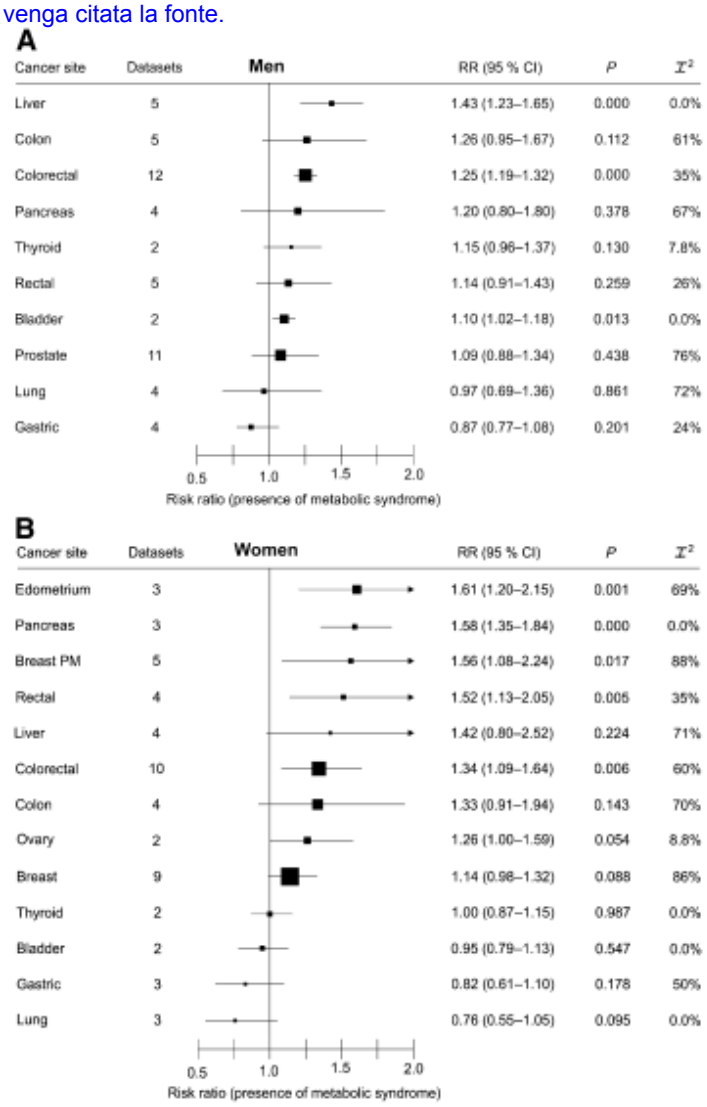


Figure 4. Meta-analyses of RR (for presence of metabolic syndrome) in men and in women.

In one study of over 33,000 men, the presence of metabolic syndrome was associated with

a 56% increased risk of cancer mortality over the following 14 years of follow-up. (13) While the Nurses' Health Study suggested that central adiposity determined by waist circumference and waist to hip ratio was associated with an increased risk of postmenopausal breast cancer, more

recent studies have claimed that premenopausal obesity is also a risk factor for breast cancer risk. (14)

Moreover from other epidemiological studies it is shown that mortality is increased in obese patients with cancer. The Million Women Study in the UK and the Cancer Prevention Study II in the USA similarly reported raised cancer mortality in obese individuals. (15)

A number of bariatric surgery studies supported the evidence for the association of obesity and cancer risk and mortality. In the Swedish Obesity Subjects (SOS) study, following more than 30% of weight loss, a decrease of cancer in women of about 41% was registered. (16) A similar effect was seen in the Utah obesity study. Moreover the Women's Intervention Nutrition Study (WINS) reported a 24% of decreasing in breast cancer after only a 4% reduction in weight over 5 years. (17, 18)

The real mechanisms involved in this effect are still undefined, but most likely reflect a correction of factors that are different in patients with obesity and metabolic syndrome.

In visceral obesity both adipocytes and filtrating immune cells produce inflammatory cytokines and create a state of chronic systemic low-grade inflammation that act as a pro-tumorogenic environment. (19)

The unbalance between pro inflammatory and anti-inflammatory cytokines operated by central obesity may contribute to IR, a key component of metabolic syndrome. The IGF-1 axis has also been linked to the progression of breast, liver, pancreatic and esophageal cancer. (20) IGF levels are influenced by circulating insulin levels in fact raised level of insulin are able

to decrease IGF-binding proteins 1 and 2 levels, thus increasing the bioavailability of IGF. (12)

Insuline Resistance and diabetes are important components of metabolic syndrome and therefore some cancerogenic pathways driven by hyperglicemia could occur in patients with metabolic syndrome.

In the contest of patients with HCC and diabetes, Ali Kamkar et al reported that the IR and the resulting inflammation environment, which are associated with the development of NASH, mediates carcinogenesis. (21) In particular the liver inflammation in a contest of diabetes, leads to the exposure of hepatocytes to increased levels of IL-6 and TNF-α, which promote the activation of JAK/STAT-3 and IKKα/NK-kβ signaling pathways, followed by lack of apoptosis, and consequently uncontrolled hepatocytes proliferation; this let the carcinogenesis begin (Figures 5 e 6).

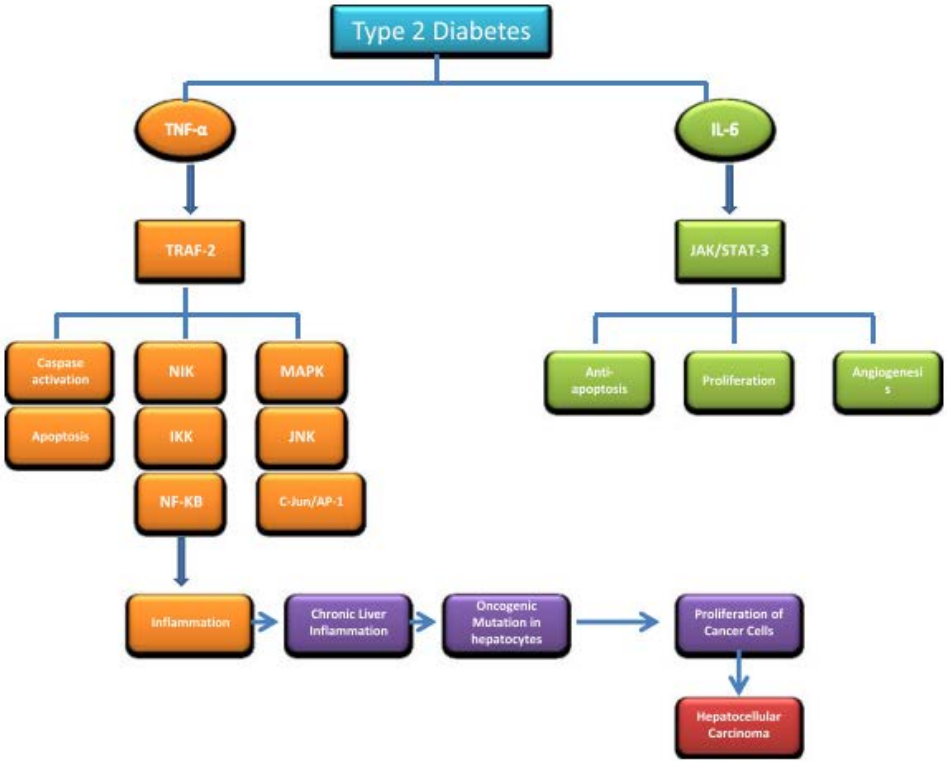


Figure 5. Oncogenic impact of IL-6/STAT-3 and TNF-α/NF-Kβ signaling pathways on the development and progression of HCC.

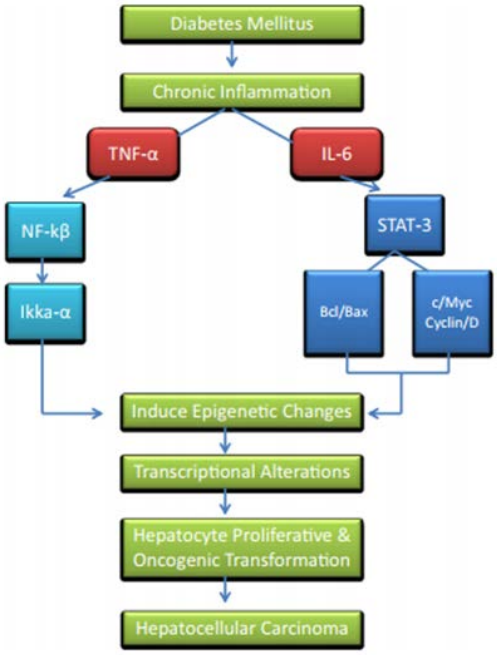


Figure 6. Overview of the possible intermediary mechanism associated with onset and progression of hepatocellular carcinoma confounded by diabetes mellitus.

HEPATOCELLULAR CARCINOMA

HCC is the most common primary liver cancer. The incidence of HCC is increasing and it has nearly tripled in Western Countries since the 1980s and is supposed to surpass colon and breast cancer by 2030.

Surgical resection and liver transplantation are the only curative therapies although not always feasible, as in most cases the diagnosis is late with a large tumor that involves both hepatic lobes or multiple nodule.

As a consequence prognosis remain poor. In order to improve survival and opportunities for curative treatment, early diagnosis is mandatory. To make a diagnosis earlier, risk factors for HCC need to be identified. (22)

Many risk factors and cofactors (23, 24) are associated with HCC that result being a complex disease. In most patients, cirrhosis precede HCC (25) and, as a direct consequence, common causes of cirrhosis have been identified as important risk factors for HCC.

Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) are important risk factors for HCC. Worldwide 50%–80% of HCCs are related to HBV infection, whereas HCV infection is considered responsible of cancer occurrence in 10%–25% of the cases. (26)

Preventing and treating viral hepatitis can significantly affect HCC incidence. In terms of prevention, the introduction of HBV vaccine for all children of the endemic areas will reduce HCC incidence.

In terms of treatment antiviral therapy resulting in viral suppression is reported to significantly reduce the risk for HCC in patients infected with

HBV and advanced hepatic fibrosis. (27,28)

Nowadays also for HCV infection several therapy are available. Interferon has been the major antiviral treatment, leading to a viral clearance in approximately half of patients. New direct-acting antivirals have significantly improved the cure rate to above 90%.

However HCC incidence is predicted to increase in the next decades even in high-resource countries because unfortunately access to viral hepatitis treatment is still limited due to the high costs and moreover under-diagnosis of viral hepatitis infection is still a problem especially in specific subpopulations, such as inmates, and injection drug users.

Moreover, cancer risk persists even after 10 years of viral cure, and therefore clinical strategies for its monitoring are urgently needed. Several risk-predictive host factors, e.g., advanced liver fibrosis, older age, accompanying metabolic diseases such as diabetes, persisting hepatic inflammation, and elevated alpha-fetoprotein, as well as viral factors, e.g., core protein variants and genotype 3, have been reported. (29)

Other environmental and genetic risk factors for HCC have been reported such as: excessive alcohol consumption, aflatoxin intake, diabetes, obesity, or hereditary hemochromatosis. (30)

Data emerging from epidemiologic studies have led to the conclusion that global variations in HCC occurrence mostly reflect geographic differences in the prevalence of different disease risk factors. (31)

For example the large Taiwan Prospective HBV Study strongly demonstrated that HBV is the primary driver of the high HCC incidence rates in regions of

high HBV endemicity such as Asia and sub-Saharan Africa where 8% of the population is chronic carriers of the HBV much higher than the 2% of HBV carriers in North America and northern and western Europe. (32)

Another potential contributor to the high incidence of HCC in Asia and sub-Saharan Africa is dietary exposure to aflatoxin (33), which is produced by a fungus of the genus Aspergillus and is a common contaminant of foods such as peanuts, grain, legumes, and corn.

Aflatoxin seems to be a cofactor for HCC acting with chronic HBV infection and increasing the risk for disease.

In sub-Saharan Africa, where 67% of all individuals is infected by HIV (34), HIV infection act as a cofactor increasing rates of HCC in HBV-infected individuals.

In countries where HBV infection is not endemic, HCV and alcoholic cirrhosis are generally considered to be the most important risk factors for HCC development. (31)

After HBV and HCV infections alcohol-related cirrhosis is considered to be the third most common cause for HCC.

Alcohol can independently cause HCC by development of cirrhosis independent of the presence of viral hepatitis. HCC carcinogenesis induced by alcohol is due to recurrent inflammation state and cycles of hepatocyte necrosis and regeneration with oxidative stress, finally resulting in cirrhosis. (35)

Alcohol can also work as a cofactor together with HBV and HCV to increase the risk of developing HCC. (36) The risk to develop HCC is high especially in

those individuals who consume more than 60 g of alcohol per day.

A study analyzing the risk of developing HCC among heavy alcohol users with chronic HCV infection showed that individuals were at 2.3 (95% confidence interval: 1.67–3.26) times higher risk of developing HCC if they were heavy alcohol users (defined as alcohol use of 210–560 g/week). (37) Similar data were reported for heavy alcohol users HBV infected where the risk for HCC increased by almost three times. (38)

Interestingly, around 40% of HCC cases were reported as idiopathic because was not possible to identify the presence of known HCC risk factors such as HCV, HBV, alcohol-induced liver disease, non specific cirrhosis, and non specific hepatitis.

This could be only in part explained by limitations in the data source, other factors, such as diabetes and nonalcoholic fatty liver disease (NAFLD), may be important contributors to the development of association in the Western Countries.

In fact, a U.S. population-based study has established that diabetes is an independent risk factor, associated with a two- to three-fold higher risk for disease. (39) Also in a Japanese case–control study diabetes and obesity (body mass index _ 25 kg/m^2) were associated with enhanced risk of HCC. (40)

Both diabetes and obesity are involved in the development of nonalcoholic steato-hepatitis (NASH), that is the severest form of NAFLD.

NASH can lead to HCC through progression in cirrhosis.

The increasing of diabetes and obesity occurrence worldwide may cause

future increases in HCC incidence, in particular in developed countries where, to date, the impact of the obesity epidemic has been most marked. (28)

An interesting population study was carried out by Kasmari et al. including adults (> 18 years of age) represented in the MarketScan Database between 2008 and 2012 who had an outpatient visit with a diagnosis code of HCC.

The total number of patients with HCC was 17,446. Because hepatitis C is the largest known risk factor for HCC in the US, these patients were included in the analysis to evaluate the contribution of hepatitis C with and without type II diabetes or metabolic syndrome. There were 7473 patients remaining after exclusions were applied. Figure 7 describes how the study sample was constructed.

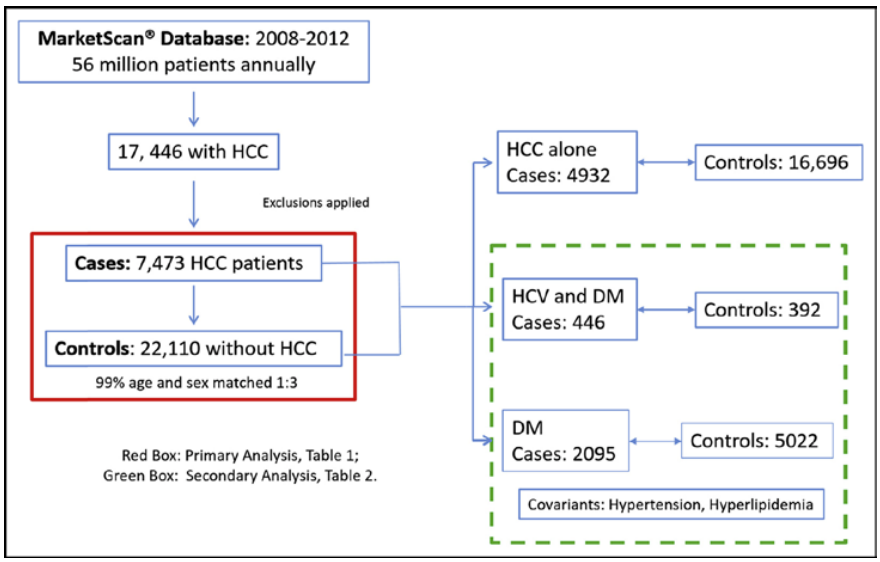


Figure 7. Study flow diagram. DM .diabetes mellitus; HCC . hepatocellular carcinoma; HCV . hepatitis C virus.

The conclusions of the study were that both type II diabetes and hypertension seem to be associated with HCC also in the absence of cirrhosis. Moreover medical treatment of diabetes can interfere with HCC developing

and in particular treatment with metformin can reduce cancer risk while insulin use was associated with increased risk of HCC in diabetic patients without cirrhosis.

This study may bring the future research into new targets for screening and therapies in order to improve survival in patients with HCC. (22)

In order to understand how metabolic syndrome and diabetes can promote liver cancerogenesis the tumor microenvironment, defined as the complex network of tumor cells and stromal cells should be considered. (41) During chronic hepatic injury, the deposition of extracellular matrix leads to poor oxygen exchange and causes fibrosis. In a hypoxic environment hypoxia-inducible factor-1α production is induced. (42) Hypoxia-inducible factor-1α is therefore stimulated by hypoxic conditions and plays a synergistic role with other angiogenic factors, such as Endothelial Vascular growth factor (VEGF) in acting against apoptosis and fostering cell proliferation. Moreover, hypoxia induces the mechanism of autophagy which promote cancer survival using the catabolic breakdown of cellular elements to generate energy for tumor cells and its surrounding environment.

In addition in a hypoxic environment stromal cells secrete pro-angiogenic factors in fact HCC is characterized by an excess of angiogenic factors produced by tumor cells, vascular endothelial cells, immune cells, and surrounding tumor microenvironment.

Therefore is clear that the process of angiogenesis plays an important role in HCC genesis. VEGF is an important mediator in tumor developing and is released by the surrounding tumor microenvironment and stromal cells.

VEGF is regulated by oncogenic gene mutations, hormones and cytokines. In addition, VEGF can interfere with the surrounding stromal environment through VEGR receptors on some cells like hepatic stellate cells and Kupffer cells. (43) Other angiogenic molecules that foster dysfunctional vascular system in HCC are angiopoietin 1, angiopoietin 2, and basic fibroblast growth factor (bFGF). Angiopoietin 2 works synergistically with VEGF on endothelial cells producing molecules that disrupt the basement membrane, further augmenting a hypoxic environment. bFGF and VEGF act together to promote angiogenesis, and platelet-derived endothelial cell growth factor induces cell migration and new vessel maturation. (38)

These factors together with transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF), endothelial growth factor (EGF), interleukin 4 (IL-4), IL-6, and IL-8 are raised in HCC patients.

In addition the fibrotic environment, through enhanced integrin signaling, can stimulate tumor growth, cells survival, and proliferation. (41) In particular there are the tumor-associated fibroblasts that can secrete factors able to promote tumor growth and angiogenesis. Tumor-associated fibroblasts are also involved in cross talk with neoplastic cells. (42)

In cirrhosis there is a chronic inflammation, mediated by cytokine and chemokine, that has a key role in development of dysplastic nodules and HCC. (44) TGF-β, HGF, and EGF are important growth factors and can regulate the immune and inflammatory process. (42) In a phenotype of more aggressive and metastatic HCC there are increased Th2-like cytokines (IL-4, IL-5, IL-8, and IL10) rather than Th1-like cytokines (IL-1α, IL-1 β, IL-2, TNF-α). (41)

The receptors are found on inflammatory, endothelial, and epithelial cells. Interactions with these receptors with cells of the tumor microenvironment mediate tumor progression, invasion, and metastasis.

Moreover an immune-tolerant microenvironment is established. The tumor microenvironment can activate a plethora of immunosuppressive mechanisms, which may act in concert to counteract effective immune responses. In fact, some immune cells, especially tumor infiltrating lymphocytes, are an important antitumor response. However, there is a predominance of circulating regulatory T-cells and myeloid-derived suppressive cells that reduce the immune response. (41) In addition, tumor-associated macrophages can release chemokines and growth factors that suppress antitumor immunity.

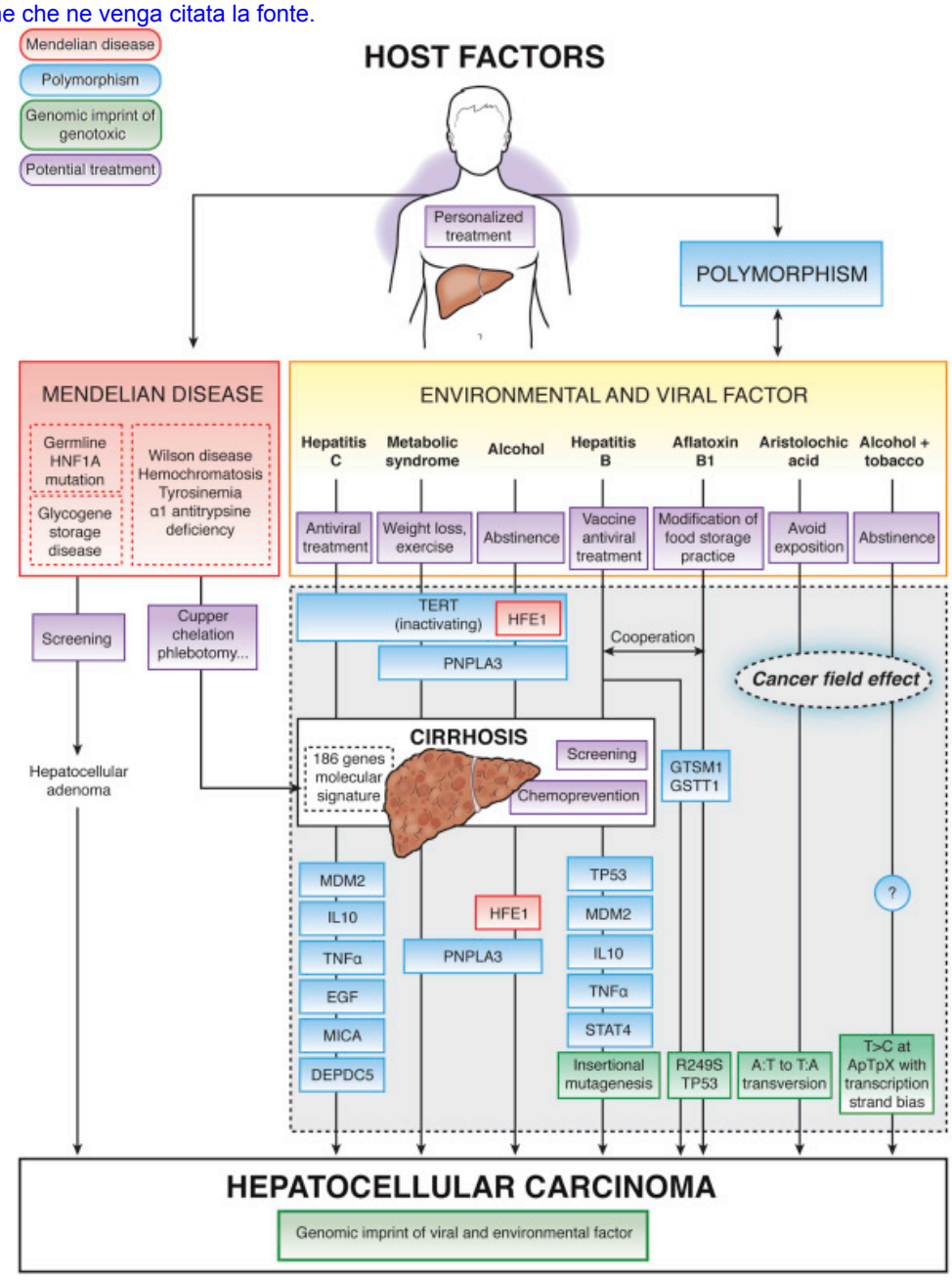


Figure 8. Final resume of risk factors and HCC pathogenesis

HEPATOCELLULAR CARCINOMA TREATMENT

Treatment strategies for HCC are commonly based on the Barcelona Clinic Liver Cancer (BCLC) a staging system that considers performance status, tumor size and location, extra hepatic spread and the underlying liver function. (45)

Treatment of HCC is often multimodal and different surgical, interventional (radiological/sonographical) and non-interventional procedures have been established.

Curative treatment options include surgical resection, orthotopic liver transplantation (OLT) or locoregional therapies and are available for early tumor stages (BCLC A).

Laparoscopic approach is being largely used, especially for small lesion. In the mini-invasive approach the post-operative complications rate decreases and there are less cases of worsening of cirrhosis that often occurred after open liver resection. In addition, the use of laparoscopic technique could facilitate reiterative surgeries and a future OLT due to the less adherence caused.

For intermediate tumor stages (BCLC B) other therapeutic option are available such as trans-arterial chemoembolization (TACE) with or without drug-eluting beads (DEB-TACE) and selective internal radiation therapy (SIRT).

In advanced tumor stages (BCLC C) systemic therapy with Sorafenib, the multikinase inhibitor, represents the current standard in patients with compensated cirrhosis. (46) Best supportive care is recommended for end-

stage HCC patients (BCLC D). (47)

Figure 9 shows treatment following the BCLC system.

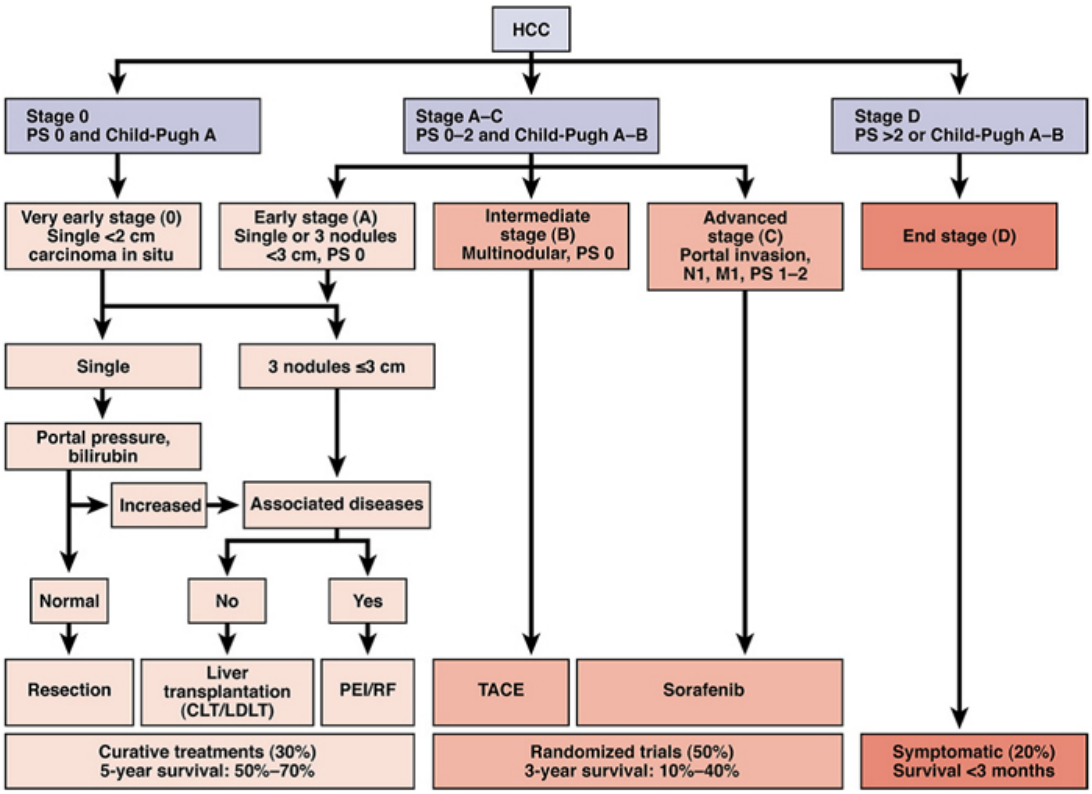


Figure 9. *Barcelona Clinic Liver Cancer staging system and treatment options*

Surgical procedures

Liver Resections

Liver resection is one of the treatment options in patients with HCC. Thypes of resection performed are based on tumor location, size and vascular rapport of the lesion.

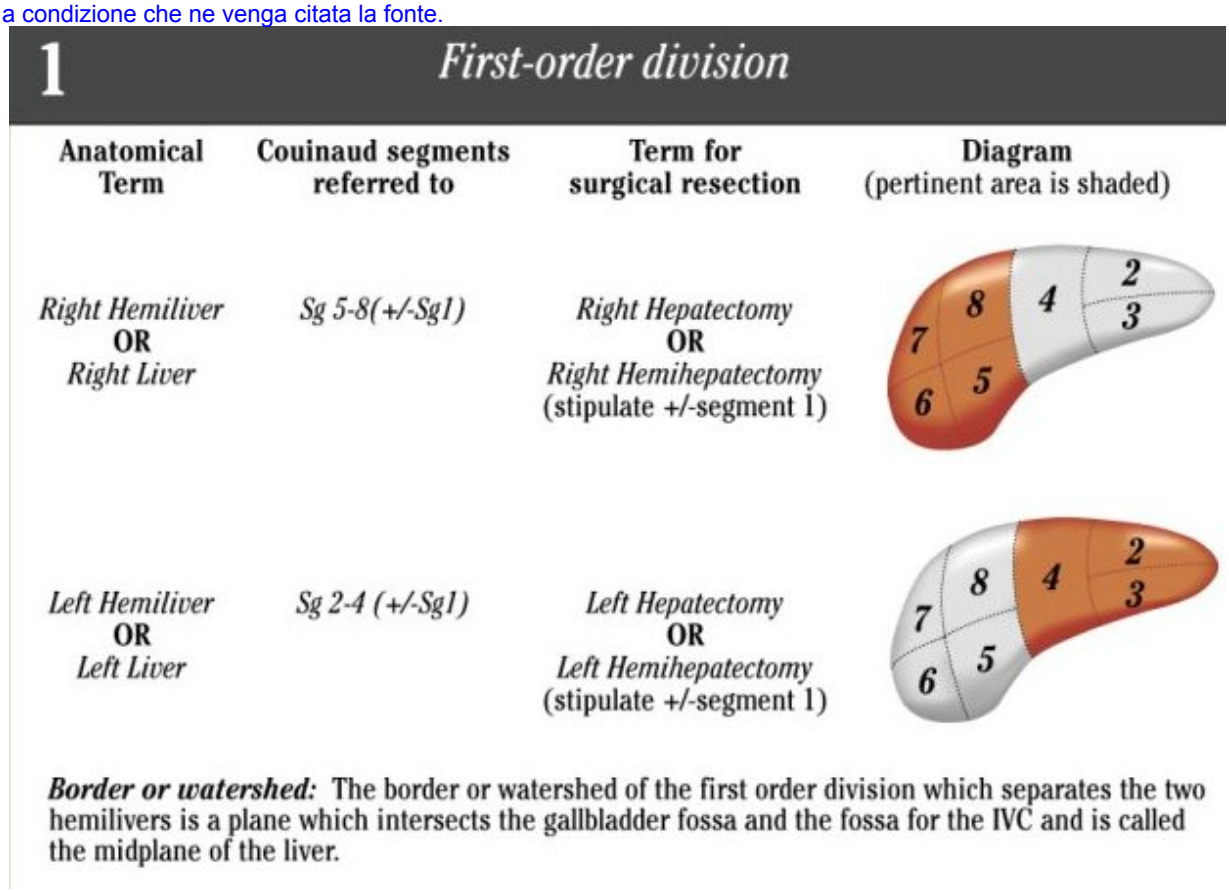
Also steps of the operation depend on number and location of the segments involved by the cancer. However common steps of the operation could be rapresent by the followings:

1. Incision: is generally a right-subcostal incision extended to the mid-line up to the xiphoid process. In some centers also a double sub-costal incision could be performed. In case of huge tumors also a thoracotomy at the level of the ninth rib is performed.
2. Mobilization of the hemiliver involved in the operation. In some case could be necessary to perform a bilateral liver mobilization.
3. Preparation of the hepatic pedicle that can be pass on a Tourniquet in order to be able to perform a Pringle's maneuver during the resection to minimize the blood loss.
4. If needed also the extra-hepatic preparation and control of the sovra-hepatic veins. This maneuver could be useful to control intraoperative bleeding or this step is propaedeutic to the section of the vein after the parenchymal resection.
5. Perform an Ultrasound of the liver to localize the lesion and to exclude the presence of other lesions.

6. The parenchymal transection could be performed in several ways such as kellyklasia, with ultrasonic dissector or other device.
7. After the resection is important to control the hemostasis and the biliostasis.
8. Place a drain or more in the site of the resection.
9. Closure of the abdomen.

The risk of this surgery can be resumed in postoperative hemorrhage, biliary leaks and post-operative liver failure in addition to the general complications that can occur after a major surgery operation.

The following images (figures 10,11,12,13) explain the different types of anatomical resections that can be performed. In addition there are the non-anatomical resections that include the resection of a part of the liver respectig the important vascular and biliary structure in order to avoid large necrosis of the remaining liver. All these procedures could be performed in both open or mininvasive approaches.



1 *First-order division*

Anatomical Term	Couinaud segments referred to	Term for surgical resection	Diagram (pertinent area is shaded)
Right Hemiliver **OR** *Right Liver*	*Sg 5-8(+/-Sg1)*	*Right Hepatectomy* **OR** *Right Hemihepatectomy* (stipulate +/-segment 1)	
Left Hemiliver **OR** *Left Liver*	*Sg 2-4 (+/-Sg1)*	*Left Hepatectomy* **OR** *Left Hemihepatectomy* (stipulate +/-segment 1)	

Border or watershed: The border or watershed of the first order division which separates the two hemilivers is a plane which intersects the gallbladder fossa and the fossa for the IVC and is called the midplane of the liver.

Figure 10. Brisbane 2000 Nomenclature of Liver Anatomy and Resections

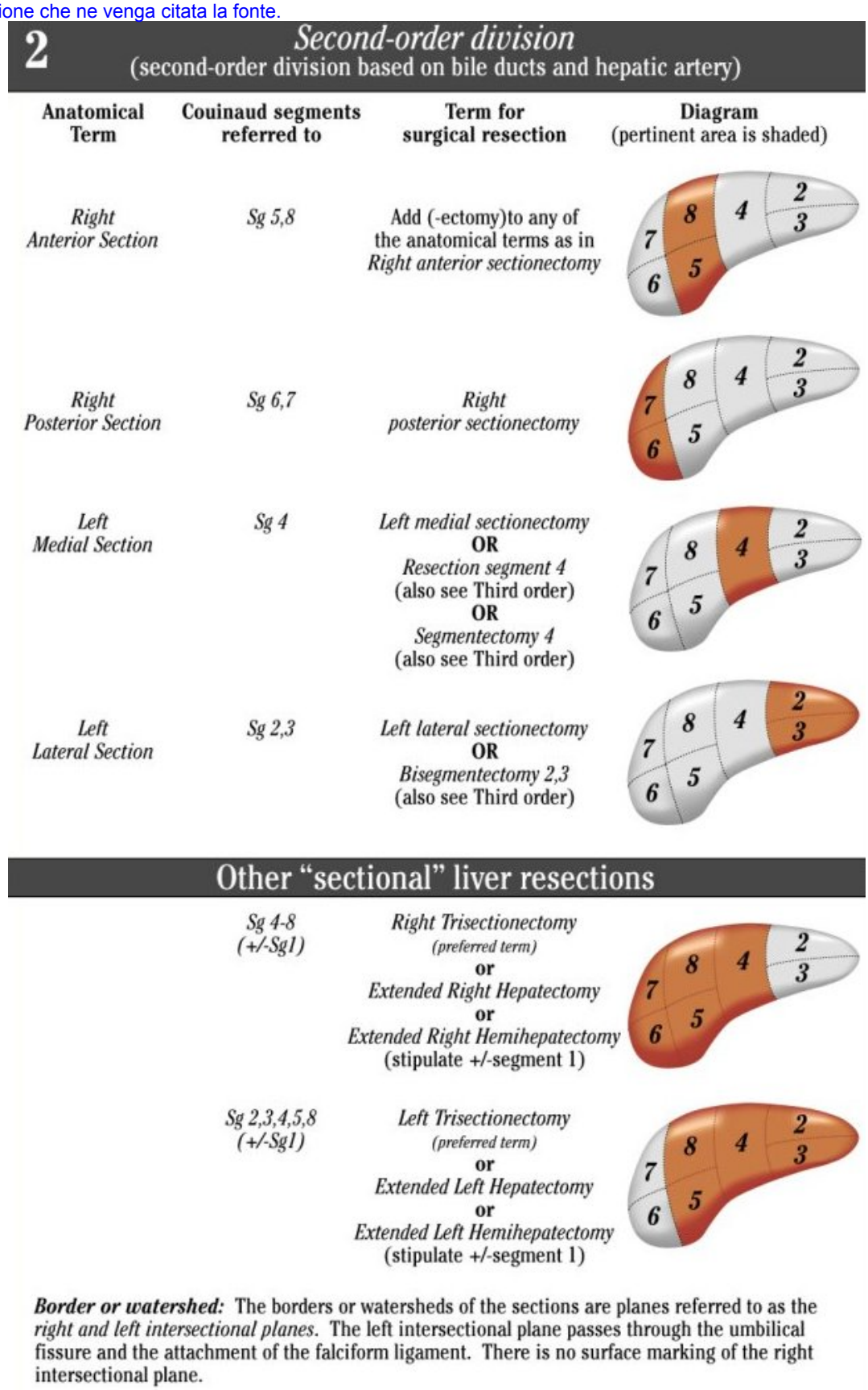
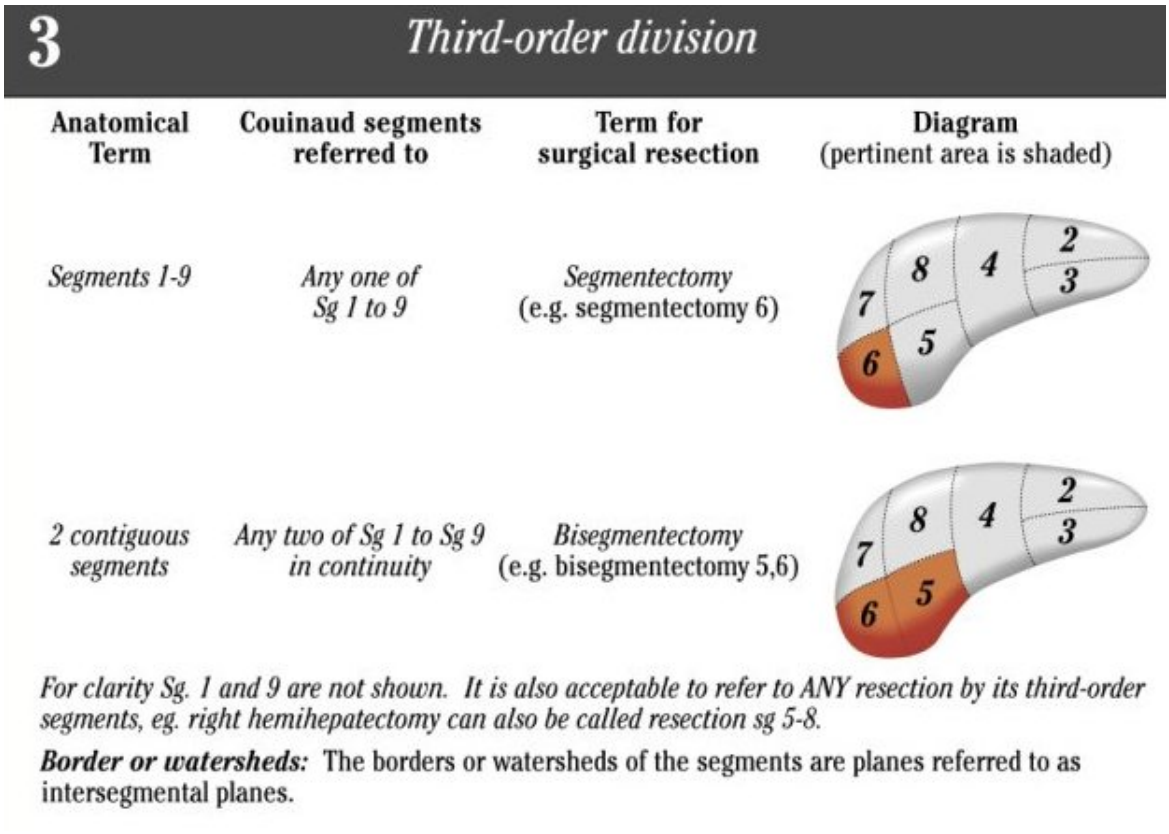


Figure 11. Brisbane 2000 Nomenclature of Liver Anatomy and Resections



3 *Third-order division*

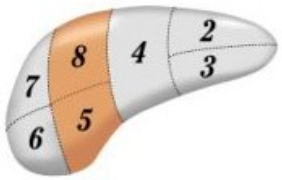
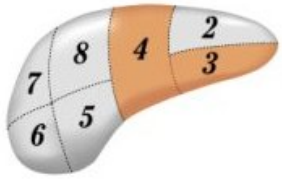
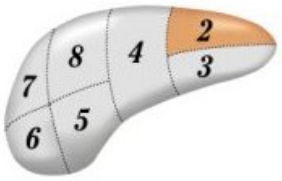
Anatomical Term	Couinaud segments referred to	Term for surgical resection	Diagram (pertinent area is shaded)
Segments 1-9	*Any one of Sg 1 to 9*	*Segmentectomy* (e.g. segmentectomy 6)	
2 contiguous segments	*Any two of Sg 1 to Sg 9 in continuity*	*Bisegmentectomy* (e.g. bisegmentectomy 5,6)	

For clarity Sg. 1 and 9 are not shown. It is also acceptable to refer to ANY resection by its third-order segments, eg. right hemihepatectomy can also be called resection sg 5-8.

Border or watersheds: The borders or watersheds of the segments are planes referred to as intersegmental planes.

Figure 12. Brisbane 2000 Nomenclature of Liver Anatomy and Resections

4 Addendum. *Alternative second-order division*
(second-order division based on portal vein)

Anatomical Term	Couinaud segments referred to	Term for surgical resection	Diagram (pertinent area is shaded)
Right Anterior Sector **OR** *Right paramedian Sector*	*Sg 5,8*	Add (-ectomy)to any of the anatomical terms as in *Right anterior sectorectomy* **OR** *Right paramedian sectorectomy*	
Right Posterior Sector **OR** *Right Lateral Sector*	*Sg 6,7*	*Right posterior sectorectomy* **OR** *Right lateral sectorectomy*	
Left Medial Sector **OR** *Left Paramedian Sector*	*Sg 3,4*	*Left medial sectorectomy* **OR** *Left paramedian sectorectomy* **OR** *Bisegmentectomy 3,4*	
Left Lateral Sector **OR** *Left Posterior Sector*	*Sg 2*	*Left lateral sectorectomy* **OR** *Left posterior sectorectomy* **OR** *Segmentectomy 2*	

Right anterior sector and Right anterior section are synonyms. Right posterior sector and Right posterior section are synonyms. Left medial sector and Left medial section are NOT synonyms and are NOT exchangeable terms. They do not describe the same anatomic areas. Left lateral sector and Left lateral section are also NOT synonyms *and are NOT exchangeable terms.*

Border or watersheds: The border or watersheds of second-order division based on PV are called right and left intesectoral planes. These have no surface markings.

Figure 13. Brisbane 2000 Nomenclature of Liver Anatomy and Resections

The Liver Transplant

A liver transplant involves the removal of and preparation of the donor liver, removal of the diseased liver, and implantation of the new organ. The liver has several connections that must be re-established for the new organ to receive blood flow and to drain bile from the liver. The structures that must be reconnected are the inferior vena cava, the portal vein, the hepatic artery, and the bile duct. The exact method of connecting these structures varies depending on specific donor and anatomy or recipient anatomic issues and, in some cases, the recipient disease.

For someone undergoing liver transplantation, the sequence of events in the operating room is as follows:

1 Incision

2 Evaluation of the abdomen for abnormalities that would preclude liver transplantation (for example: undiagnosed infection or malignancy)

3 Mobilization of the native liver (dissection of the liver attachments to the abdominal cavity)

4 Isolation of important structures (the inferior vena cava above, behind, and below the liver; the portal vein; the common bile duct; the hepatic artery)

5 Transection of the above mentioned structures and removal of the native diseased liver.

6 Sewing in the new liver: First, venous blood flow is re-established by connecting the donor's and the recipient's inferior vena cava and

portal veins. Next, arterial flow is re-established by sewing the donor's and recipient's hepatic arteries.

7 Finally, biliary drainage is achieved by sewing the donor's and recipient's common bile ducts.

8 Ensuring adequate control of bleeding.

9 Closure of the incision.

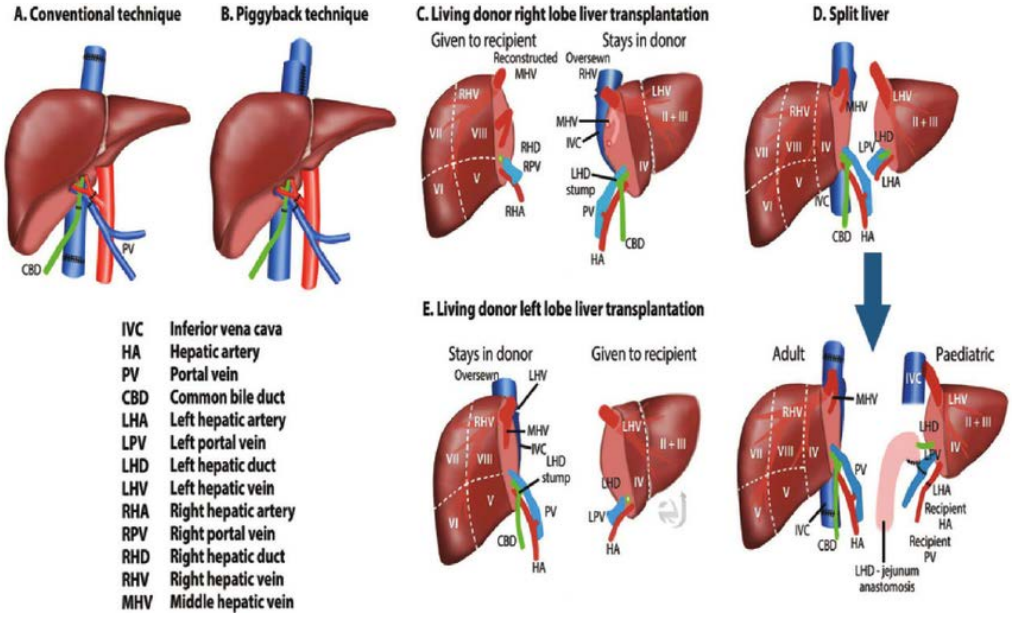


Figure 14. Type of possible liver transplant and vascular anastomosis

As with any surgical procedure, complications related to the operation may occur, in addition to the many possible complications that may happen to any patient who is hospitalized. Some of the problems specific to liver transplantation that may be encountered includes:

Primary non-function or poor function of the newly transplanted liver occurs in approximately 1-5% of new transplants. If the function of the liver does

not improve sufficiently or quickly enough, the patient may urgently require a second transplant to survive.

Hepatic artery thrombosis, or clotting of the hepatic artery (the blood vessel that brings oxygenated blood from the heart to the liver) occurs in 2-5% of all deceased donor transplants. The risk is doubled in patients who receive a living donor transplant. The liver cells themselves typically do not suffer from losing blood flow from the hepatic artery because they are primarily nourished by blood by the portal blood flow.

In contrast, the bile ducts depend strongly on the hepatic artery for nutrition and loss of that blood flow may lead to bile duct scarring and infection. If this occurs, then another transplant may be necessary.

Portal vein thrombosis or clotting of the large vein that brings blood from the abdominal organs (the intestines, the pancreas, and the spleen - the organs that belong to the portal circulation) to the liver occurs infrequently. This complication may or may not require a second liver transplant.

Biliary complications: In general, there are two types of biliary problems: leak or stricture. Biliary complications affect approximately 15% of all deceased donor transplants and up to 40% of all living donor transplants.

Biliary leak means that bile is leaking out of the bile duct and into the abdominal cavity. Most frequently, this occurs where the donor and recipient bile ducts were sewn together. This is often treated by placing a stent, or plastic tube, across the connection through the stomach and small intestine and then allowing the connection to heal. In the case of living donor or split liver transplants, bile can also leak from the cut edge of the liver. Typically, a

drain is placed and left during the transplant operation along the cut edge to remove any bile that may leak. As long as the bile does not collect in the abdomen, the patient does not become ill. Leaks will often heal with time, but may require additional treatment procedures.

Biliary stricture means narrowing of the bile duct, resulting in relative or complete blockage of the bile flow and possible infection. Most frequently, the narrowing occurs at a single site, again where the donor and recipient ducts are sewn together.

This narrowing can often be treated by dilating the narrowed area with a balloon and/or inserting a stent across the stricture. If these methods are unsuccessful, surgery is often done to create a new connection between the liver's bile duct and a segment of intestine. Rarely, biliary strictures occur at multiple or innumerable sites throughout the biliary tree. This occurs most frequently because the biliary tree was poorly preserved during the period when the liver was not in either the donor or recipient circulation. Livers procured from cardiac death donors are at higher risk than those from brain dead donors. Alternatively, diffuse biliary strictures may occur if the biliary tree has inadequate blood supply because of an abnormality with the hepatic artery.

Bleeding is a risk of any surgical procedure but a particular risk after liver transplantation because of the extensive nature of the surgery and because clotting requires factors made by the liver. Most transplant patients bleed a minor amount and may get additional transfusions after the operation. If

bleeding is substantial or brisk, return to the operating room for control of bleeding is often necessary.

In general, approximately 10% of transplant recipients will require a second operation for bleeding.

Infection - Infections can occur during the healing of the wound created by any operation. Liver transplant recipients are also at risk for infections deep within the abdomen, particularly if there is a collection of blood or bile (from a bile leak). The immunosuppressive medications along with the history of liver failure increase the liver transplant recipient's risk for developing an infection after transplantation.

NON ALCOHOLIC FATTY LIVER DISEASE & STEATOHEPATITIS

Nonalcoholic fatty liver disease (NAFLD) could be defined as the hepatic manifestation of metabolic syndrome .

Typically, most patients with metabolic syndrome-related NASH present with characteristic features of metabolic syndrome such as central obesity, impaired glucose tolerance, high triglycerides, and low high-density lipoprotein [HDL] . (48)

In addition patients with NAFLD have an unbalanced lipid metabolism characterized by higher low-density lipoprotein (LDL) particle concentration and lower LDL particle size resulting in abnormalities in lipoprotein profile. (49)

While concurrent components of metabolic syndrome increase the risk of developing NAFLD, the presence of NAFLD also increases the risk of developing complications such as dyslipidemia and IR. (50)

From an epidemiological point of view NAFLD has emerged as an epidemic in the Western Countries as well as in the Asia Pacific region. NAFLD has an estimated prevalence of~ 20– 30% in the USA (51) and is also the most common cause of persistently elevated liver enzymes. (52,53). The rising incidence of NAFLD is not unexpected because of the increasing trends in obesity and obesity-related diseases. However, both prevalence and incidence trends must be interpreted with caution as diagnosis of NAFLD may also be influenced by misclassification biases and selection biases. In addition, the prevalence estimates of NAFLD can differ depending on the population studied and the accuracy of the diagnostic test.

From an histological point of view NAFLD is a clinical disease characterized by the finding of 5% or greater of macrovesicular steatosis of hepatocytes in a patient without significant alcohol use or other known causes of chronic liver disease. (54)

Histologically, it covers a wide spectrum of liver disease ranging from isolated steatosis (without or with only minimal inflammation) to severe nonalcoholic steatohepatitis (NASH),

characterized by inflammation, cell necrosis (ballooning), perilobular fibrosis, and

eventually cirrhosis (Figure 15).

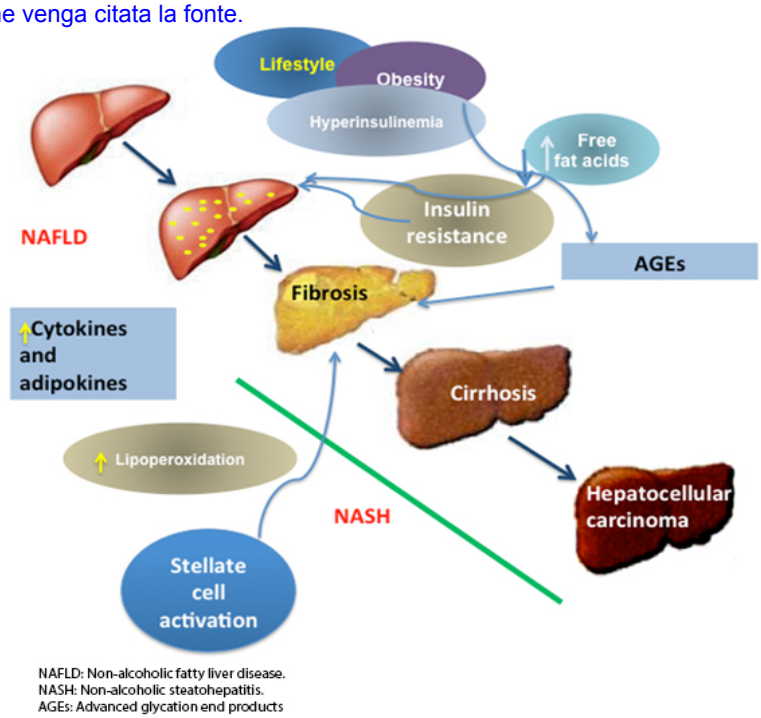


Figure 15. Progression from NAFLD to HCC

The risk of NASH progressing to cirrhosis has not been clearly defined, but it has been estimated to range from 21% to 26% over 8.2 years. Up to 30% of NASH patients with compensated cirrhosis develop liver decompensation in 8 to10 years. (55)

Fibrosis stage is the strongest predictor for disease specific mortality in NAFLD. Angulo et al performed a retrospective international longitudinal study of 619 NAFLD patients and showed that increasing severity of liver fibrosis on biopsy was associated with increasingly higher risk of death, liver-related events, or Liver Transplantation (LT). (56) A recent study using registry data from the United Network for Organ Sharing/Organ Procurement Transplant Network(UNOS/OPTN) demonstrated that in 2013, NASH became the second leading etiology of liver disease among adults awaiting LT in the U.S. and is predicted to become the leading indication for

LT in the near future. (57) In addition, NASH is currently the second leading etiology and the most rapidly growing indication among adults with HCC undergoing LT in the U.S. (58)

While NAFLD is not entirely a diagnosis of exclusion, its diagnosis require a careful investigation of alcohol consumption, as alcoholic liver disease itself can show similar histological features to NAFLD. Current guidelines recommend that, in order to make a diagnosis of NAFLD, alcohol exposure should be less than 30 g/day for male sex and less than 20 g/day for female sex.

As already discussed, diabetes and obesity are two conditions of metabolic syndrome that act as independent risk factors for the development of HCC. Both of them are strongly associated with NAFLD. Recent studies have suggested the association between NAFLD and HCC, supporting the role of NAFLD in liver carcinogenesis. (59) Increased risk of HCC in NAFLD seems to affect especially patients with cirrhosis and is generally due to NASH. Furthermore, it has recently been suggested that also in non-cirrhotic livers, the presence of NAFLD and/or metabolic syndrome is associated with the development of HCC. (60) The calculated risk of HCC is roughly 7% over 6.5 years. (50)

In most series of patients with HCC , the prevalence of NAFLD-related HCC ranges from 4% to 22%, however the incidence is expected to increase in the future considering the obesity epidemic worldwide. (61)

Previous studies have described a steatohepatitis variant of HCC (SH-HCC), which is characterized by features resembling steatohepatitis in the tumor

similar to the non-tumoral liver . This phenotype has been reported to occur in the setting of underlying steatosis, steatohepatitis, and metabolic syndrome. (60)

Despite the stability of new cases of viral hepatitis and alcohol-related cirrhosis, HCC incidence is increasing in areas traditionally considered as low incidence. This increased incidence of HCC follows the epidemic of obesity and metabolic syndrome within Western Countries. In addition it has been suggested that NASH may become the most frequent cause of HCC in an era with improving treatment strategies for chronic viral hepatitis. Moreover cases of HCC traditionally diagnosed as secondary to criptogenetic or idiopatic are now being classified as NASH-related cirrhosis due to overwhelming clinical evidence linking criptogenetic or idiopatic with the metabolic syndrome seen in NASH. The presentation of patients with NASH-related HCC tends to be in the older of age and mostly in women, and they generally have better overall survival. Recently, PNPLA3 has been demonstrated as a potential biomarker involved in the development of HCC in patients prone to NASH. However, the actual physiological mechanism of PNPLA3 is still under investigation. (62)

In addition an important aspect under evaluation is if survival outcome is different in HCC NASH-related patients rather than in HCC non NASH-related patients.

In a paper published by Weinmann et al (63) the median survival of all patients with HCC was 15.3 months (range 0– 131 months) while overall

survival in NASH-HCC was 4.22 months shorter compared to non- NASH-HCC (median [range]: 11.28 [0.7-127.6] vs. 15.5 [0– 131.3], p = 0.287) (Figure 12). In HCC, overall survival is strongly depended on liver function. In the absence of cirrhosis, NASH-HCC patients showed an increased overall survival compared to non-NASH HCC patients (43.4 vs. 25 month, p = 0.748) (Figure 16). (63)

Both, compensated cirrhosis and decompensated cirrhosis were associated with a decreased survival in NASH-HCC compared to non-NASH-HCC. Patients with NASH-HCC in Child–Pugh–Turcotte (CTP) stage A exhibited a reduced overall survival (15.5 vs. 24.2 months, p = 0.268). The difference in overall survival in CTP stage B achieved statistical significance (5.55 vs. 10.6 month, p< 0.05). In this study there were no NASH-HCC patients with CTP stage C (Figure 16). BMI was the second factor that was identified to contribute to the overall survival in these patients. A higher BMI was associated with longer survival in all groups of HCC even independent of the underlying cause (Figure 16).

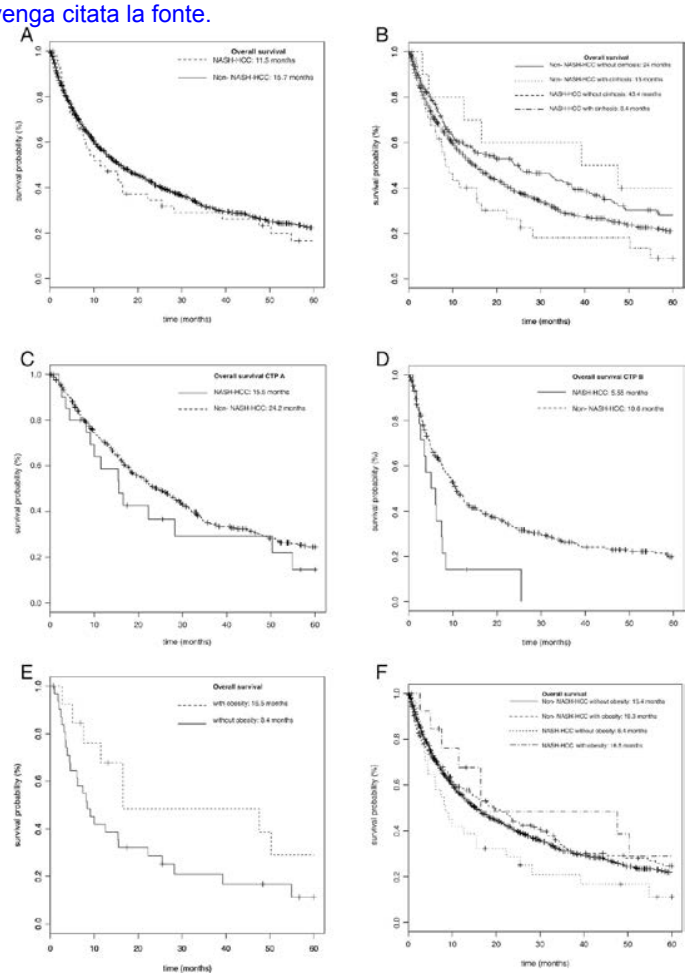


Figure 16. Kaplan-Meier survival curves. Kaplan-Meier survival curves comparing overall survival in NASH-HCC and non-NASH-HCC patients; A for all patients; B for all patients regarding presence of liver cirrhosis; C for all patients with Child Pugh stage A; D for all patients with Child Pugh stage B; E for all patients regarding obesity; F for NASH-HCC and non-NASH-HCC patients regarding obesity.

In the Weinmann report LT was not performed as primary treatment in any of the 45 cases of NASH-HCC . This might be related to the tendency of NASH-HCC to have a large and multifocal tumor at diagnosis possibly restricting surgical approach. (63)

Over the last years, much information has come to find out the association

between metabolic syndrome, NASH and HCC. However there are still many queries on this topic including whether hepatic steatosis and NASH represent different disease states or a continuum, and what is the actual incidence of NASH-related HCC in Westerns Countries. In the future it is desirable to better understand the optimal management for NASH-related HCC, as well as determining the role of genetic markers of NAFLD and NASH in the diagnosis and prognosis of NASH-related HCC. (62)

In the paper written by Bril (64) an algorithm for the diagnosis of NAFLD and NASH in patients with prediabetes or T2DM has been suggested (Figure 17).

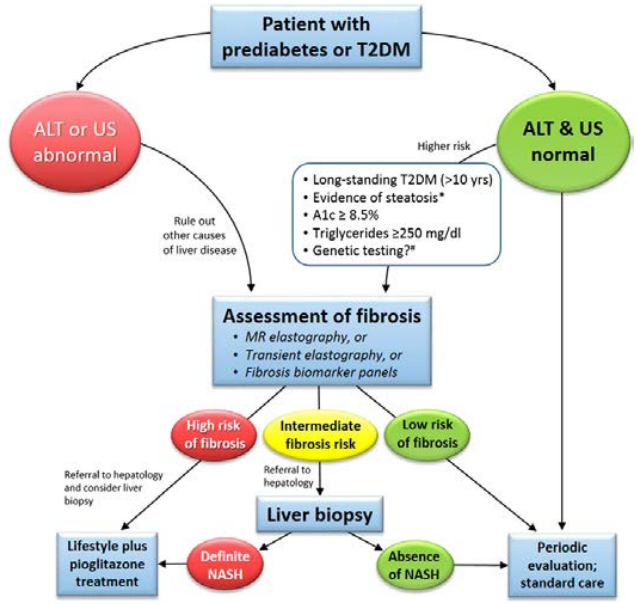


Figure 17. Algorithm for the diagnosis of NAFLD and NASH in patients with prediabetes or T2DM in clinical practice. This suggested algorithm is based on the authors' interpretation of available evidence.MR, magnetic resonance; US, ultrasound. *Based on results from more sensitive tests such as liver 1H-MRS, MRI-proton density fat fraction, or CAP. #Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M and/or transmembrane 6 superfamily member 2 (TM6SF2) E167 K.

PANCREATIC CANCER

Pancreatic cancer is a devastating disease characterized by an increased incidence in Western Countries, an extremely poor median survival of 4-6 months after diagnosis (65) and limited therapeutic options. (66)

The incidence and number of deaths caused by pancreatic cancer have been gradually increased, despite incidence and mortality of other common cancers has been declined along the last decades. (67) The difficulty to detect pancreatic carcinoma at an early stage, its aggressive progression, and the lack of effective systemic treatment are the main reasons of the poor prognosis of patients with pancreas cancer. So far many risk factors for this disease have been identified such as cigarette smoking, family history of chronic pancreatitis, advancing age, male sex, diabetes mellitus, obesity, non-0 blood group, high-fat diet, diets high in meat and low in vegetables and folate content, possibly Helicobacter pylori and hepatitis B virus infections, alcohol consumption and periodontal disease. (68)

Smoking is the most important environmental factor for pancreatic cancer. The carcinogens deriving from tobacco can extend to the pancreas by two modalities. The first one is by the direct oropharyngeal absorption the second is by the blood flow, and can indirectly damage the pancreas, facilitating duodenopancreatic reflux. Several papers showed a significantly higher risk in smokers than in nonsmokers, highlighting a direct connection with tobacco smoking especially in the 15 years before evaluation. (69) In the PanC4 studies, risk for those who smoked ≥35 cigarettes per day was more than 3 times that of never smokers, and those who smoked ≥30 years were at

more than double the risk In. the cohort studies, risk was highest in those with ≥30 cigarettes per day, >50 years of smoking, and >40 pack years.

Family history, defined as having at least one affected first degree relative, is another risk factor for pancreatic cancer. (70) A family history of pancreatic cancer is described in 5–10% of patients with this disease and the most commonly found mutations in families with pancreatic cancer are in BRCA2, with about twice the risk found in those with mutations in this gene.

From a genetic point of view, pancreatic cancer is fundamentally caused by inherited (germline) and acquired (somatic) mutations in oncogenes. The germline changes associated with ductal adenocarcinoma of the pancreas mainly involve BRCA2 gene. The exomes of ductal adenocarcinoma and of all of the most common types of tumors of the pancreas have been recently sequenced, providing new insight into the somatic mutations in these neoplasms. (71) This insight will change management of this tumor and forms the basis for new approaches to the early detection and treatment of pancreatic cancer. The sequencing of infiltrating ductal adenocarcinomas of the pancreas revealed that 4 genes, KRAS, p16/CDKN2A, TP53 and SMAD4, are each somatically altered in >50% of the cancers. KRAS, an oncogene on chromosome 12, is activated by point mutation in 95% of invasive ductal adenocarcinomas. (72) The protein coded for the KRAS gene is a small GTPase that plays an important role in cell signaling through the mitogen-activated protein kinase (MAPK) and other pathways. The point mutations in KRAS occur early in pancreatic neoplasm, and almost exclusively target 3 codons (codons 12, 13, and 61), making them relatively easy to identify and,

suggesting that KRAS mutations could form the basis for gene-based tests to detect early curable pancreatic neoplasm. (73)

Unfortunately, most pancreatic cancers present with no specific symptoms and are diagnosed late in the course of the disease when the cancer has already spread to other organs. Common symptoms include abdominal pain and in particularly epigastric pain that radiates to the back, unexplained weight loss, jaundice, clay-colored stools, nausea, and in around 10%, migratory thrombophlebitis (Trousseau's syndrome). (74) Patients with pancreatic cancer sometimes may present at diagnosis with new-onset diabetes mellitus or with signs and symptoms of chronic pancreatitis. Of interest, depression is common in patients with pancreatic cancer, and in some instances the diagnosis of depression is established before the patient is found to have the cancer. (75)

PANCREATIC CANCER TREATMENT

Pancreatic cancer is a complex and highly lethal disease that is best treated in the multidisciplinary setting. (76) The optimal treatment depends on accurate staging. Patients with Stage I/II disease should undergo surgical resection followed by adjuvant therapy. Neoadjuvant therapy should be considered in this patient population but is controversial, whereas patients with Stage III borderline resectable cancers should undergo neoadjuvant

therapy prior to resection. (77) Patients with stage III locally advanced disease should be treated with chemotherapy and/or chemoradiotherapy. Most of these patients develop metastatic disease; however, select patients can still be considered for surgical resection. Patients with Stage IV and good performance status may receive systemic therapy and those with poor overall health should be given supportive therapy. (78)

In Figure 18 is shown a flow chart diagramming a general approach to the treatment of pancreatic cancer.

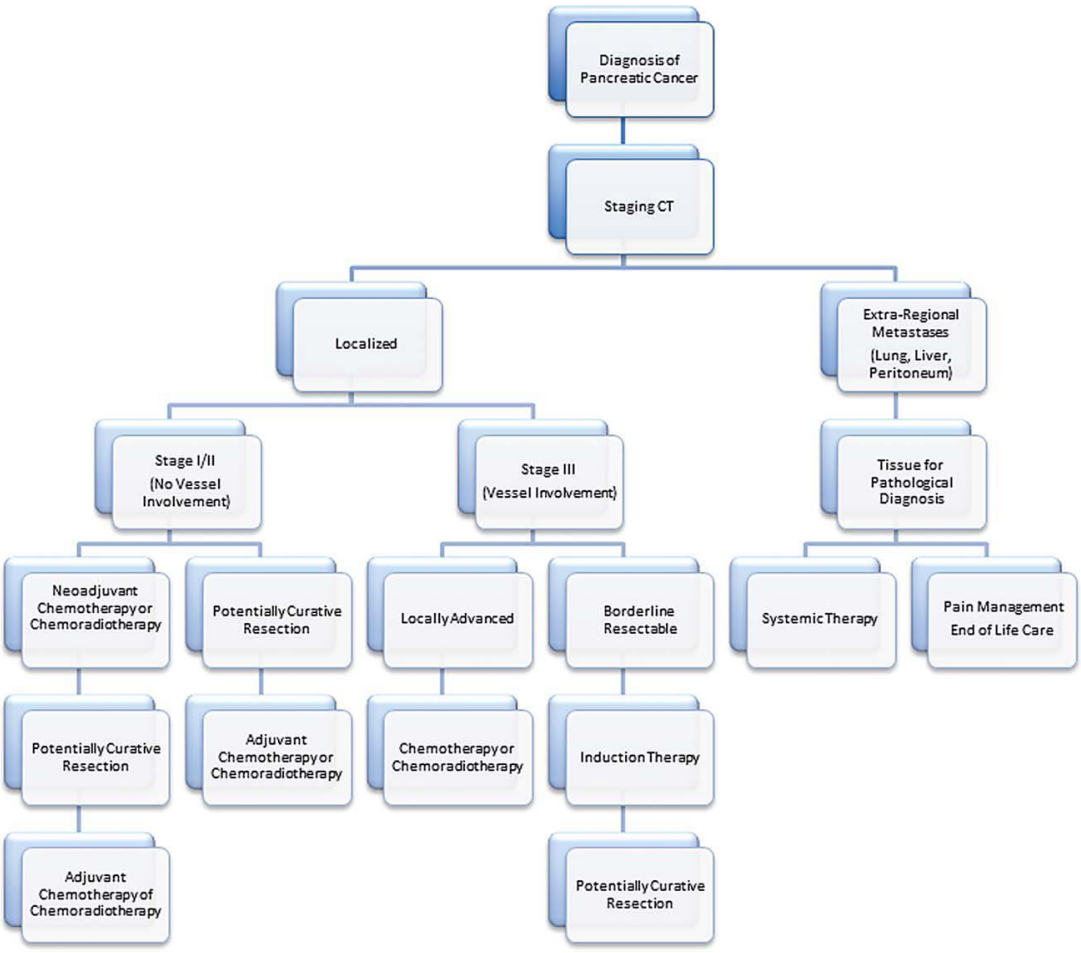


Figure 18. Treatment of pancreatic cancer © Johns Hopkins University

The surgical approach depends on the location of their tumor. Cancers arising in the head of the pancreas require a pancreaticoduodenectomy (Whipple operation), whereas those in the tail require a distal pancreatectomy with an en bloc splenectomy. Lesions located in the neck and body may require a pancreaticoduodenectomy, distal pancreatectomy or, rarely, a total pancreatectomy. Other partial resections, such as central pancreatectomy or enucleations do not result in an adequate lymphadenectomy and should not be considered curative resection for pancreatic cancer. The surgical resections are more often performed with a minimal invasive surgical approach. In the last years several papers have been published to demonstrate the feasibility and safety of the laparoscopic approach in pancreatic surgery.

Despite all the efforts prognosis is still very poor for this neoplasm.

However there are a number of bright spots on the horizon, including individualized therapies and the prevention of an invasive pancreatic cancer. (79)

Surgical procedures

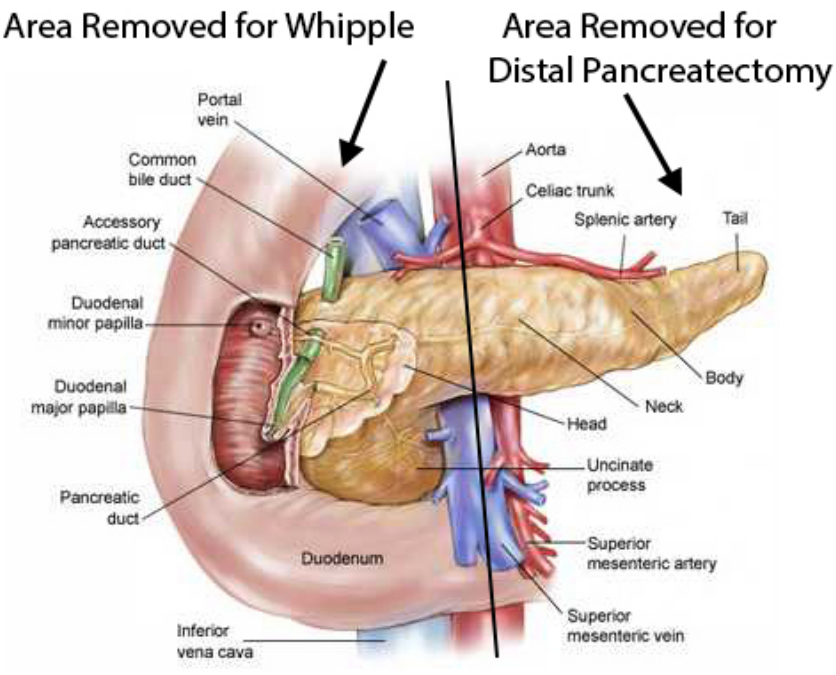


Figure 19. Resection areas for pancreatoduodenectomy or distal pancreatectomy

Pancreatoduodenectomy

Pancreaticoduodenectomy is a technically challenging operation with postoperative morbidity rates of 30 - 80 % even when performed at high-volume, tertiary referral centers.

The incision preferred for this operation could be a double sub-costal or a mid-line incision.

The peritoneal cavity is then visually inspected. If the indication for the procedure is malignancy, it is critical to assess the peritoneum and the liver for any radiographically-occult metastatic disease. The procedure steps could be summarize in:

1. Kocher maneuver to mobilize the duodenum-pancreatic complex

2. The gastrocolic ligament is incised just off of the gastroepiploic vessels and the lesser sac is entered. This dissection is carried up along the greater curvature of the stomach and is stopped at the level of the short gastric vessels. The dissection is then carried medially toward the origins of the right gastroepiploic vessels / Henle's trunk. It can be difficult to maintain the correct plane in this portion of the procedure. Maneuvers to facilitate this dissection include dividing any posterior attachments of the stomach to the transverse colon mesentery and pancreas, and mobilizing the hepatic flexure of the colon. The right gastroepiploic vessels are then ligated with a stapler and/or clips.
3. Attention is then turned to the region of the hepatic pedicle. The triangle of Calot is dissected and the cystic duct and artery are ligated and the cholecistectomy performed.
4. The peritoneum and fatty tissue in the supraduodenal region is then divided, layer-by-layer, in the region of the right gastric artery, to the left of the porta hepatis. The stomach is then elevated and the posterior aspect of the pylorus / proximal duodenum is dissected. The duodenum is then transected with a stapler just distal to the pylorus. If standard pancreaticoduodenectomy is preferred, the stomach is transected at the level of the incisura. The stomach is then tucked away in the left upper quadrant.
5. The next step is to identify the common hepatic artery (CHA) cephaled to the pancreas, which is usually evident by its pulsation. The CHA lymph node is dissected out and removed to provide exposure of the

gastroduodenal artery (GDA). The GDA is then dissected circumferentially. A silk tie is passed around the vessel and is tied down to ligate it proximally. Four metal clips are then applied, two proximally to reinforce the tie and two distally. The GDA is then divided sharply. There are often small branches posterior to the vessel that bleed during the course of this dissection.

6. Attention is then turned to the common bile duct (CBD). It is dissected away from the portal vein (PV) and proper hepatic artery (PHA) from medial to lateral, just above the superior border of the pancreas. Once the distal CBD is circumferentially dissected, it is temporarily occluded with a small bull-dog clamp. This is placed as far proximally as possible. The distal CBD is then divided sharply with laparoscopic scissors.
7. Next, attention is turned to the inferior border of the pancreas. The neck of the gland is identified and the inferior border is carefully mobilized with hook electrocautery. The superior mesenteric vein (SMV) is identified. The space between the SMV and the overlying pancreas is then carefully dissected until a tunnel is created. A Penrose drain through the tunnel is pass. Once this is complete, it is helpful to dissect out the right lateral aspect of the SMV until the first branch is exposed.
8. Now the ligament of Treitz (LOT) is identified. The mesentery to the proximal jejunum is divided a point approximately 15-cm distal to the LOT. The jejunum itself, however, is not transected yet. Once the LOT

itself is reached it is similarly divided. It is helpful to perform as much of the LOT dissection as is safely possible from this side of the superior mesenteric vessels, however, care should be taken to avoid injury to them or to the inferior mesenteric vein (IMV) during this dissection.

9. The distal duodenum / proximal jejunum is gently passed from left to right under the superior mesenteric vessels. The proximal jejunum is then transected with a stapler at the site of demarcation of the previously de-vascularized segment.
10. The duodenum is elevated and the plane between the posterior aspect of the uncinate process and the retreo-peritoneum is dissected with the harmonic scalpel.
11. The pancreatic neck is now diveded
12. The uncinate processi s now progressively free from caudal to cephal and the spicimen removed.
13. Pancreatic anastomosis is now performed in different possible fashion such as pancreato-jejunostomy or pancreato-gastrostomy.
14. Hepatico-jejunostomy is performed.
15. And final duodenal-jejunostomy or gastro-jejunostomy is performed.
16. Drains are placed.
17. Closure of the abdomen.

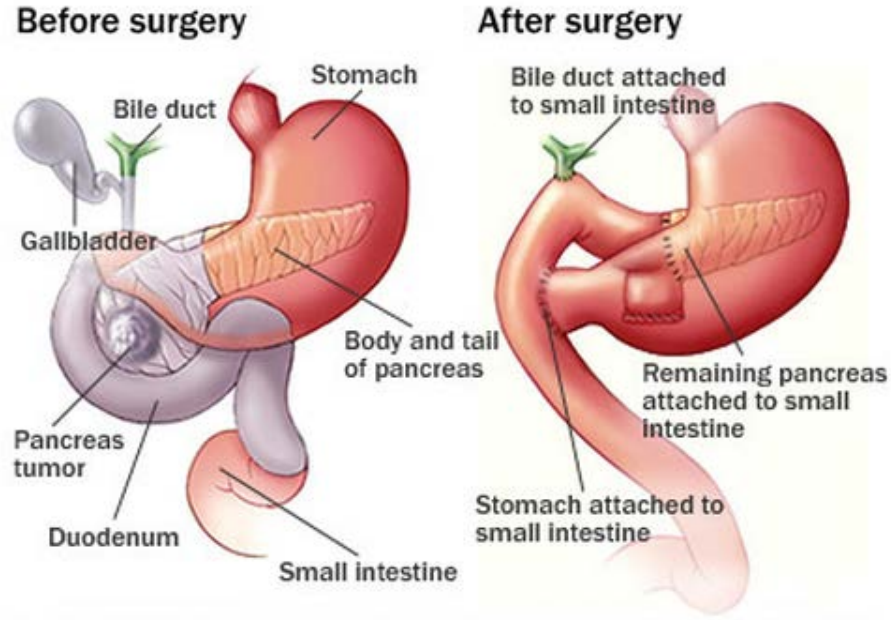


Figure 20. Pancreatoduodenectomy with classical Traverso-Longmire reconstruction

The most common complications of this procedure are the postoperative pancreatic fistula, the delayed gastric empty and the hemorrhage.

Distal Pancreatectomy

For tumor located in the body and tail of the pancreas the surgical treatment proposed is the distal pancreatectomy. This procedure is done with en bloc splenectomy in order to perform a correct lymphadenectomy taking out all the lymph nodes long the splenic vessels and around the splenic hilum. Several approach to this procedure are performed like the "clockwise technique" or the "posterior radical antegrade modular pancreatospenectomy" (RAMPS).

Here reported some common steps of the procedure:

1. Mobilization of the splenic flexure of the colon and exposure of the pancreas
2. Dissection along the inferior edge of the pancreas and choosing the site for pancreatic division.
3. Pancreatic parenchymal division and ligation of the splenic vein and artery.
4. Dissection along the superior edge of the pancreas.
5. Mobilization of the spleen and specimen removal.

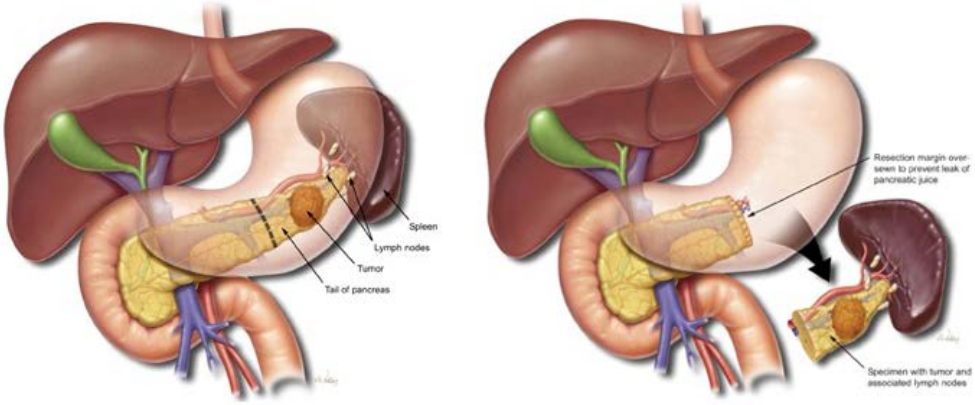


Figure 21. Distal Pancreatectomy

Postoperative complications are similar to the Pancreatoduodenectomy but in a minor percentage and with a most favorable post-operative course.

NON ALCOHOLIC FATTY PANCREAS DISEASE & STEATOPANCREATITIS

Evidence that obesity is linked to pancreatic cancer is developing and (80) as pancreatic steatosis is related to obesity, non-alcoholic fatty pancreas disease

(NAFPD) has been supposed to be involved in the development of pancreatic cancer. (81) NAFPD has also been suggested to cause pancreatic cancer through non-alcoholic steatopancreatitis (NASP), a concept analogous to non-alcoholic fatty liver disease (NAFLD), which can cause hepatic cancer via non-alcoholic steatohepatitis(NASH) and hepatic cirrhosis. In summary, it appears that pancreatic inflammation associated with obesity (pancreatic steatosis) and substantial pancreatic fibrosis, making this organ more susceptible to pancreatitis, may increase the risk of pancreatic cancer. Interestingly, many studies reported a positive link between pancreatic cancer and more elements of the syndrome such as abdominal obesity, hyperinsulinemia and elevated serum glucose level. (82)

It was recently reported by Li D (83) that the exact mechanism that explain the link between diabetes and pancreatic cancer is difficult to find but is known to include some metabolic, hormonal and immunological alterations that influence tumor growth. Some of the most hypothesized mechanisms underlying the association between type 2 diabetes mellitus and pancreatic cancer are IR and compensatory hyperinsulinemia as well as elevated levels of circulating IGFs. Insulin is a growth promoting hormone with mitogenic effects that promotes cell proliferation and increases glucose use, both of which being important to tumor development and progression. (84) Furthermore, as already discussed, insulin reduces the hepatic production of IGF-binding proteins increasing the bioavailability of IGF.

Figure 14 shows the effect of IR on endocrine production of insulin and GH effect on hepatic IGF-I production as well as autocrine and paracrine

production of IGF-I and IGF-II by tumor cells. (85)

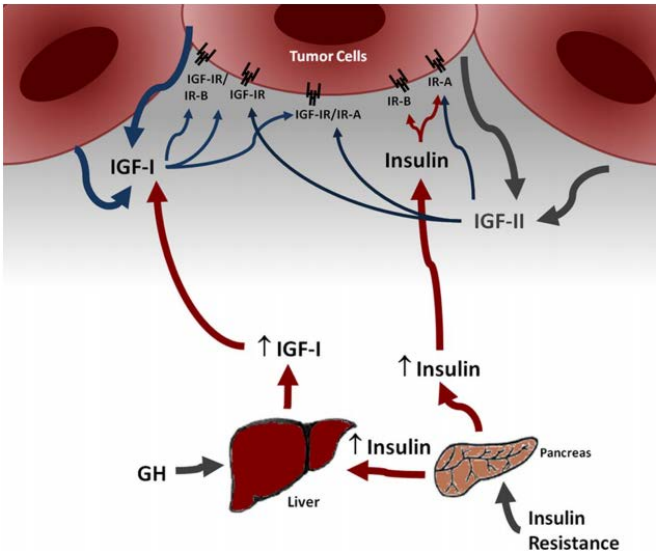


Figure 22. Endocrine, autocrine, and paracrine signaling of insulin, IGF-I, and IGF-II in tumor cells. Schematic representing the effect of IR on endocrine production of insulin and GH effect on hepatic IGF-I production as well as autocrine and paracrine production of IGF-I and IGF-II by tumor cells. Arrows from insulin, IGF-I, and IGF-II demonstrate their interactions with the IR isoforms (IR-A and IR-B), the IGF-IR, and the hybrid receptors (IGF-IR/IR-A and IGF-IR/IR-B)

IGF-1 has a more potent mitogenic and antiapoptotic activity than insulin and may act as growth factor in cells expressing insulin and the IGF-1receptor (IGF1R). (83) Importantly, pancreatic cancer cells highly express IGF-1 and IGF1R and IGF-1-mediated signaling transduction increases proliferation, invasion and expression of angiogenesis mediators and decreases apoptosis in pancreatic cancer cells. (86) Data from animal studies also indicate that the islet cell turnover, which is associated with IR, is critical for pancreatic carcinogenesis. (83) For example, in hamsters, stimulation of islet cell proliferation, enhanced pancreatic ductal carcinogenesis, and destruction of islet cells by treatment with streptozotocin or alloxan inhibited pancreatic

cancer induction. (87)

Another possible mechanism includes the effect of adiponectin, a protein secreted by adipocytes that is lower in diabetics and in patients with metabolic syndrome. Adiponectin levels are negatively related with plasma glucose and insulin concentration, although it is not clear whether this is a cause or a consequence of IR. Moreover, hypoadiponectinemia may be considered an independent risk factor for hypertension and also has been associated with an atherosclerotic lipid profile, as it is an independent predictor of high-density-lipoproteins. (88) In addition, adiponectin has been shown to inhibit endothelial cell proliferation and migration and has been involved in the etiology of several cancers, particularly pancreatic cancer. (89, 90)

Circadian clock is another important aspect to consider in pancreatic carcinogenesis. It is an important regulator of metabolism and disruption of this clock has been implicated in various pathologies, ranging from obesity, diabetes and metabolic syndrome to cancer such as pancreatic cancer. (91)

Recent research also suggests that cardiovascular abnormalities, neurodegeneration and pancreatic carcinogenesis can be prevented by the intake of several dietary antioxidants (e.g., coenzyme Q, vitamin C and E,selenium) and phytochemicals (e.g., ellagic acid, curcumin, lycopene, epigallocatechin gallate, and resveratrol) that are present in fruit, vegetables, herbs and medicinal plants. (92) Several studies indicate that the presence of metabolic syndrome or a greater number of its components are associated with a significantly higher risk for all-cause cancer mortality, as well as

pancreatic cancer mortality.

Diabetes, a major public health problem worldwide, is currently considered the key component of metabolic syndrome in pancreatic carcinogenesis and understanding of the pathological association between diabetes and pancreatic cancer would help to the develop novel preventive and therapeutic strategies for pancreatic tumor. (87)

SECTION 2

INTRODUCTION TO SECTION 2

As already discussed in the first section of my thesis diabetes and metabolic syndrome are indipendent risk factors for the development of both hepatic and pancreatic cancers.

Therefore diabetes can increase the risk of hepatic cancer occurance but is still unknown if diabetes can also influence tumor progression.

In order to understand the behaviour of hepatic cancer in a setting of diabetes an in vivo study on a murine model was performed.

This part of my thesis was realized under the supervision of Professor Annarosa Arcangeli, Full Professor of Medical Pathology at Careggi University Hospital in Florence.

The experimental part was carried out in collaboration with Dr. Angela Guerriero.

METHODS USED IN THIS THESIS

THE TRANSFECTION

The gene transfer is a powerful tool enabling study of the function of genes and protein expression. The terms transformation, transfection and transduction all refer to the insertion of foreign DNA in the cell nucleus, but in the first case the host is generic cell, in the second it is an eukaryotic cell and in the third case the transfer is via the mediation of virus.

The discovery of transformation is a milestone in biology history as it has shown that DNA is the genetic material.

At the end of the 1920s Fred Griffith, working on Streptoccus Pneumoniae, showed that its virulence was due to the presence or absence of a polysaccharide capsule of which strain S (smooth) was provided, but not strain R (rough). Mice infected with S-type cells die in just two days, while nothing happens with type R. Griffith saw, however, that in the case of injected smooth cells, previously killed using heat, along with rough-type cells, the infection was still lethal and the bacteria isolated from the dead mouse were of S.

In order to get more evidence, several types of strain S with different compositions of the Polysaccharide capsule were injected together with strain R and the detected bacteria in the killed mice presented that specific structure (93).

The process was defined as transformation and was explained from a molecular point of view only in 1944 by Avery, MacLeod and McCarthy at the

institute Rockefeller in New York after a decade of experiments: they demonstrated that the phenomenon was reproducible in vitro as in vivo and that was just the extract of the cells killed by heat to induce transformation. This was purified and it was seen that it was DNA; later, other researchers at the Rockefeller Institute showed that other characters beyond the capsular capability could be transmitted with transformation (94).

"If we are right, and of course that is not yet proven, then it means that nucleic acids are not merely structurally important but functionally active substances in

determining the biochemical activities and specific characteristics of cells and that by means of a known chemical substance it is possible to induce predictable and

hereditary changes in cells. This is something that has long been the dreams of geneticists."

Oswald T. Avery, 1943

The discovery was not accepted unanimously: many scientists believed that the process of transformation was due to some protein associated with nucleic acids.

In the past decades the idea that only proteins possessed the structural complexity to carry hereditary information was widespread; the DNA was considered only a monotonous chain of four repeated nucleotides.

Bacterial transduction was discovered by American scientist Norton Zinder (95) while studying with Lederberg, who already had discovered bacterial conjugation, on genetic recombination in Salmonella Typhimurium. Zinder wanted to demonstrate that conjugation could take place in others organisms in addition to E.Coli.

During the experiments, however, he noticed that mutants were generated without the need of cell to cell contact as was expected in the conjugation process.

At that point a transformation process seemed to has been conduct but it was not inhibited by the use of DNase and the transfer factor seemed like a bacteriophage.

It was also shown that the transduction process occurred only with recipient cells carrying the virus in question on the outer membrane (96).

Zinder and Lederberg coined the term to define any process of genetic recombination that was fragmentary and did not require cell to cell contact, then it was used to indicate only a gene transfer mediated by a virus.

The term transfection was coined in 1964 by Foldes and Trautner and derives from the combination of the two words transformation and infection. The term transfection is mainly used when we refer to the introduction of exogenous nucleic acids into eukaryotic cells without the support of viruses; cells that incorporate DNA are defined transfected. This technique has been expanded during time thanks to the development of reporter genes, the ability to make transfections stable and to express transfected DNA.

Insertion of DNA fragments into eukaryotic cells plays an important role both in research and in clinical practice, in the field of gene therapy. In fact this technique is used *in vitro* to detect regulatory sequences, gene structures and functions and protein production (97). While *in vivo*, thanks to transfection, the production of transgenic animals to be used as models for the understanding of molecular mechanisms of human pathologies has been used (98).

In the last year scientists developed many transfection techniques, but during the process not all cells are transfected, in fact it is very important to start with a very high number of cells because the process itself is inefficient, only a small percentage of them can receive DNA, and an immortalized cell line free of contamination is needed.

Transfer in non-viral transfections occurs via a carrier that needs to have three fundamental features: origin of replication, region where it begins DNA replication, multi cloning site, needed to insert the DNA of interest, and selection markers, which allows the operator to let only transfected cells to grow.

Two types of transfections can be performed:

- Transient: genes are expressed by the receiving cell for a limited time, usually 24-96 h; It is useful for short-term experiments.

- Stable: cells incorporate exogenous DNA into the genome, so it is possible to isolate and propagate transfected clones.

As a general mechanism, transfection needs to neutralize or overstep the negative charges of cell membrane to allow DNA, as a negatively charged molecule, to entry into the cell.

Different methodological approaches can be classified into three categories:

- Chemical methods,
- Physical methods and
- Viral methods.

CHEMICAL METHODS

Chemical methods include:

- Dietiloamminoetile (DEAE)
- Calcium Phosphate
- Activated dendrimers
- Magnetofection
- Liposomes

I will focus on the liposome technique that was the one used for the experiments.

LIPOSOMES

The term liposome refers to a layer of lipids that, in a liquid fluid, forms colloidal particles (99); for the first time in 1980, artificial liposomes were created to transfer DNA into the cells (100). Next, synthetic cationic lipids have been developed (101) in which the part of the head is associated with

the phosphate groups (negatively charged) of nucleic acids. These are often mixed with neutral lipids such as L-dioleoyl phosphatidylethanolamine (DOPE) that increase the capacity of gene transferring of cationic lipids (102, 103).

The lipid-DNA complexes merge with the cell membranes and release spontaneously their content in the cells. Following its internalization, the complex takes place first at endosomal stage and then at a nuclear stage. The DOPE is considered a "fusogenic" lipid allowing both the release of complexes from endosome and facilitating the fusion of lysosoma / nucleic acid complex with the outer membrane of the cell.

This technique has high efficiency and is effective with cell types that are resistant to DEA and calcium phosphate. Moreover can mediate the transfer of amount of DNA higher than those brought by viruses, from oligonucleotides to yeast chromosome (101, 102, 104,105), is able to convey DNA (106) and proteins (107); can be used for *in vivo* transfer of DNA and RNA to animals and humans (108).

Cells transfected with this method integrate the exogenous DNA within the chromosomes or keep it as episoma. An example of liposomes are lipofectamines.

PHYSICAL METHODS

They were developed for the first time in the early 1980s, using techniques that allow direct entrance of DNA into the cytoplasm without the aid of any tool.

They are:

- Electroporation (which will be explained below)
- Microinjection
- Gene-Gun

Electroporation

It was the first technique used for gene transfer in murine cells (109). At the moment is one of the most advanced methods used in laboratory because of its effectiveness even in the most difficult cells to transfect. The cells are exposed to a high intensity electric field that destabilizes cell membrane: at this time it becomes highly permeable to molecules that are in the outer culture field. The electrical pulses produce temporary pores through which the nucleic acids can enter into the cytoplasm (110). When the electric field is switched off, the pores in the membrane are closed and the DNA remains caught inside the cell.

Electroporation requires a sophisticated power supply unit because the pulses must be precisely controlled and pulses should last for only few milliseconds.

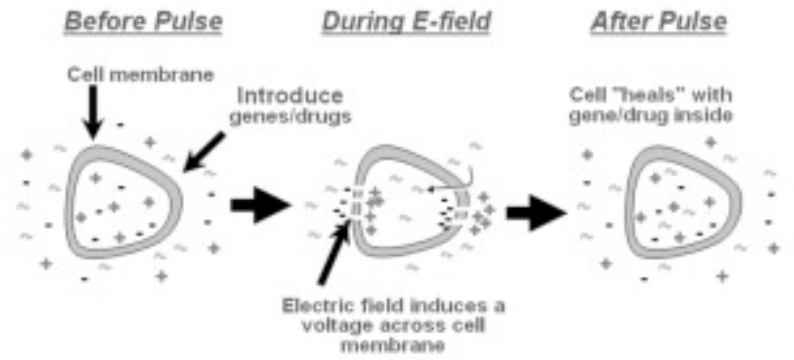


Figure 23: Elettroporation effect on cell membrane.

The pulses with which the electric field is applied may be exponential decay or square waves forms. For this technique effectiveness it is crucial to know the cellular line that is being handled in order to optimize power, durability and number of pulses. The right combinations of the various parameters are needed to allow the carrier to enter without a massive stress for the cell.

Electroporation requires a very high number of cells compared to that required by chemical-based method. The technique is indeed very invasive and, even if a high optimization for the cellular type is achieved, a high mortality is registered and relatively few cells manage to internalize the carrier.

Overall it is a high efficient, easy-to-perform technique that can be applied to many cell types and, being not a chemical method, does not alter biological functions or cellular structure.

There are two parameters that determine the various types of electroporation: the power of the electric field and time.

The electric field, E, is measured in volts / cm and describes the electric environment that it is generated in the room where electroporation occurs. In the cuvettes used the electrodes are two parallel blades separated by a distance, d, among which is applied a voltage, V:E = V/d

With time it is considered how much the cells are exposed to the electric field. If this is applied with exponential decay the duration cannot be set, in the case of square wave, the time parameter can be set.

The exponential decay pulse is generated by discharging the condenser: the voltage between the electrodes increases rapidly until reaching a peak and decays at a time t dependent on the circuit resistance, R, expressed in ohms, and from its capacity, C, expressed in microfarad, according to the equation:

$$Vt= V0 [e-(t/RC)]$$

An impulse of square waves instead comes from blocking this after the condenser discharge: from a theoretical point of view the same voltage at the beginning as well at the end of the pulse is achieved, in practice the initial voltage is always higher than the final one. The wider is the length of application of the electric field, indicated by T, the more marked is this difference. Square wave decay is expressed by the following formula:

$$\ln (V0 / Vt)= t / (R C)$$

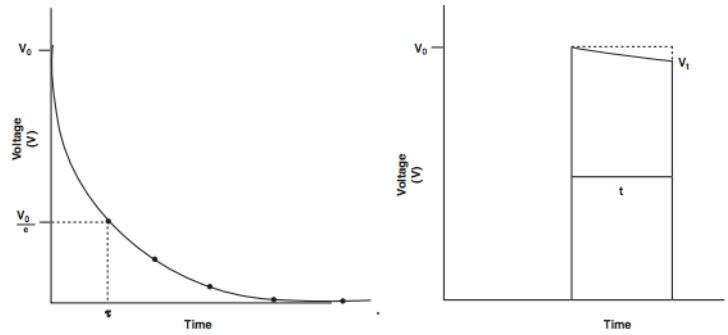


Figure 24: To the left, the graph of the exponential decay function. The point τ, on the absciss axis, indicates the time at which a voltage decay of 37% from the nitial value V0. To the right, the graph showing square wave decay. The difference between the initial and the final voltage is defined as droop and is described by the formula Droop = (V0 - Vt) / V0.

VIRAL METHODS

Although transfection has always been effective as a technique for gene transfer, how to use viruses in this field has begun to be evaluated both as an alternative method and for their possible use *in vivo.*

Many classes of retroviruses, adenoviruses, lentivirus and adeno-associated viruses have been

used for the development of recombinant vectors with the aim of obtaining lines stably transfected (111).

SELECTION MARKERS

In order to verify that exogenous DNA has been inserted in the target cell, a gene that allows the selection of cells that have incorporated the nucleotide sequence accompanies the carrier. The gene selection will give resistance to a certain substance that will be present in the cell culture medium; in this way, the operator can select positively transfected cells. Selective pressure has a twofold meaning: on the one hand it is necessary to isolate the small

percentage of cells that have incorporated DNA at the moment of transfection (approximately 1 out of 104 cells), on the other hand it allows to obtain a stable transfection while maintaining the cellular line over time. The most common selection markers are genes that confer resistance to antibiotics, such as puromycin and geneticin, G418. Geneticin resistance is conferred by the aminoglycoside phosphotransferase gene (APH). The Pac gene gives puromycin resistance that encode for n-acetyl transferrin puromycin. Even the NeoR gene is very used in plasmids and has the peculiarity to confer resistance to geneticin, neomycin and kanamycin, all aminoglycosides antibiotics. While the APH gene is not very effective in bacteria, the gene NeoR is optimal in both these and eukaryotic cells. It's important to consider that bacterial cells are sensitive to all three antibiotics above, while eukaryotic cells only to the geneticin.

REPORTER GENE

The purpose of the various transfection methods is to study the function of a gene and / or to evaluate its level of expression. In the first case the gene of interest is preceded by a standard promoter, in the second one there is the fusion of the promoter of the gene of interest with a reporter gene, which makes possible to quantify their activity.

Reporters have been important in the study of gene expression and eukaryotic cell regulation. Their role has been significant in both *in vivo* and *in vitro*. Generally, the reporter gene is inserted inside the carrier that must be transferred to the cells. Since this is useful to follow the presence of

exogenous DNA, it is crucial that it is not expressed at the endogenous level in the cell of interest. Moreover is important that protocols to evaluate its presence are available and sensitive, fast, reproducible, safe and able to give quantitative information.

Another aspect to consider among the features of the reporter gene is a possible interference with physiologic cellular metabolism. All the pathways of signaling and protein functions must remain unchanged. Proteins derived from reporter genes can be quantified by using their properties as enzymatic activity, spectrophotometric characteristics or indirectly with antibody-based assays.

Enzyme tests are sensitive due to the fact that a small amount of reporter enzyme is needed to allow readings. However enzymes have the limit of not being usable if they are present at an endogenous level (eg β-galactosidase). Antibody tests are less sensitive, but they have the advantage of being able to detect if the enzyme has been synthesized and if the enzyme is or not active.

Examples of translated and transcribed proteins from reporter genes are:

- β-galactosidase, E.Coli's LacZ gene. Converts into colored compounds substrates such as

X-Gal (producing blu colored colonies of bacteria), and o-nitrophenyl-β-D-galactopyranoside (ONPG) (making the soluble compound yellow);

- GFP, green fluorescent protein. Expressed in the medusa Aequorea Victoria. In nature produces bioluminescence in a Calcium-dependent process due to an energy transfer. Ultraviolet light exposure of the protein produces

autofluorescence of green color in the absence of aequorina or any cofactor or substrate. There are several variants that differ only in punctiform mutations and emit light at different waves lengths, such as CFP (Cyan) and YFP

(Yellow);

- Luciferase, which oxidizes its substrate, a luciferin, with the production of bioluminescence.

One of the best analysis methods of reporter genes and / or their products is the one based on the production of photons by the reporter system.

The use of selection markers can be:

• MONITORING OF SIGNALING PATHWAYS

• STRUCTURAL ANALYSIS OF PROMOTORS

• PROMOTOR POLYMORPHISM ANALYSIS

• RNA INTERFERENCE

• DOUBLE IBRIDO

• IMAGING *IN VIVO*

In our experiments we used Imaging *in vivo*. The ability to express the luciferase gene in tumor cells is a powerful tool in animal models of human neoplasms. In particular is useful for studying tumor growth and testing new therapies. The light emission produced by the exogenous enzyme is transmitted through the tissues even at low intensity and there are tools able to detect photon emission used to follow *in vivo* the tumor.

The expression of reporter genes allows following tumor growth and regression in animals with a non - invasive method without the need to sacrifice them. Instruments currently available have very high sensitivity and are able to identify even 1000 tumor cells transformed with the luc gene within the peritoneal cavity of a mouse.

LUCIFERASE

Luciferase genes have been cloned from bacteria, from insects like the fireflies, from Renilla, Aequorea, Vargula and Gonyaulax. Among these only the first three are used as indicators of gene expression. Bacterial luciferases are not effective for mammalian cell analysis and are used to give autonomous luminescence in bacteria.

FIREFLAY LUCIFERASE

Firefly, luciferase is the most widely used reporter. It's a 61kDa monomeric enzyme catalyzing an oxidation composed of two reactions that generate light at waves-lengths of 550-570 nm, in the region from the green to yellow. In the first step of the reaction, the luciferin is

carboxylated by ATP and a reactive adenylate is product. This adenylate reacts with oxygen creating two oxidized products:

Oxyluciferin and CO2. During the reaction, a first emission of light is initially produced. This light declines in 15 seconds to a lower level of luminescence that last longer.

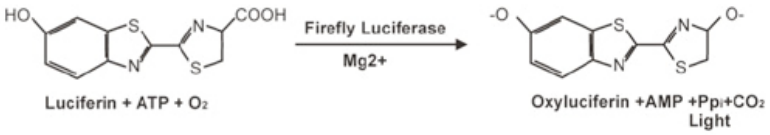


Figure 25. Mechanism for fireflay luciferase

Various strategies have been suggested to modify the enzyme in order to obtain a stable luminescence: the most effective was the one that included incorporation of coenzyme A to get a luminescence with maximum intensity that decayed slowly after few minutes. It is not clear how coenzyme A intervenes in the reaction, although probably the two enzymes have had a common evolution: the amino acid sequence of firefly luciferase has a connection with several families of acetyl- coenzyme A-synthetase with which it shares catalytic mechanisms. In experiments with coenzyme A, luminescence is generated in less than 0.3 seconds with one enzyme concentration below 10-20 mols of enzyme.

Fireflay luciferase is encoded by luc gene, does not need post-transcriptional modifications and is found in the enzymatic form directly following mRNA translation. Catalytic power is immediately active from ribosomes release. Moreover, luciferase has a very short half-life in cells, about 3 hours.

STUDY AIM

The aim of the study was to investigate HCC growth in mice affected by diabetes.

In particular the objectives of the experiments performed were:

- Production of murine models for human HCC that is traceable in vivo with optic imaging.
- Evaluation of the effects of hyperglycemic diet on these murine models of human HCC.

MATERIAL AND METHODS

CELL CULTURES

Defrosting the cell lines

The cell lines are stored in liquid nitrogen and at the time of need is left again in culture. The vial containing the cells is transferred to a becker glass filled with water, brought about 45º C, and inside this is transferred under the hood. Under cellular sterility conditions, the contents of the vial are centrifuged, 1200 x g 5' to eliminate DMSO and the pellet is suspended in complete serum medium then place it in a plate or in a flask depending on the condition of cell line growth. Depending on the culture conditions, cells are then passed, usually after 24-48 h, and kept in culture.

Keeping the cell lines

The cell lines used for the experiments performed in this study have all been kept in incubator at 37° C with 5% CO2.

HEPG2 characteristics

Hep G2 is a human liver cancer cell line. Hep G2 is an immortalized cell line that was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. These cells are

epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice. The cells secrete a variety of major plasma proteins, e.g., albumin, transferrin, and the acute-phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin, and plasminogen. They have been grown successfully in large-scale cultivation systems.

HepG2 cells and their derivatives are used as a model system for studies of liver metabolism and toxicity of xenobiotics, the detection of environmental and dietary cytotoxic and genotoxic (and thus cytoprotective, anti-genotoxic, and cogenotoxic) agents (112) to understand hepatocarcinogenesis and for drug targeting studies. HepG2 cells are also employed in trials with bio-artificial liver devices.

METHODS OF CELL TRASFECTIONS

Lipid mixture

We used two different products both based on the use of lipid mixture: X-tremeGene HP DNA Transfection Reagent and X-tremeGene 9 DNA Transfection Reagent, both products by Roche brand.

Both kits contain a reagent made up of a mixture of lipids and others components dissolved in 80% ethanol, filtered with membranes equipped with filters of 0.2 microns. Their action explores forming a complex with the DNA that comes this way carried inside the cells.

It can be used both for stable and transient transfections, both in presence or in absence of serum. To get the best results from the kit, the carrier used

must have a higher ratio near possible to the value of 1.8, given the ratio between the absorption at 260 nm and that at 280 nm, must have been prepared by using sterile TE buffer or water to an internal concentration of 0.1-2.0 µg /µl. As for the cells it is advisable to seed them the day before so that they are growing and must be aware that they are not contaminated by Mycoplasma. In our case, all these conditions were respected and our DNA was dissolved in H2O sterile.

Before transfection it is good to remove from the cell growth medium any type of additive, such as antibiotics and fungicides, and provide a method for verifying the actual presence of the carrier and select the cells. In our case the reporter gene provides the resistance to the G418. The selection of stable clones was started 24 hours later by adding G418 to the fresh complete medium. The two reagents have two very similar protocols, but differ in some steps.

Elettroporation

The electroporation system is an instrument that exposes cells to a field of high electrical voltage to obtain a temporary rearrangement of the cell membrane to allow the solutes of the external solutions to enter into the cytosol: among them the endogenous DNA.

The instrument used was the Gene Pulser XcellTM (Biorad), able to generate both exponential and square waves impulses. It's composed from a main unit generating the electric field and an external room where electroporation takes place and where the appropriate cuvette is housed with electrodes.

Figure 26: Elettroporation system Gene Pulser XcellTM

DETERMINATION OF CELLULAR EMISSIONS WITH BIOLUMINESCENCE

In order to evaluate, as a result of transfection, that the gene of our interest was properly inserted and the translated protein was active and functioning the following luciferase test was performed: Luciferase Assay Systems (Promega).

300,000 cells were plated at 70-90% confluence and centrifuged in their medium at 2000 x g for a time of 5' at room temperature; the supernatant liquid and the pellets were kept dry at -80ºC until reading. The Promega kit consists of three components: Luciferase Assay Substrate, lyophilized, Luciferase Assay Buffer, in a volume of 10 ml, Cell Culture Lysis Reagent, in a volume of 30 ml at 5X concentration. The buffer is composed as follows: 125 mM Tris, 10 mM EDTA, 10 mM DTT, 50% glycerol and 5% Triton X-100. In the first step of the protocol, it is necessary to prepare the Luciferase Assay Substrate, adding 10 ml of Luciferase Assay Buffer (the unused portion of the new compound will be stored at -80 ° C), and dilute the Cell Culture Lysis Reagent with water, to a 1X concentration. The protocol differs according to the cell type used for the experiments.

Regarding the lysate of mammalian cells, proceed as follows:

- Add 50 µl of CCLR 1X to the lysate and re-suspend the pellet;

- Transfer 20 µl of lysate into the multiwell plate provided with the instrument of reading "Glomax 96 microplate luminometer" (Promega);

- Place the Luciferase Assay Reagent consisting of the substrate and the buffer in the appropriate position and insert the canula inside it: the instrument then will take the amount indicated by the operator using the software "Glomax".

Before starting, the luminometer will take a small amount of reagent to get the preliminary data required for reading;

- Set the sample and reagent amounts, respectively 20 and 100 µl in the software;

- Start reading process.

The data provided by the software is RLU / s (relative light unit).

IN VIVO TECNIQUES

Stabulation

All mice of the NOD-SCID strain were used in this study.

The NOD-SCID strain, Non-Obese Diabetic SCID, in addition to the hematopoiesis mutation, develops insulin dependent diabetes mellitus autoimmune (113).

Cause their immune deficiency, the animals were isolated and kept in a sterile room. Their cages, as well as water dispensers, were autoclaved

before use, with the CISA autoclave model 640, which is emptied directly into the sterile area. The cages were placed inside a cabin with controlled airflow. Access to the sterile area was limited and was allowed only to the staff of the lab that must wear at the entrance, in the anteroom, mask, shirts, headphones, covers and sterile gloves.

Monitoring of human HCC in mice affected by diabetes

For all the experiments cellular lines type HEPG2 were used. The cells were transfected in our laboratory with luciferase gene, *luc*-2.

We used 6 males and 6 females aged 16-weeks of which liver was surgically inoculated with 2 x 10^6 cells of HEPG2-*luc*-2. The trend of the disease was than monitored by optical imaging (see next paragraph).

Monitoring of human HCC in mice affected by diabetes after high sugar diet administration

Six SCID females aged 16 weeks were subdivided into 2 different treatment groups. All animals were surgically inoculated, with 2 x 10^6 cells of HEPG2-*luc*-2, in their liver. From the 1st day after the inoculum 4 animals were fed with 35 grams of glucose dissolved in 250 ml of water per day. The remaining 2 animals were fed with standard diet.

OPTIC IMAGING

The cell lines transfected with the luciferase enzyme gene were surgically and intraepathically inoculated and were followed along with the course of the disease, by imaging technique.

The luciferase enzyme, in the presence of D-Luciferin substrate, makes one bioluminescence reaction.

D-Luciferin was purchased by Caliper LifeScience. The full name of the product is XenoLight Rediject D-Luciferin; in a concentration of 30 mg/ml and 150 mg/kg intraperitoneal administration is recommended for *in vivo* analysis on mice.

After adding the substrate, the signal produced by the luciferase enzyme present in the tumor cells was acquired through Photon Imager (Biospacelabs), an instrument able to read both in bioluminescence, as our interest, or in fluorescence.

The instrument is equipped with CCD sensors, which convert light intensity into electrical signal. Since it has been designed to record also very weak signals it uses the intensified charging-coupled device (iCCD) which amplifies the signal 106 times thanks to the association of an image intensifier with the sensor. Photons are focused and, through a series of lenses, arrive at photocathode level and at the end the photoelectric effect converts photons into free electrons.

The number of electrons released depends on the intensity of the signal; these are then amplified through a microcanal plate, MCP, which plays a central role in improving signals of weak intensity. The electrical signal comes then again converted into photons by a phosphorescent screen so that

the camera equipped with an iCCD sensor can record a bright spot. To evaluate the signal in the animals at each reading measurement the mice were weighed to determine the volumes to be injected. They were first sedated with 9 µl / g of anesthetic avertina 2.5% and then 5 µl / g of D-Luciferin were injected via intraperitoneal.

The mice were placed on the plate adapted for the PhotoImager; during the measurement reading this is heated to balance the physiological decrease in body temperature during anesthesia. After 5' from the injection of the luciferase substrate (this time is needed for its distribution in the body) the reading starting and it has a duration of 3'. Images of animals in the ventral position were acquired. The acquisitions were analyzed with the program provided with the instrument, M3 VISION, which allows, among its various functions, to derive the cpm, counts per minute, emission in every area of the image, then in each mouse, and to change the range of the image.

You can change the range of photons displayed, from a minimum to a maximum, and the smoothing, a filter that allows increasing or decreasing the size of the pixel corresponding to the photons emission.

Both parameters serve to eliminate background noise in the image and serve to choose the most accurate range depending on emission level of mice bioluminescence.

In our study measurements were made after 6-10-17-24 days.

In the first experiment measurements were made even after 30 days.

RESULTS AND DISCUSSION

In order to study the behavior of human HCC, both *in vitro* and *in vivo* techniques can be used. The information that can be obtained from cell line culturing tests remains, however, limited and preliminary, and although crucial to address subsequent studies, there is no evaluation of the experimental variables that occur when tumor growth is studied within the more complex living system of a guest, as in this case the mouse.

The use of genetically modified animals has allowed the study of innumerable human neoplasms where it was possible to test new therapeutic drugs and then translate it to the clinic. In the recent years, in this field, an advanced technique allows to track tumor growth and evolution directly on the live animal. This technique is not invasive; in fact the animal is inoculated under anesthesia with tumor cells previously modified in order to express a gene reporter responsible for their traceability. This technique is called optical imaging on small animals and it has the ability to use fluorescent or luminescent reporters, as in the experiments conducted in this study.

During the study two experiments were performed.

In the first experiment 6 males NOD-SCID mice, aged 16 weeks, were used in order to develop a model for human HCC growth.

All mice were injected surgically into the liver with HEPG-*luc*-2 cells. None of the animals reported complications and / or mortality in relation to the surgical procedure. No complications or mortality occurred during the 24 days of the experiment.

At 6 days after injection, 50% of mice showed a satisfactory liver uptake without apparent signs of peritoneal dissemination. In 2 mice liver uptake was almost absent, in 1 mouse there was a "splenic-like" uptake probably related to an injection into the left lobe of the liver. (Image 1)

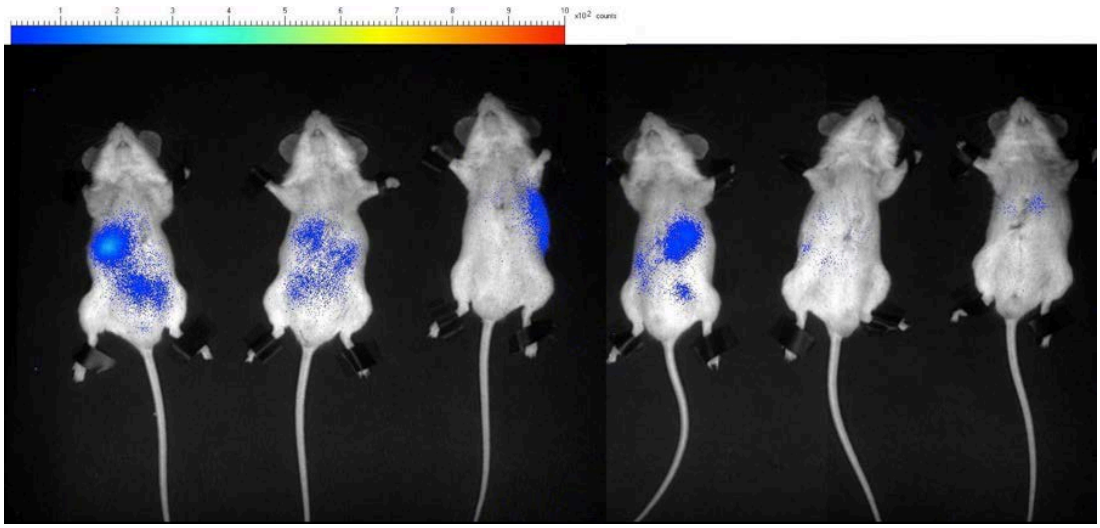


Image 1. Control after 6 days.

At 10 days, there was a progressive increase of the signal even in the 2 mice previously with no uptake. (Image 2)

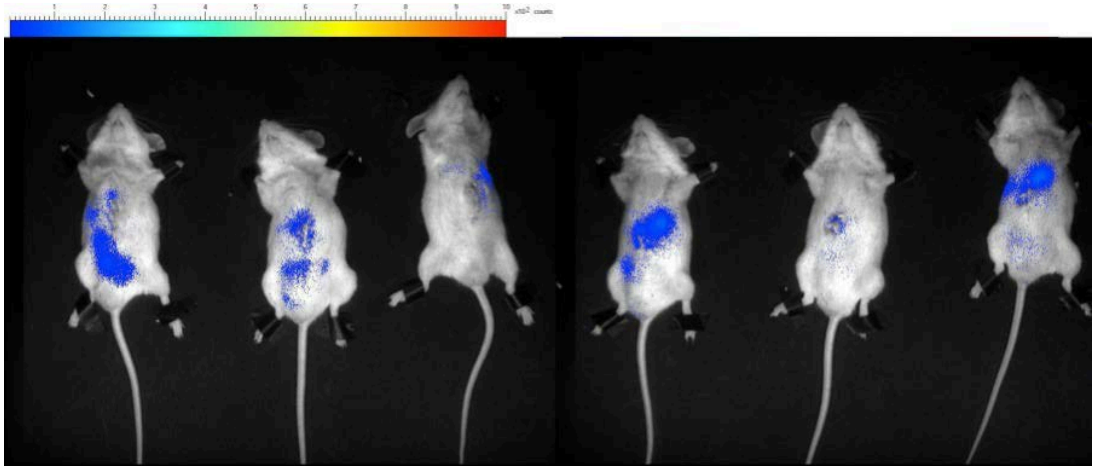


Image 2. Control after 10 days.

At the end of the experiment in 5 mice, the uptake increased further in intensity as for the spread of extraepathic disease. (Image 3)

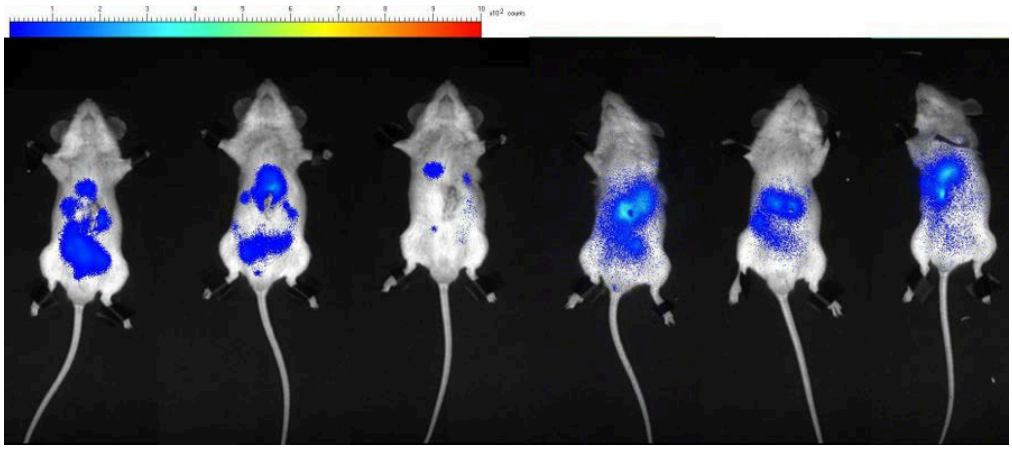
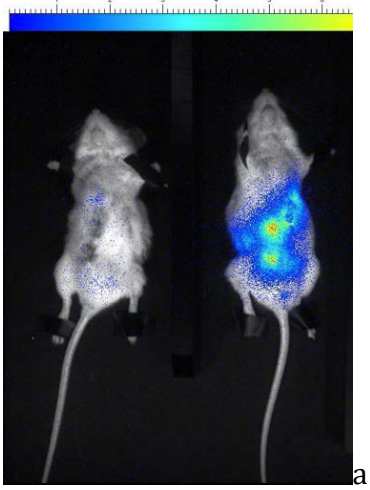


Image 3. Control after 17 days.

Being in a study with murine model we decided to treat with surgery 4 of the 6 mice. The mouse with a more localized uptake and the mouse with a higher level of uptake were not treated with surgery. During the 4 operations the exact localization of the tumor was shown. In all 4 mice the liver tumor was spread into the diaphragm, and in all cases the surgical approach of the diaphragmatic disease lead to the death of mice due to respiratory failure.
The remaining 2 animals had imaging test also after 24 and 30 days and they both showed an exponential increase of the uptake. (Image 4)


a

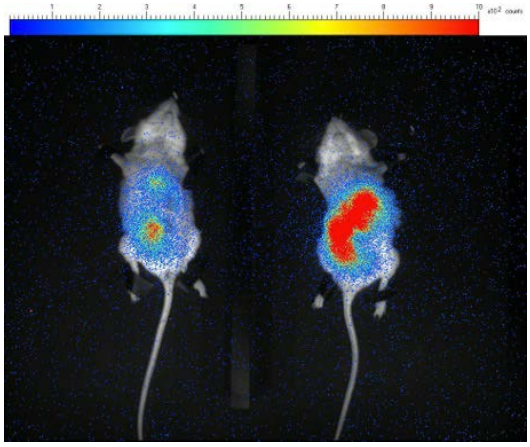

b

Image 4. a: control after 24 days; b: control after 30 days.

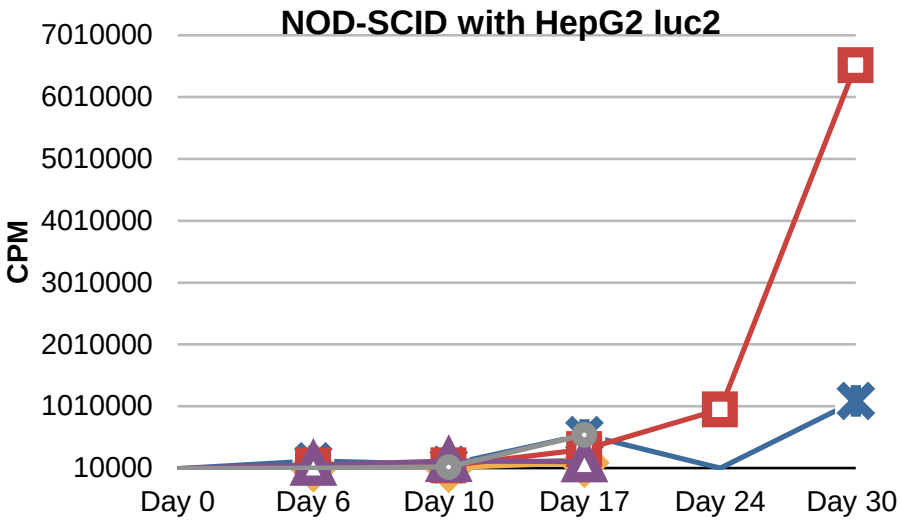


Figure 27. Graphical view of the cells emissions during the experiment.

After acquiring the last images, the other 2 mice were sacrificed and the autopsy study showed massive hepatic and diaphragmatic involvement in the absence of macroscopic signs of peritoneal and / or pulmonary lesions. In

figure 27 is show the graphical evolution of the cell emission during the experiment.

In the second experiment 6 NOD-SCID females mice aged 16-weeks were used. The aim was to evaluate the effects of a hyperglycemic diet on HCC growth.

After six days a higher uptake has seen in mice on hyperglycemic diet. This high increasing of uptake was steady in all measurements. (Image 5, 6, 7, 8) By focusing on tumor growth in the 2 groups of mice, it is clear that the mice on hyperglycemic diet had a faster and more rapid tumor progression. (Figure 28)

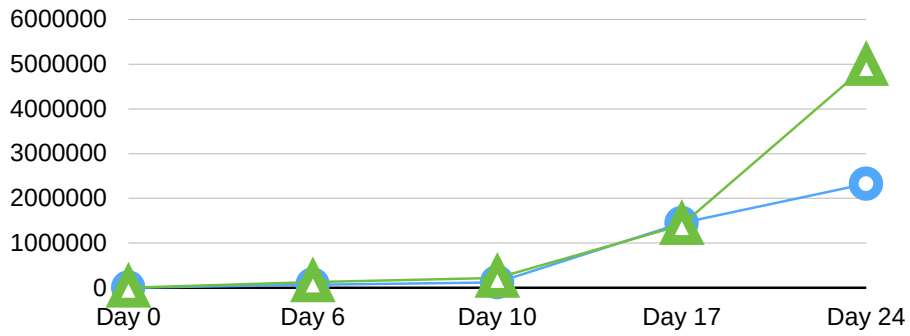


Figure 28. Green triangle hyperglycemic diet; blue circle normal diet

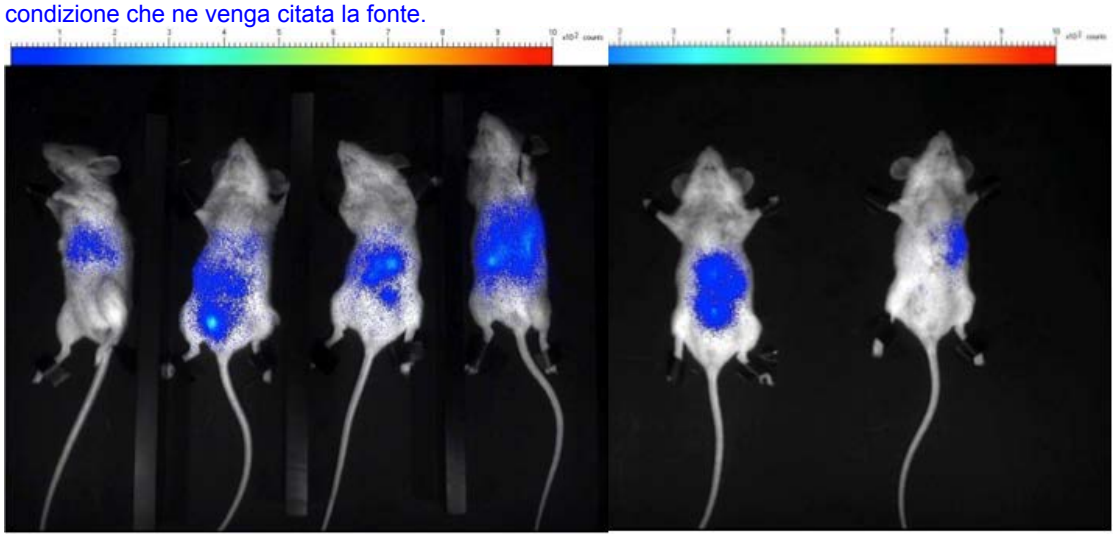


Image 5. Control after 6 days.

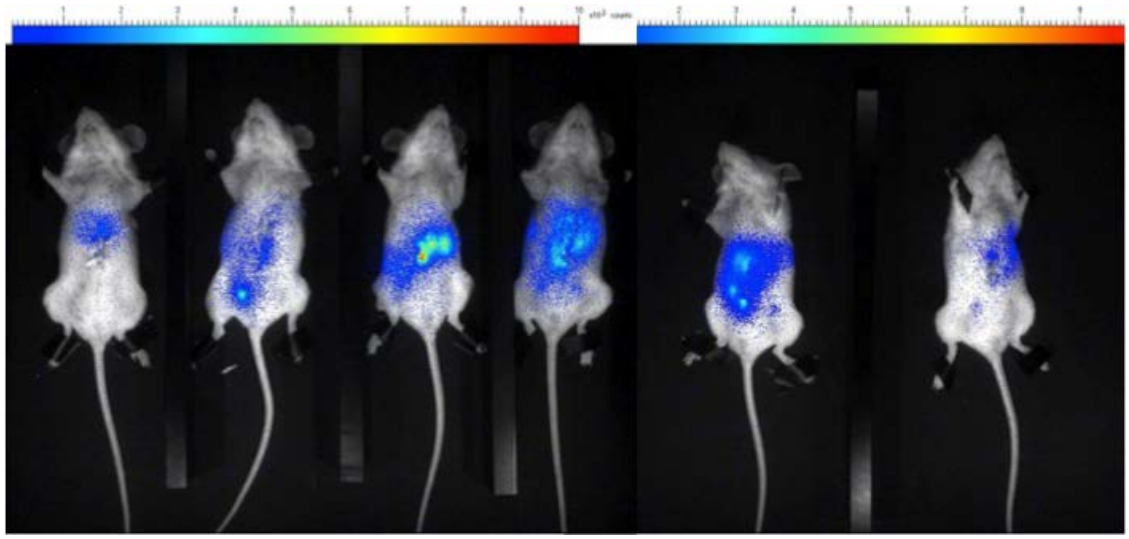


Image 6. Control after 10 days.

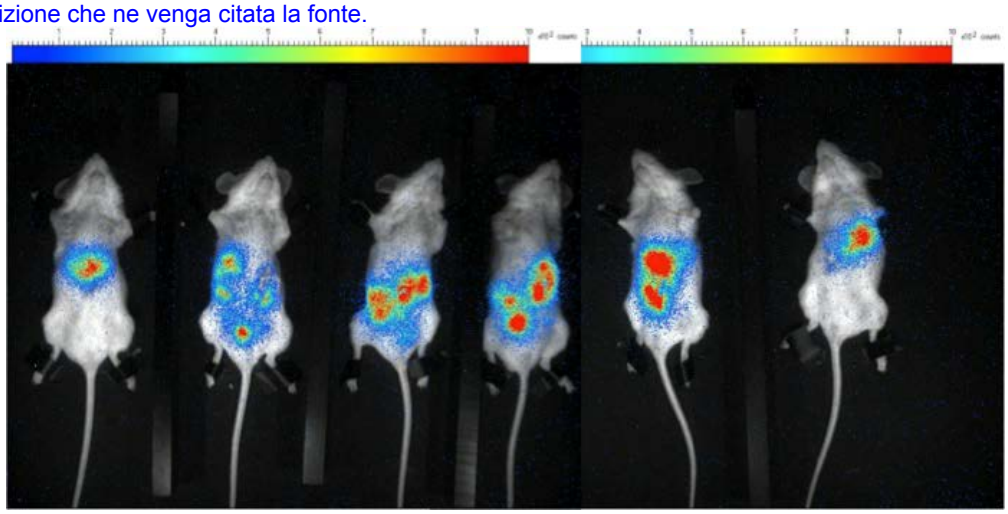


Image 7. Control after 17 days.

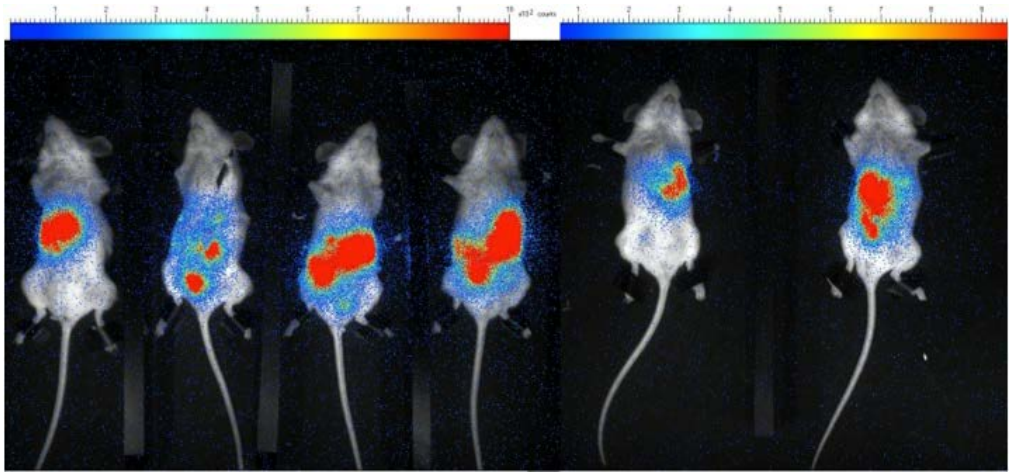


Image 8. Control after 24 days.

However analyzing tumor growth singularly is showed that 1 mouse fed with normal diet had a greater increase in tumor growth than two mice on hyperglycemic diet. (Figure 29)

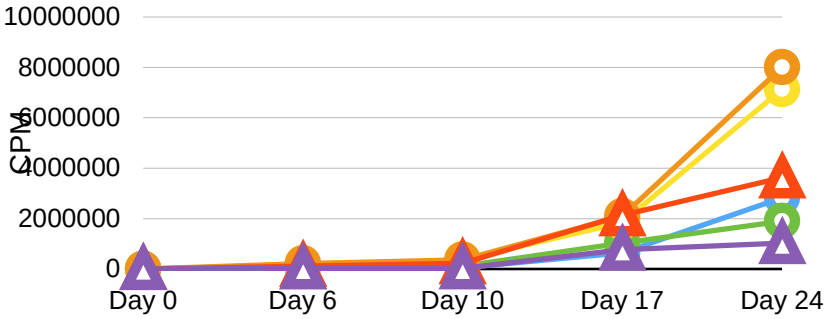


Figure 29.

CONCLUSIONS

The most relevant limit of our study is the small sample evaluated, although from our data some conclusions can be drawn.

From a technical point of view, the direct and surgical inoculation of cells in the liver exposes a high incidence of diaphragmatic disease.

The presence of diaphragmatic disease is more likely caused by the direct contact of the inoculated cells with the diaphragm surface rather than a secondary invasion.

Hepatic resection surgery after 3 weeks has a high mortality rate mainly due to the tumor aggressiveness. Therefore, if the protocols on relapse after hepatic resection needed to be set, it may be better to perform the resection in the first 15 days after inoculation in order to not intervene on a too widespread disease.

The most interesting data emerging from both experiments is that, with the worsening of diabetes, tumor growth increases.

Indeed, in the first experiment, the worsening of the metabolic state is due to the advancement of weeks while in the second experiment to the diet rich in sugars.

These experiments can provide basic knowledge for future studies and protocols involving more complex murine models, such as mice with metabolic syndrome or type 2 diabetes, and who want to investigate HCC carcinogenesis in the interesting NASH setting.

SECTION 3

INTRODUCTION TO SECTION 3

The use of vascular access devices is an integral aspect of health care for neonates, children, and adults and has moved beyond the acute care setting to chronic, long-term care. Vascular devices have a paramount role throughout the management of oncology patients, as they are needed in the initial phases for surgery or chemotherapy, in the advanced stages for chronic treatment, and in the last stages for palliative cares. For these reasons I had a large experience in clinical placement and managment of vascular access.

During the period of the experiments in Florence, along with my lab colleagues the unsolved problem of central venous access in rat has been emerged. My laboratory colleagues for repeated infusions of nanoparticles or blood samples would like to use vascular catheter similar to the ones that I am usual to manage.

The problem related to the venous access was composed of two main parts. On one side the venous catheter should be cheaper than those available on market; on the other side the need of an easy implant technique.

My surgical skills allowed me to realize and implant a new model of central catheterization for laboratory rats. As following there is the all description of the project, as part of a major project, currently under submission for a publication.

Finalizing an economic surgical procedure of jugular vein catheterization in laboratory rat

Introduction:

Catheterization of laboratory rats is a common procedure used in several application, especially in farmacieutical field; this technique is essential to have a proper and convenient procedure that allows repeated administration, of drugs and, at the same time, blood sampling to control different blood values in animals.

This model is frequently used to examine the sistemic toxicity of new injectable drugs; the application of this procedure is also suggested to analize all the possible changes in plasma during any pharmacological trials. In the preclinical tests, one of new most important parameter to be tested is the repeated toxicity for long time. These requirements for injectable drugs are fulfilled by frequent intravenous injection and blood withdrawal from rats, leading to an intense stress and suffering of the animals. This surgical procedure allows repeated intravenous infusion and blood collection from laboratory animals through a catheter without needing of anesthesia; for this reason is considered one of the less painful and stressful method (Hau *et al.* 2001, Morton & Hau 2002). Although vessel catheterization requires microsurgical skills, it reduces the variability due to the different expertise of the operators working during the experiment; the repeated intravenous injection and the blood withdrawal from a catheter implanted in the animals is a standard procedure which can be performed by different operators

reducing the variability generated by several traditional intravenous injection with a syringe.

On the one hand this techinque is reliable and effective, on the other hand results to be very expensive. Animals with an intravenous catheter surgically implanted, are usually housed in specific cages, and the animal's catheter is generally connected to an osmotic pump connected to the cage; these features bring the costs of the procedure to an higher level than the common injection and withdrawal methods by using traditional syringes.

In the last decades different techniques for rats vessels cannulation have been developed, but the origin of each specific method is not clear because they have been developed by many scientist from different fields of study. One of the first method was developed by Terkel and Urbach in 1974.

Our aim was to modify the catheterization procedure to obtain a cheaper catheterization model in terms of housing and materials used, keeping a good catheter positioning and patency such as the traditional techniques.

Materials and methods:

Due to the simplicity of this technique, there are few tools and equipments needed for the surgical operation:

- Animals:
    - 25 Sprague Dawley Rats purchased from Harlan Laboratories, Inc.
- Surgical instruments:
    - Blade number 24

  - Two Pick up
  - Surgicryl 3-0, DS 19 mm needle, 75 cm purple
  - Surgicryl 5-0, DS 19 mm needle, 75 cm purple
  - Scissors
  - Two Micro-Mosquito
  - Needle driver

- Other materials:
  - Avertin 2.5%
  - Heparin Coated catheter model CBAS® PU Cath 3Fr, Solomon Scientific
  - *Locking Solution* (50U of heparin/ml of sterile PBS)
  - Heparin
  - Ethanol
  - PBS sterile
  - Elettric Razor
  - Depilatory cream (Veet®)
  - Agocanula
  - 10% povidone-iodine solution
  - Heating pad
  - Ibuprofen (80mg/Kg)

Surgical procedure

All experiments on live vertebrates have been carried out in accordance with the Principles of Laboratory Animal Care (directive 86/609/ EEC).

In order to ensure an asepsis condition, the surgical procedure had been performed in the sterile room of Ce.S.A.L. (stabulation centre for laboratory animals of the University of Florence).

The operating table is carefully cleaned with ethanol 70% and covered with a sterile surgical sheet.

The preliminary step involves the rats anesthesia with an intraperitoneal injection of Avertin 2,5% (10 µl/gr). Subsequently we begin to shave the area of rats neck, followed by the right shoulder and the right and middle part of the back. This procedure must be done very carefully to avoid future infections of the skin (i.e. surgical wound infections) or on the catheter. The rat is placed in supine position trying to recreate the Trendelenburg position. The four extremities of the rat are ligated with an adhesive band.

The catheter has to be assembled as described in *Fig.1* before starting the surgical operation.

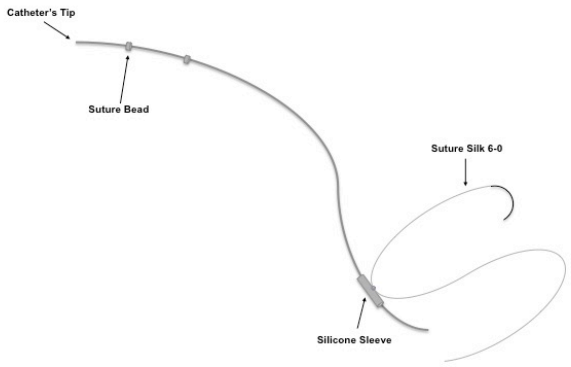


Figure 1. The catheter is assembled by adjusting the silicon beads at the right length (2 and 4 cm from the catheter tip). The next step is the insertion of the suture needle in the silicon sleeve of the catheter and the wash of the lumen with the locking solution.

A vertical incision of 2 to 4 cm is performed in the right lateral portion of the neck, followed by a careful dissection of the neck with the exposure and preparation of the right jugular vein. The jugular vein preparation is carried from cephalic to caudate position in order to create enough space for the catheter insertion (i.e. ~ 2 cm). The vein is now surrounded by two ties of suture silk 5.0 and a needle is inserted in the vein, following the orientation of the vessel and right in the space between the two ties. Now the Teflon guide is inserted trough the needle until there is some resistance, meaning that the guide is stationed in the correct position (right atrium); the teflon guide now has to be retracted for 1-2 mm. The jugular vein is then carefully ligated cephalic from the needle insertion. Once the guide is secured and stable inside the vein, the needle can be removed. The heparin coated catheter is now inserted on the guide until loss of resistance and then pulled back 1-2 mm. During this operation we must be sure that at least one bead

enters the vein. The vicryl loop can be tied now around the vein paying attention not to close completely the catheter; to avoid this complication is important to keep the Teflon guide inserted. Carefully remove the Teflon guide and clamp the catheter to avoid blood loss and air embolism. Close the muscle fibers around the vein with a 5-0 vicryl. Now perform an incision from the previously incision made up to the shoulder to arrive to the back for a length of 8-10 cm. The incision must be done between the two scapula. Put some stitches to fasten the silicone sleeve of the catheter to the subcutaneous tissue; these stitches are important to avoid traumatic removal from the rat. Performing a single stitches skin suture allows the catheter to come out from the back and secure the pin port. In the end skin disinfection with 10% povidone-iodine solution. Insert then the *pin-port* in the catheter and inject 50 µl of locking solution to prevent clots formation and blood loss from the catheter.

After the surgery we have to dissolve ibuprofen (80 mg/Kg) in the water in the cages of the animals. The rats have to be frequently observed for at least 4 hours to identify potential signs of pain or suffer (in case of animal suffering, the interested rat has to be sacrificed by cervical dislocation). The administration of ibuprofen is stopped after 48 hours from the surgery.

After two days from the catheterization, we started testing the patency and the stability of the catheters implanted by measuring the length of the silicon tube outside the animals and by the withdrawal of peripheral blood. See the picture below for the detailed of the surgical procedure.

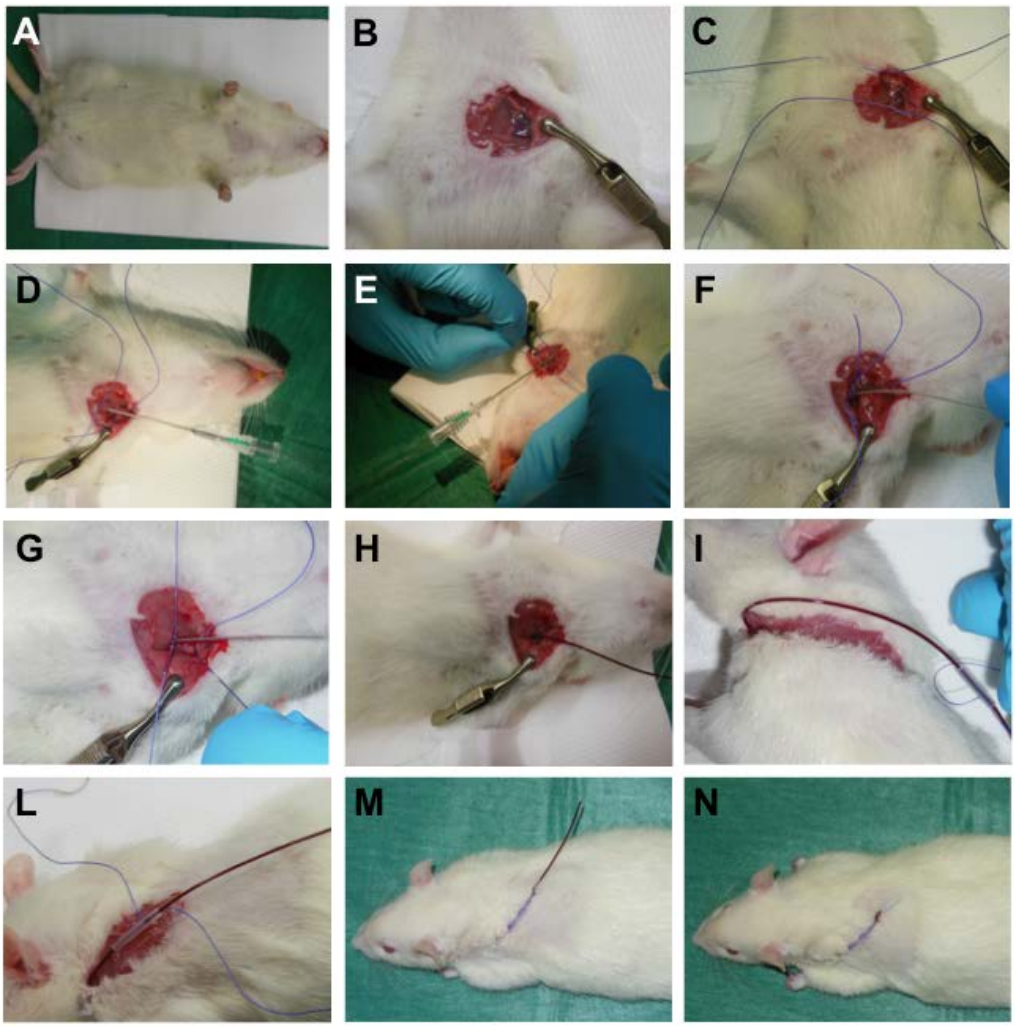


Figure 1S. **(A)** Shave the interested area. **(B)** Pass two suture silk threads under the jugular vein. **(C)** Knot slightly the two threads around the jugular vein. **(D)** Gently insert the needle of the catheter (with the guide inserted) into the jugular vein. **(E)** Secure the upper knot around the vein, manteining the needle inserted. **(F)** Insert the catheter tip into the jugular vein until the first beads it's in the lumen. **(G)** Close the other knot around the catheter with the teflon guide still inserted. **(H)** Pull out gently the teflon guide from the vein leaving the catheter inserted. The animal blood should start flowing into the catheter. **(I)** After suturing the peritoneum and the skin of the insertion area, cut the animal skin to create a subcutaneus accomodation for the catheter. **(L)** Suture the silicone sleeve to the internal skin

of the animal to secure the position of the catheter. **(M)** Suture the wound completely. **(N)** Cut the catheter excess leaving just 4/5 mm from the closed wound; close the catheter with the pin port®.

Results:

Mortality evaluation:

We perfomed this kind of surgery on 25 Sprague Dawley rats and only 2 animals died during the operation, before the awakening from the anesthetic phase.

After the surgery, every rat was perfectly conscious and healthy; we didn't find any trace of stress and/or pain in the animals. All these animals survived until the day of sacrifice, 25 days after the surgery procedure. Rats didn't show any signs of infection and/or swelling of the surgical wound.

The 26th day after the surgery, all the animals were euthanized by cervical dislocation; we didn't find unexpected macroscopic signs in the zone we secured the catheter under the skin of the animals.

Fixation of catheters evaluation:

The first thing we wanted to do was the evaluation of the stability of catheters under the skin of the rats. We investigated daily the efforts of the animals to pull out catheters measuring the leght of the silicon tubes exposed out of the skin. We decided to consider a good secure posizion of the catheters a distance of 1 cm from the *pin-port* at to the rats' skin; in case we found an animal without the vein access or with a catheter pulled out for more than 1 cm of lenght, this rat was counted as "rat without vein access". We daily controlled the good fixation of the catheters for 25 days after the

surgery; during the period of observation, we found that the 88% of rats had their catheters firmly positioned in the right position (*Fig2 A)*. We thoroughly investigated these data after we euthanized the rats, controlling the correct position of catheters under the skin of the animals (from the intrascapulae region to the heart).

Patency evaluation:

Every two days after the surgery, we whitdrawed 100 µl of blood through the *pin-port* access and immediately after we injected 50 µl of locking solution to prevent formation of blood clots inside the lumen of the catheters. We also slowly injected 500 µl of saline solution through the access every 4 days, to simulate the intravenous administration of injectable drugs.

When we couldn't get access to the jugular vein neither in the withdrawal phase nor in the injection phase, we considered that catheter as non patent.

These are the data of 25 days of patency evaluation after the surgery; our data show that 87% of the catheters secured under the animals'skin remained in a patency condition during the entire period of observation (*Fig2 B*).

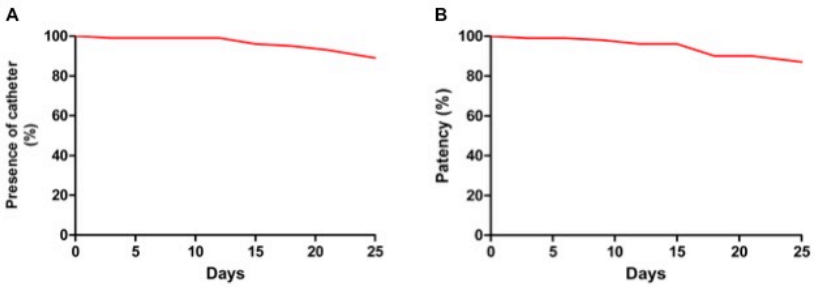


***Figure 1*: (A)** Monitoration of the correct position of the catheters implanted in the animals during the perdiod of observation (25 days). **(B)** Patency evaluation performed every two days for the entire period of observation (25 days).

Histological examination:

After 25 days after the surgery, the animals were sacrificed by cervical dislocation. The first thing we evaluated was the site of catheter insertion under the skin of the animal; we didn't observe any sign of infection of the wounds. Then we performed a necropsy of all the animals and we didn't see any abnormal sign on the internal organs of the rats. The last thing we wanted to analyze was the histological condition of the hearts; we surgically removed the hearts and we fixed them in formaline solution 4%. We investigated the histological condition of the organs by an hematoxylin-eosin staining and we didn't see any sign of alteration in the cardiac tissue. See the supplementary informations for the pictures of the cardiac tissues analyzed (*Fig 2S)*.

Discussion:

Our technique resulted very effective, comparable with the others vessel cannulation procedures. The number of catheters accidentally removed is very low, allowing the housing of the animals in traditional cages without the use of tethers or similar items. Thanks to the high level of patency reached with this technique, is possible to house the animals for long time, allowing any kind of repeated treatment. We were able to perform both injection and withdrawal of blood fast and easily, without stressing or harming the animals.

Furthermore, thanks to the avoid of the use of specific dedicated cages it's possible to save an high amount of economic resources, expecially when the number of animals to be used is rather high. Our post mortem examination didn't show any sign of wound infection and the histological investigation didn't reveal any alteration of the cardiac tissue after the insert of the catheter for a long period. This clearly indicates that our technique is safe for the animals and it doesn't affect their health, which is a crucial point to set before starting any *"in vivo"* test.

SECTION 4

Introduction to Section 4

In the previous two chapters of my thesis I described the strong relation between HCC and pancreatic adenocarcinoma with Metabolic Syndrome and Diabetes. Moreover patients affected by liver or pancreatic cancer and Metabolic Syndrome or Diabetes have some problems manly related to the surgical technique.

In literature is demonstrated that these patients have a major risk to develop intraoperative and postoperative complications due to their metabolic conditions.

The large surgical incision, needed for conventional open liver and pancreatic surgery, exposes these fragile patients to several complications such as wound infections or postoperative pain. These complications although can seem minor complications they can lead patients to death.

In fact large wound infections often require high dose of antibiotics with an increased risk of toxicity and organ failure. Moreover a severe wound infection, if not well controlled, can lead to sepsis that is a well known serious and deadly complication.

On the other side postoperative pain plays a key role during postoperative course. Patients with a strong pain stop breathing adequately and they spend more time being bed-ridden. This less patient mobility can increase the risk to develop pneumonia or other respiratory disorders. In addition being bed-

ridden is a major cause to develop deep vein thrombosis with consequent risk of pulmonary embolism.

Since '90 laparoscopic technique has proposed a less invasive approach that can lead to a faster recover of patients.

Laparoscopic surgery, also called minimally invasive surgery (MIS), is a modern surgical technique in which operations are performed through small incisions (usually 0.5–1.5 cm) in the abdomen.

There are several advantages for patient treated with laparoscopic surgery in comparison of patients treated with the more common open procedure. Several studies demonstrated that both pain and intraoperative blood loss are reduced. Furthermore recovery time is shorter in patients who underwent to laparoscopic surgery.

During laparoscopic surgery the abdomen is usually insufflated with carbon dioxide gas (CO2). This gas elevates the abdominal wall above the internal organs creating a working and viewing space. CO2 is the gas used because is normally present in the human body and can be absorbed by tissue and removed by the respiratory system. CO2 is also non-flammable, which is important because electrosurgical devices are commonly used in laparoscopic procedures.

As already written, there are several advantages in patients who underwent to a laparoscopic procedure if compared to patients treated with an open surgical procedure.

These advantages include:

- Less estimated blood loss with a consequence lower need of blood transfusions.

- Smaller incision, which reduces pain and shortens recovery time, as well as resulting in less post-operative scarring.

- Less pain, leading to a less needing of painkillers and better postoperative motility.

Although procedure times are usually slightly longer, hospital length of stay is shorter, often with a same day discharge that leads to a faster return to everyday living.

Furthermore in laparoscopic surgery the reduced exposure of internal organs to possible external contaminants reduces the risk of acquiring infections.

At the beginning of the laparoscopic era the effort of surgical studies was directed to demonstrate the safety and feasibility of the laparoscopic approach. For this reason only benign or functional disease were approached laparoscopically. Once this goal was reached and almost all the abdominal surgical procedures were recognized eligible for the laparoscopic approach, the attention was focus on the possibility to adopt the laparoscopic technique for malignant diseases. Then the role of laparoscopic approach was investigated for the treatment of several abdominal malignancies such as colon, stomach and esophageal cancers. Several study demonstrated the non-inferiority in terms of oncological outcomes in patients treated with laparoscopic surgery over standard open approach. In addition some

advantages were found in the laparoscopic approach such as the highest number of retrieval lymphnodes or less transfusion rate that is demonstrated to be an important factor for survival in oncologic patients. Another important emerging data is the faster recovery after laparoscopic procedure that allows a precocious beginning of adjuvant chemotherapy.

Liver and pancreatic surgery were the last to be approach laparoscopically due to the high complexity that these types of surgery need. However in recent years several reports of hepatobiliary and pancreatic laparoscopic surgery were published.

In this fourth part of my thesis I would like to describe some results deriving from my two years job as a Research Collaborator in General Surgery at the Mayo Clinic, Jacksonville, FL, USA, under the direction of Prof. H.J. Asbun. During this collaboration, we tried to investigate the role of laparoscopic surgery in pancreatic adenocarcinoma treatment.

Recent advances in laparoscopic technique allowed performing almost all the surgical procedures. However pancreatic surgery remains one of the last fields of application of this approach. In addition there are some concerns about the oncological radically that the mini-invasive approach can obtain. If safety, feasibility and oncological adequacy of laparoscopic technique will be all demonstrated laparoscopic approach could represent a great opportunity especially for patients with pancreatic cancer and diabetes and metabolic syndrome.

In our papers we demonstrated the technical safety and feasibility of the laparoscopic approach. Also the oncological outcomes are demonstrated to

be similar in laparoscopic and open approach with slightly more advantages of the laparoscopic technique.

This work is issued in three papers published on different surgical journals.

In the first paper entitled "Laparoscopic versus open pancreaticoduodenectomy for pancreatic adenocarcinoma: long-term results at a single institution" (114) we reported data of patients with pancreatic adenocarcinoma of the pancreatic head treated with laparoscopic pancreatoduodenectomy (LPD) performed at Mayo Clinic Jacksonville,FL. It is interesting to point out that in our population almost 1/3 of patients were diabetic and almost 50% of the entire population had a diagnosis of metabolic syndrome. Our data confirmed the high incidence of pancreatic cancer in patients with metabolic syndrome and/or diabetes. Therefore the laparoscopic approach could be a extremely good solution for these patients.

The second paper entitled "Laparoscopic Versus Open Distal Pancreatectomy for Pancreatic Adenocarcinoma" (115) was about the treatment of pancreatic adenocarcinoma of the body and tail of the pancreas. In these cases, laparoscopic distal pancreatectomy (LDP) was the procedure under evaluation. In this paper, patients had similar percentage of diabetes and metabolic syndrome and in addition the median BMI of the open and laparoscopic series was 26.1 and 28.3 respectively. This data again underlined the high incidence of diabetes and metabolic syndrome in patients affected by pancreatic adenocarcinoma.

The third paper entitled "Laparoscopic pancreatoduodenectomy: current status and future directions" (116) was a review with the aim to investigate

the safety and feasibility of LPD in larger series published worldwide. The final message of the paper was that LPD is a safe and feasible procedure. Moreover the initial surgical learning curve of the center should be taken into account as a factor that can influence results much more then a standard open surgical procedure.

The fourth paper entitled "Laparoscopic Distal Pancreatectomy with En Bloc Splenectomy" was a thecnically paper on the methods and indications of laparoscopic distal pancreatectomy with the en bloc splenectomy

FIRST PAPER

Laparoscopic versus open pancreaticoduodenectomy for pancreatic adenocarcinoma: long-term results at a single institution

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Abstract

Background: Pancreaticoduodenectomy remains as the only treatment that offers a chance for cure in patients with pancreatic ductal adenocarcinoma (PDAC) of the head of the pancreas. In recent years, laparoscopic pancreaticoduodenectomy (LPD) has been introduced as a feasible alternative to open pancreaticoduodenectomy (OPD) when performed by experienced surgeons. This study reviews and compares perioperative results and long-term survival of patients undergoing LPD versus OPD at a single institution over a 20-year time period.

Methods: From 1995 to 2014, 612 patients underwent PD and 251 patients were found to have PDAC. These latter patients were reviewed and divided into two groups: OPD (n = 193) and LPD (n = 58). LPD was introduced in November 2008 and performed simultaneous to OPD within the remaining

time period. Ninety-day perioperative outcomes and long-term survival were analyzed.

Results: Patient demographics were well matched. Operative time was significantly longer with LPD, but blood loss and transfusion rate were lower. Postoperative complications, intensive care unit stay, and overall hospital stay was similar. OPD was associated with larger tumor size; LPD was associated with greater lymph node harvest and lower lymph node ratio. LPD was performed by hand-assist method in 3 (5.2 %) patients and converted to open in 14 (24.1 %). Neoadjuvant therapy was performed in 17 (8.8 %) patients for OPD and 4 (6.9 %) for LPD. The estimated median survival was 20.3 months for OPD and 18.5 months for LPD. Long-term survival was similar for 1-, 2-, 3-, 4-, and 5-year survival for OPD (68, 40, 24, 17 and 15 %) and for LPD (67, 43, 43, 38 and 32 %), respectively.

Conclusion: LPD provides similar short-term outcomes and long-term survival to OPD in the treatment of PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer mortality in the USA, with more than 30,000 deaths annually [1]. Despite a variety of neoadjuvant and adjuvant treatment protocols, surgical resection remains the only opportunity for cure in patients diagnosed with PDAC. Since publication of the first series of laparoscopic cholecystectomy in the late 1980s, the field of minimally invasive surgery has expanded

dramatically and is now utilized for treatment of almost all gastrointestinal malignancies. Many oncological procedures have been proven not only feasible and safe, but also oncologically equivalent to traditional open procedures regarding both immediate operative benchmarks (margins, lymph nodes retrieval, and morbidity) and long-term outcomes (survival). The role of laparoscopy in pancreatic surgery for PDAC had previously been primarily used for staging and palliation. Laparoscopic staging was shown at the time to be superior to radiographic cross-sectional imaging for visualizing small, occult liver, and peritoneal metastases and aided in avoiding non-therapeutic laparotomy in patients with advanced disease [2]. While the adoption of laparoscopic pancreatic surgery is still not universal, distal resections have been proven to be safe and feasible with significant advantages over the open counterpart. Laparoscopic distal pancreatectomy is routinely performed for PDAC at many centers [3]. For proximal resections, however, the complexity of the procedure and lack of data regarding clear benefits have appropriately resulted in slow adoption of the technique [4, 5]. Pancreaticoduodenectomy (PD) is one of the most complex gastrointestinal surgical procedures, including resection of the duodenum, head and uncinate process of the pancreas, and distal common bile duct, with reconstructive anastomoses involving the stomach, pancreatic remnant, and biliary tract. From the first description written by Gagner and Pomp in 1994 [6] that questioned its feasibility, several studies were published with an increasing number of laparoscopic PD (LPD). In the beginning, the majority of patients underwent PD for benign or borderline lesions. In 2007, a series reported by

Palanivelu and colleagues of 45 patients with benign and malignant disease undergoing LPD demonstrated that the procedure might not only be feasible, but it might have advantages compared with open PD (OPD) [7]. An increasing number of studies also showed that patients with PDAC could be treated with minimally invasive techniques with similar oncological results [8]. For this reason, the oncological outcomes of the laparoscopic approach in pancreatic surgery have now become a major focus of studies [3, 8–11]. Other recent studies regarding LPD with vascular resections were published with similar outcomes to OPD [9, 12]. The aim of our study was to review and compare the perioperative results and long-term survival of patients with PDAC who underwent laparoscopic versus OPD at a single institution over a 20-year period.

MATERIAL and METHODS

From 1995 to 2014, information regarding patients undergoing pancreatic resection was collected and retained in an institutional review board-approved database, including demographics, operative variables, postoperative outcomes, pathologic findings, and extended follow-up. Preoperative characteristics included age, sex, comorbidities, body mass index, American Society of Anesthesiologists (ASA) score, and use of neoadjuvant treatments. Operative details included operative time (incision to close of the wound), estimated blood loss, and blood product transfusion (obtained from the anesthesia record). Use of laparoscopy, vascular resection, and concomitant resection was obtained from the operative report.

Postoperative outcomes were tracked for 3 months (90 days) after surgery in our comprehensive electronic medical record, and all complications were recorded and graded according to the Clavien system [13]. Final overall patient complication grade was given to the highest rated complication grade in the postoperative time period. Minor complications included grades I and II, while major complications included grades III–V. Pancreatic fistula (PF) [14], delayed gastric emptying [15], and postpancreatectomy hemorrhage [16] were scored and graded according to standard international consensus definitions. Length of stay was recorded and did not include the day of the operation, but included the day of discharge, while readmission was tracked for all patients to any hospital for 90 days after surgery. Reoperation and readmission were defined as any unplanned operation or admission, respectively, within 90 days of the primary procedure related to the pancreatic resection. Final pathology, margin status, and lymph node involvement were recorded as well. These recorded data were compared in an intent-to-treat analysis and reported below. Preoperative workup included appropriate cross-sectional imaging to exclude distant metastases. All patients underwent a preoperative medical examination, but no specific criteria including age or comorbidity were used to determine eligibility for surgery. Similarly, no objective patient criteria were used to exclude patients from undergoing an LPD. Radiographic evidence for vascular involvement or a history of previous surgery or altered foregut anatomy was taken into consideration, but were not absolute contraindications for an attempted minimally invasive approach. The surgical procedures were performed by

multiple high-volume experienced hepato-pancreato-biliary or transplant surgeons over the course of the study period. However, since 2008, PD (including all LPD in this study) were performed only by two surgeons (J.A.S. and H.J.A.). LPD was performed as previously described [4]. Briefly, the abdomen was entered through a supraumbilical 12-mm incision, and abdominal exploration was performed to exclude metastatic disease. Five (3–12-mm trocars and 2–5-mm trocars) accessory port sites were used to perform the resection in a standard fashion. The specimen was extracted by extending the small midline incision and sent to pathology to confirm negative margins by frozen section analysis. Pneumoperitoneum was reinstated, and the reconstruction was performed laparoscopically. A single layer hepaticojejunostomy was constructed first, followed by a two-layer duct to mucosa pancreaticojejunostomy. Lastly, gastrointestinal reconstruction was performed with an end-to-side duodenojejunostomy. A single drain was placed behind the pancreaticobiliary reconstructions. Descriptive statistics for categorical variables were reported as frequency and percentage; continuous variables were reported median (range). Categorical variables were compared between OPD and LPD patients using Chisquare test or Fisher's exact test, and continuous variables were compared using two sample t test or Wilcoxon rank sum test, where appropriate. Kaplan–Meier method was used to calculate 1- to 5-year survival statistics. Cox regression model was used to identify univariate and multivariate risk factors for survival. All univariately significant risk factors,

together with OPD/LPD, were included in multivariate model. All statistical tests were twosided with the alpha level set at 0.05 for statistical significance.

RESULTS

All patients

In the study time period, 612 patients underwent PD. Of these, 251 patients underwent PD for curative intent for the diagnosis of PDAC, including OPD (n = 193) and LPD (n = 58). Prior to October of 2008, all PD for PDAC were performed by open techniques (n = 120). After this time, both OPD (n = 73) and LPD (n = 58) techniques for PDAC were performed simultaneously. Table 1 shows the demographic data. Patient sex, age, body mass index, and medical comorbidities were similar but there were significantly more patients with ASA of IV in the OPD group.

Variable	OPD (n = 193)	LPD (n = 58)	p value
Male	96 (49.7 %)	32 (55.2 %)	0.468
Hypertension	128 (66.3 %)	33 (56.9 %)	0.189
Diabetes	62 (32.1 %)	19 (32.8 %)	0.928
Cardiac disease	64 (33.3 %)	16 (27.6 %)	0.411
Pulmonary disease	47 (24.4 %)	10 (17.2 %)	0.257
ASA			**0.007**
I/II	39 (20.3 %)	16 (27.6 %)	
III	131 (68.2 %)	42 (72.4 %)	
IV	22 (11.5 %)	0 (0.0 %)	
Age (years)*	68.9 (33.3–86.9)	69.9 (40.6–84.8)	0.950
Body mass index*	25.6 (15.0–46.1)	25.9 (17.7–49.6)	0.368

Bold values are statistically significant p values

ASA American Society of Anesthesiologists score

* Values are median (range)

Table 1 *Demographics for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy(LPD) for pancreatic ductal adenocarcinoma*

Operative variables are given in Table 2. LPD was associated with significantly longer operative time (median 375 vs 518 min, p\0.001), but decreased blood loss (median 600 vs 250 mL, p\0.001) and decreased use of packed red blood cell transfusions (46.6 vs 25.9 %, p = 0.005). The need for vascular resection or total pancreatectomy was similar for both groups. Use of a hand-assist technique was required in 3 (5.2 %) patients, and conversion from LPD to open was required in 14 (24.1 %). All conversions were performed for venous involvement requiring vein resection (n = 11) or adherence to the underlying vasculature from the result of pancreatitis/ desmoplastic reaction (n = 3). Twenty patients (34.5 %) underwent LPD with vein resection, including 11 patients with conversion to open as mentioned previously, and nine patients underwent a totally laparoscopic vein resection.

Variable	OPD (n = 193)	LPD (n = 58)	p value
Operative time (min)*	375 (159–681)	518 (313–761)	**<0.001**
Estimated blood loss (mL)*	600 (50–7800)	250 (50–8500)	**<0.001**
No. of pts pRBC transfusion	90 (46.6 %)	15 (25.9 %)	**0.005**
Vascular resection	60 (31.1 %)	20 (34.5 %)	0.627
Total pancreatectomy	31 (16.1 %)	7 (12.1 %)	0.457

Bold values are statistically significant p values
pRBC intraoperative packed red blood cell transfusions
* Values are median (range)

Table 2 Operative variables for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductal adenocarcinoma

Table 3 contains the postoperative outcomes for both groups. Overall, complications were similar between the two groups. Median hospital (9 vs 6 days, p\0.0001) and intensive care stay (1 vs 0 days, p = 0.016) were

significantly decreased in the LPD group. Major morbidity (30.1 vs 22.4 %, p = 0.170) and mortality (5.2 vs 3.4 %, p = 0.737) over 90 days was similar for both OPD and LPD, respectively.

Variable	OPD (n = 193)	LPD (n = 58)	p value
Cardiac complication	22 (11.4 %)	11 (19.0 %)	0.135
Pulmonary complication	27 (14.0 %)	5 (8.6 %)	0.282
Pancreatic fistula‡	20 (12.3 %)	6 (11.8 %)	0.912
A	6 (3.7 %)	2 (3.9 %)	
B	9 (5.5 %)	4 (7.8 %)	
C	5 (3.1)	0	
Postpancreatectomy hemorrhage	8 (4.1 %)	4 (6.9 %)	0.481
A	1 (0.5 %)	1 (1.7 %)	
B	3 (1.5 %)	1 (1.7 %)	
C	4 (2.1 %)	2 (3.4 %)	
Delayed gastric emptying	28 (14.5 %)	10 (17.2 %)	0.611
A	12 (6.2 %)	4 (6.9 %)	
B	6 (3.1 %)	3 (5.2 %)	
C	10 (5.2)	3 (5.2 %)	
Wound infection	30 (15.5 %)	5 (8.6 %)	0.182
Renal insufficiency	10 (5.2 %)	5 (8.6 %)	0.333
Hepatic insufficiency	8 (4.1 %)	2 (3.4 %)	1.000
Readmission	41 (21.2 %)	13 (22.4 %)	0.849
Reoperation	12 (6.2 %)	1 (1.7 %)	0.309
Intensive care stay (days)*	1 (0–51)	0 (0–48)	**0.016**
Length of stay (days)*	9 (4–71)	6 (4–68)	**<0.001**
Morbidity (Clavien grade I–V)	129 (66.8 %)	31 (53.4 %)	
None	64 (33.2 %)	27 (46.6 %)	
I	15 (7.8 %)	3 (5.2 %)	
II	56 (29.0 %)	15 (25.9 %)	
IIIa	32 (16.6 %)	7 (12.1 %)	
IIIb	6 (3.1 %)	1 (1.7 %)	
IVa	1 (0.5 %)	1 (1.7 %)	
IVb	9 (4.7 %)	2 (3.4 %)	
V	10 (5.2 %)	2 (3.4 %)	
Morbidity minor (Clavien grade I and II)	71 (36.8 %)	18 (31.0 %)	0.170
Morbidity major (Clavien grade III–V)	58 (30.1 %)	13 (22.4 %)	

Bold values are statistically significant p values

* Values are median (range)

‡ Excluding total pancreatectomy

Table 3 Postoperative outcomes (90-day) for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductal adenocarcinoma

Table 4 contains the pathologic details. PDAC stage, node involvement, and negative margin rates were similar between the two groups. LPD was associated with more T1 tumors and a smaller tumor size, but a higher number of lymph node harvests. The rate of initiation of adjuvant therapy (73.5 vs 75.9 %, p = 0.858) and time to start of adjuvant therapy (55 vs 54 days, p = 0.578) were similar between OPD and LPD, respectively.

Variable	OPD (n = 193)	LPD (n = 58)	p value
T stage[#]			**0.022**
T_1	8 (4.2 %)	8 (13.8 %)	
T_2	26 (13.8 %)	3 (5.2 %)	
T_3	151 (79.9 %)	47 (81.0 %)	
T_4	4 (2.1 %)	0 (0.0 %)	
N stage[#]			0.567
N_0	63 (33.3 %)	17 (29.3 %)	
N_1	126 (66.7 %)	41 (70.7 %)	
Stage			0.100
IA	5 (2.6 %)	6 (10.3 %)	
IB	11 (5.8 %)	1 (1.7 %)	
IIA	44 (23.2 %)	10 (17.2 %)	
IIB	124 (65.3 %)	40 (69.0 %)	
III	4 (2.1 %)	0 (0.0 %)	
IV	2 (1.1 %)	1 (1.7 %)	
Margin status			0.426
R_0	154 (79.8 %)	49 (84.5 %)	
$R_{1/2}$	39 (20.2 %)	9 (15.5 %)	
Tumor size (cm)*	3.5 (0.3–14.0)	2.5 (0.3–10.0)	**0.003**
Number of lymph nodes resected*	17 (1–63)	27 (9–70)	**<0.001**
Lymph node ratio*	0.098 (0.000–1.000)	0.060 (0.000–0.583)	0.468
Receipt of adjuvant chemotherapy[€]	122 (73.5 %)	41 (75.9 %)	0.858
Time to start adjuvant therapy (days)[€]	55 (27–209)	54 (23–187)	0.578

Bold values are statistically significant p values

* Values are median (range)

[#] T and N staging unavailable for four patients

[€] Data available for OPD (n = 166) and LPD (n = 54) patients only

Table 4 Pathologic findings for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductal adenocarcinoma

Table 5 and Fig. 1 show similar estimated 1- to 5-year survival for both groups. Figure 2 shows similar stage-for-stage survival for patients with stage II PDAC groups by excluding all those with stage I PDAC in both groups.

Survival	OPD (n = 193) Estimated survival (95 % CI)	LPD (n = 58) Estimated survival (95 % CI)	p value
1 year	67.50 % (61.05, 74.64)	66.51 % (54.96, 80.48)	0.249
2 years	40.22 % (33.41, 48.40)	43.29 % (30.99, 60.48)	
3 years	24.31 % (18.39, 32.13)	43.29 % (30.99, 60.48)	
4 years	17.09 % (11.89, 24.57)	38.48 % (25.63, 57.78)	
5 years	15.34 % (10.35, 22.71)	32.07 % (18.66, 55.11)	

Table 5 Kaplan–Meier estimated survival for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductal adenocarcinoma

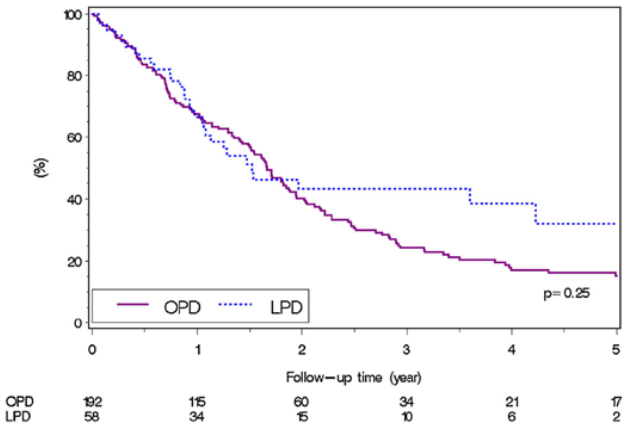

Fig. 1 Kaplan–Meier estimated 5-year survival graph for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductal adenocarcinoma

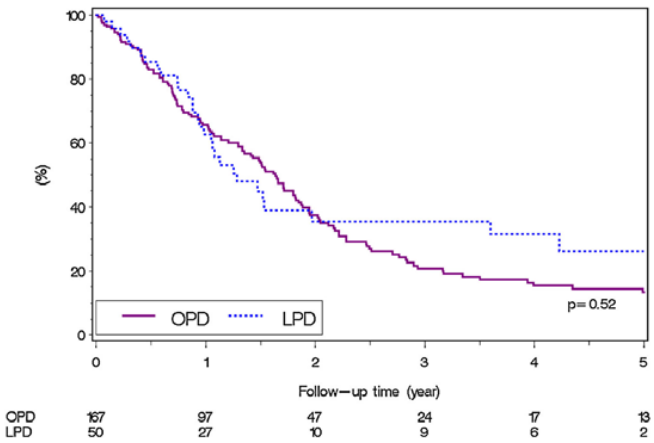

Fig. 2 Overall survival compared between OPD and LPD patients (stage II only)

On the univariate Cox proportional hazard model (Table 6), significant predictors of survival included increased tumor size ([3 cm), major complications (Clavien III–V), significant blood loss ([500 mL), positive margins (RI/R2), and elevated lymph node ratio [(LNR)[0.2]. All other factors including LPD versus OPD were not significant predictors. On the multivariate Cox proportional model, only increased major complications, positive margins, and an elevated LNR remained significant.

Variable	Univariate		Multivariate	*p* value
	HR (95 % CI)	*p* value	HR (95 % CI)	
Age > 65 years	1.2 (0.87, 1.65)	0.263		
BMI (kg/m^2)	0.99 (0.96, 1.02)	0.385		
Time period 1/2*	1.07 (0.76, 1.5)	0.694		
Tumor size (>3 cm)	1.51 (1.12, 2.03)	**0.007**	1.37 (0.99, 1.88)	0.055
Complications (minor)	1.13 (0.79, 1.62)	0.499		
Complications (major)	1.74 (1.19, 2.54)	**0.004**	1.77 (1.18, 2.65)	**0.006**
Operative time (>6 h)	1.4 (0.87, 2.26)	0.163		
Estimated blood loss (>500 mL)	1.54 (1.12, 2.1)	**0.007**	1.31 (0.93, 1.85)	0.117
Positive margins (R_1/R_2)	1.94 (1.38, 2.72)	**<0.001**	1.56 (1.06, 2.3)	**0.025**
Lymph node harvest (<20)	0.92 (0.68, 1.24)	0.567		
Lymph node ratio (>0.2)	2.11 (1.55, 2.89)	**<0.001**	1.91 (1.36, 2.7)	**<0.001**
LPD versus OPD	0.79 (0.53, 1.18)	0.25		

Bold values are statistically significant *p* values

BMI body mass index

* Time period 1 = 1995–2004, Time period 2 = 2005–2014

Table 6 Univariate and multivariate Cox proportional hazard model predicting overall survival for 251 patients undergoing open pancreaticoduodenectomy (OPD) or laparoscopic pancreaticoduodenectomy (LPD) for pancreatic ductaladenocarcinoma

DISCUSSION

Effective treatment of PDAC has proven to be a challenging endeavor for the medical and surgical community. Non-surgical treatments concentrate on palliation while operative interventions can often be associated with high

morbidity and likely eventual recurrence. Therefore, multimodality approach to the treatment of PDAC is encouraged, emphasizing treatments that maximize quality and quantity of remaining life. Significant attempts are being made to improve the performance and overall recovery from the surgical intervention with an additional focus on factors that may also improve the overall survival from PDAC. Improving the safety of the operation will potentially allow a higher percentage of patients to undergo operative treatment and gain the survival advantages, or even cure, associated with surgical intervention. LPD has been performed at our institution with the intent to improve outcomes and the overall patient's experience while recovering from surgery. Furthermore, given the improved access and visualization of the laparoscopic approach, LPD may, in experienced hands, have the potential of resulting in a better oncologic operation. Definitive evidence of this, however, will be difficult to show due to the relatively small series and low survival in a pathologic process such as PDAC. In our previous publication, the operation was associated with similar morbidity and mortality to OPD, but with improvements of surrogate oncologic markers such as blood loss, transfusion rates, lymph node retrieval, and LNR for patients with malignant disease [4]. Therefore, this study was performed in order to evaluate the long-term survival of patients undergoing LPD versus OPD focusing on PDAC. As noted previously [4], the patients undergoing LPD and OPD in this study were well matched. Prior to 2008, all PD were performed open, but after 2008, both LPD and OPD were performed simultaneously without any specific inclusion/exclusion criteria other than

extensive vascular involvement. LPD was also associated with longer operative times, lower estimated blood loss, and fewer overall blood transfusions than OPD. This finding was highlighted by our previous report [4] and in a meta-analysis of LPD in 2014 [5]. Similar to our study, decreased blood loss and need for subsequent blood transfusions have been shown to be independent predictors of survival after resection for PDAC [17–19]. Therefore, LPD may optimistically be associated with improved survival compared with OPD in those with PDAC. Of course, this would be best evaluated by a randomized controlled study to exclude the possibility of selection bias. A subgroup analysis of all OPD performed from 2008 to 2014 was performed to assess whether there were any changes to our comparison to the LPD group. In this analysis, we did not find any differences in the demographics of the patients or the overall survival. However, there were small changes and improvements in this contemporary OPD group that included decreased blood transfusions, decreased intensive care and hospital stay, and increased lymph node resections than now equal LPD for the above-mentioned operative variables and outcomes. All other complications and tumor selection comparisons did not have any significant changes. This likely reflects the use of updated pancreatic protocols and pathways as well as operative technique that rarely lead to changes in long-term survival. Similarly, others have identified postoperative complications as factors that may decrease the overall survival after pancreatectomy for PDAC [20]. This finding was confirmed in our study that showed major complications to be an independent predictor of worsened survival. Clearly, major postoperative

complications will at least delay the administration of chemotherapy or the ability to tolerate the full course. In this study, postoperative outcomes were quite similar between the two groups, although hospital stay was shorter for LPD. Despite this, LPD was not associated with a shorter time interval to initiating chemotherapy or with a higher percentage of patients beginning adjuvant therapies. Another group reported a significantly higher proportion of patients who had a delay or lack of adjuvant therapy after undergoing OPD compared to those undergoing LPD [21]. Nationwide, however, it appears that minimally invasive PD is not associated with an increased use or earlier initiation of adjuvant therapy according to a recently published National Cancer Database study [10]. This may be related more to the established practice protocols of time to initiate chemotherapy after PD regardless of if the operation was performed by OPD or LPD. At our institution, adjuvant therapy is aggressively recommended for all patients within 6–8 weeks of the operation as long as the patient has sufficiently recovered from the operation, regardless of the operative approach. Therefore, it stands to reason that time to initiate or rates of receiving adjuvant therapy will only be enhanced once improved complication rates from the operation are realized. LPD was associated with significantly increased lymph node harvest. The corresponding LNR was lower for LPD but did not reach statistical significance. LNR has been shown to predict overall survival from PDAC by our multivariate analysis and several other groups [22–25], but it is likely that overall survival is not truly affected by removing additional peripancreatic lymph nodes. Rather, a low LNR is more likely to represent

more accurate lymph node staging by increasing the denominator of resected lymph nodes and potentially, a reflection on a complete and adequate oncologic resection for an aggressive malignancy such as PDAC. This factor, similar to a high negative margin rate, low blood loss, reasonable complication rate, and high adjuvant therapy use among others, is just one part of the contemporary multidisciplinary treatment of PDAC. Overall survival, for both groups and matched stage for stage, was not statistically improved for those undergoing LPD versus OPD. Nevertheless, LPD was associated with an impressive 32 % 5-year survival. It remains to be seen whether this will continue with the accrual of more patients and further follow-up of current patients. Due to incomplete disease recurrence data in this study, it also remains to be seen whether LPD is associated with a significantly longer progression-free survival as others have found[21]. Similar to Croome et al., we also found that the technique of LPD did not reach statistical significance as a predictor of improved or worsened overall survival for PDAC after PD using univariate and multivariate analyses. Recently, French surgeons have reported on 46 patients undergoing LPD and compared them to the same number of OPD in the same time period. They note that LPD is a long and technically difficult procedure that is associated with increased rate of PF, bleeding, and need for reoperation. They concluded that LPD is not indicated for the treatment of resectable periampullary tumors, mainly due to the increased risk of PF [11]. In our study, PF was seen at an equal and acceptably low rate for both LPD and OPD. Similarly, upon further analysis, PF was not associated with decreased overall survival as

was also previously noted by other centers [26]. Overall, the results of this study conclude that LPD has comparable short-term results and is clearly not associated with worse long-term survival after PD for PDAC. Despite our findings, this study had limitations that must be acknowledged. First, our study was a retrospective analysis. We performed an intent-to-treat analysis and based on our current practice, there are very few patients that are absolutely excluded from undergoing LPD. Nevertheless, LPD was associated with a smaller tumor size, more T1 tumors, and fewer ASA IV patients. Secondly, this study was performed over the course of a 20-year time period. While the practice has remained fairly stable, there have certainly been improvements over this time frame in preoperative imaging studies, surgical technique, perioperative care, as well as neoadjuvant and adjuvant therapy administration. However, time period analysis did not show any difference in survival for patients treated in the first half of the series compared to the second half. Thirdly, many patients undergoing PD at our institution undergo adjuvant therapy at outside facilities. Therefore, full adjuvant therapy details including completeness of treatment, tolerance of dull dose treatments, and recurrence data are limited. In fact, a recent sub analysis of the ESPAC-3 study suggests that the completion of adjuvant therapies rather than the earlier initiation was a more important independent factor for survival prognosis [27]. Certainly, a comprehensive, prospective, multicenter, randomized study would be optimal to better underline the role of a minimally invasive approach in the treatment of pancreatic adenocarcinoma. In conclusion, this study shows that LPD appears to be safe and feasible with

similar short-term outcomes and long-term survival to OPD in the treatment of PDAC. Moreover, as LPD is often characterized by less intraoperative blood loss, lower transfusion rates, and improved lymph node harvests, a survival benefit for this technique may be conceivable if analyzed in greater numbers over a long period of time. Nonetheless, in an attempt to optimize all surgeon-related factors, focused efforts brought on by concepts acquired during performance of LPD conveyed to OPD have also resulted in improvements in OPD seen in the past few years.

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SECOND PAPER

Laparoscopic Versus Open Distal Pancreatectomy for Pancreatic Adenocarcinoma

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Abstract

Background: Laparoscopic distal pancreatectomy (LDP) has been shown to have short-term benefits over open distal pancreatectomy (ODP). Its application for pancreatic ductal adenocarcinoma (PDAC) remains controversial.

Methods: From 1995 to 2014, 72 patients underwent distal pancreatectomy for PDAC at a single institution and were included in the study. Postoperative and long-term outcomes of patients undergoing LDP (n = 44) or ODP (n = 28) were compared.

Results: LDP was associated with less blood loss (332 vs. 874 mL, p = 0.0012) and lower transfusion rates than ODP (18.2 vs. 50 %, p = 0.0495). Operative time was similar (254 vs. 266 min) for LDP and ODP; five patients (11.4 %) required conversion to ODP. Pancreatic fistulas (13.6 vs. 7.1 %) and major complications (13.6 vs. 25 %), were similar between LDP and ODP, respectively. Length of hospital stay (5.1 vs. 9.4 days, p = 0.0001) and time to

initiate adjuvant therapy (69.4 vs. 95.6 days, p = 0.0441) was shorter for LDP than ODP. Tumor characteristics were similar but LDP was associated with more resected lymph nodes than ODP (25.9 vs. 12.7, p = 0.0001). One-, three-, and five-year survival rates were similar between LDP (69, 41, and 41 %, respectively) and ODP (78, 44, and 32 %, respectively).

Conclusion: LDP is associated with less blood loss and need for blood transfusion, shorter hospital stay, and faster time to initiate adjuvant therapy than ODP for patients with PDAC. Postoperative outcomes and long-term survival are similar between the two groups. LDP appears to be safe in the treatment of patients with PDAC.

INTRODUCTION

Laparoscopic distal pancreatectomy (LDP) has been shown [1–8] to have significant advantages over open distal pancreatectomy (ODP). Many large, single institutional or multi-institutional studies have demonstrated the safety and feasibility of this approach for malignant indications [9–13]. However, some still question its appropriateness for pancreatic adenocarcinoma (PDAC) [14]. A recent meta-analysis of LDP for PDAC showed less blood loss, shorter hospital stay, longer operative times, and smaller tumor size than for ODP for PDAC [15]. Oncologic outcomes, such as margin status and lymph node harvest, as well as pancreatic fistula rate, morbidity, mortality, and administration of adjuvant therapy were similar. However, the overall number of patients analyzed in this study was small and only surrogate markers of oncologic equivalence were analyzed. Similarly, a

study using the National Cancer Data Base to examine the outcomes of LDP for PDAC over a two-year time period demonstrated similar advantages over ODP without compromising perioperative oncologic outcomes [16]. Prognosis from PDAC is notoriously poor, even after complete surgical resection. Therefore, surgeons must closely evaluate each intervention to ensure that both quality and quantity of each patient's survival is not deleteriously affected. Additionally, in studies of adjuvant therapy for pancreatic adenocarcinoma, up to 38 % of patients are not able to complete full adjuvant therapy regiments, at least in part due to the effects of surgical morbidities [17]. Surgical techniques that help reduce morbidity may help increase the proportion of patients completing therapy and thus may have some beneficial effect on survival. It is clear that LDP has short-term advantages over ODP. However, reports regarding long term survival outcomes that compare LDP to ODP for PDAC are scarce and underpowered. This study was performed to compare short- and long-term outcomes of LDP and ODP for PDAC at a single institution over a 20-year time period.

MATERIAL and METHODS

Information was collected on all patients undergoing pancreatic resection from January 1995 to December 2014. This study used an institutional review board (IRB)-approved prospective database. The data points that were collected from the database included demographics, operative variables, postoperative outcomes, pathologic findings, and extended follow-up. Preoperative characteristics included age, sex, comorbidities, body mass

index (BMI), American Society of Anesthesiologists (ASA) score, and the use of neoadjuvant treatments. Operative details included operative time (incision to close of the wound), estimated blood loss (EBL), and blood product transfusion obtained from the anesthesia record. Use of laparoscopy, type of distal pancreatectomy, vascular resection, and concomitant resections were also recorded. Postoperative outcomes were tracked for 3 months (90 days) after surgery and were graded according to the Clavien system [18]. A final overall patient complication grade was given to the highest-rated complication grade experienced by patients in the group. Clinically significant complications (morbidity) were defined as grade III-V complications. Pancreatic fistula (PF) [19] and post-pancreatectomy hemorrhage (PPH) [20] were scored and graded according to standard international consensus definitions. Length of stay (LOS) was recorded and was defined as postoperative day 1 through day of discharge. Readmission to any hospital was defined as any unplanned admission and was tracked for all patients through 90 days after surgery. Reoperation was defined as any unplanned operation within 90 days of the primary pancreatic resection. Final pathologic details were recorded and included margin status and lymph node harvest. Positive margins were considered positive if microscopic (R1) or macroscopic (R2) disease was noted at the surface of any surgical margin. There was no use of separate diagnostic laparoscopy with peritoneal cytology or intraoperative peritoneal cytology in this study. Adjuvant therapy (chemotherapy and/or radiation) is administered as indicated to all eligible patients at a time frame of 4–8 weeks postoperatively. Adjuvant therapy data

were collected retrospectively from our institution as well as outside facilities in which the therapy was administered for patients who received treatment elsewhere. Patients included in the study were those patients with PDAC undergoing consecutive distal pancreatectomy for curative intent during the study time period. Those patients with the finding of unresectable metastatic peritoneal disease at the time of the index operation were excluded from analysis. This finding is rare as the routine use of high quality magnetic resonance imaging and endoscopic ultrasound by a dedicated HPB team is performed for all patients. NCCN guidelines were used to determine resectability based on these studies. Distal pancreatectomy for PDAC was approached by open technique for the first 15 years of the study with only one exception and approached by laparoscopic technique during the last five years of the study with only two exceptions due to the arrival of a minimally invasive HPB surgeon at the end of 2008 (H.A.) and the addition of another in 2011 (J.S.) who was trained at our institution and therefore, LDP was performed in the same manner by both surgeons as previously described [21]. Parenchymal transection was performed by stapled transection in the large majority of ODP and LDP. However, since 2008, LDP parenchymal transection has been performed using a slow compression technique with staple line reinforcement. Categorical data are reported as number with percentage of the whole with significance tested by 2-tailed Fisher's exact test. Continuous data are reported as a mean with range with significance tested by a t test. Survival analysis was performed using Kaplan–Meier estimated survival. Analyses were completed in an intention-to-treat manner.

RESULTS

All patients

During the study time period, 351 patients underwent distal pancreatectomy. Of these, 72 patients underwent distal pancreatectomy for curative intent for the diagnosis of pancreatic ductal adenocarcinoma. Open distal pancreatectomy (ODP) was performed in 28 patients, while laparoscopic distal pancreatectomy (LDP) was performed in 44 patients. The demographics of the patients are shown in Table 1, and the majority of patients in both groups had at least one major comorbidity. The patients undergoing LDP were older, but the patients were otherwise well matched between the two groups.

	ODP $n = 28$ (%)[a]	LDP $n = 44$ (%)[a]	p value
Male	16 (57.1)	26 (59.1)	1.0000
Hypertension	16 (57.1)	31 (70.5)	0.3123
Diabetes	8 (28.6)	16 (36.4)	0.6105
High cholesterol	15 (53.6)	32 (72.7)	0.1290
Cardiac disease	13 (46.4)	19 (43.2)	0.8122
Pulmonary disease	9 (32.1)	10 (22.7)	0.4194
ASA[b]			1.0000
II	5 (17.91)	5 (11.4)	–
III	22 (78.6)	35 (79.5)	–
IV	1 (3.6)	4 (9.1)	–
Mean age (years)*	67.3 (44–85)	72 (55–90)	0.0534
Body mass index*	26.1 (17–43)	28.3 (17–63)	0.2121

ASA American Society of Anesthesiologists score

*Values are mean (range)

[a] Values in parenthesis are percentages unless otherwise indicated

Table 1 Demographics for 72 patients undergoing open distal pancreatectomy (ODP) or laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma

The hand-assisted method was used in four patients (9.1 %) from the LDP group, while the conversion to open surgery was necessary in five (11.4 %) patients from this group (Table 2). Conversion to open surgery was performed when multivisceral resection could not be completed laparoscopically (n = 2), when there was extensive tumor involvement near the celiac trunk (n = 1), when there was recurrent positive pancreatic margins (n = 1), and for control of hemorrhage (n = 1). Regarding operative variables (Table 2), LDP compared with ODP was associated with significantly less blood loss [332 vs. 884 mL (p = 0.0012)] and intraoperative blood transfusion [18.2 vs. 50 % (p = 0.0495)]. A multivisceral resection was required in 32.1 and 38.6 % (p = 0.6229) of patients undergoing ODP and LDP, respectively, and most commonly included partial gastrectomy, colectomy, or left adrenalectomy. Vascular resections included portal vein or hepatic arterial resections, and their incidences were very similar between groups, occurring in approximately 7 % of patients in each group. Ninety-day postoperative complication rates (Table 2) were largely similar between the two groups. Postoperative monitoring in the intensive care setting was needed much less frequently in the LDP patient group (16 %) compared with those undergoing ODP (44 %) (p = 0.0150). Morbidity and readmission rates were also lower for LDP but did not reach statistical significance. Length of stay was significantly shorter for the LDP group (5.1 days) versus the ODP group (9.4 days) (p = 0.0001). One patient experienced both the single postoperative hemorrhage and mortality in the LDP group. This patient had variant hepatic artery anatomy with subsequent injury eventually resulting

in hepatic infarction, pancreatic fistula, and hemorrhage eventually resulting in multiorgan dysfunction and death. The one patient that required reoperation experienced an abdominal fascial dehiscence at the specimen extraction site resulting in the need for wound exploration and fascial closure.

Variable	ODP $n = 28$ (%)[a]	LDP $n = 44$ (%)	p value
Operative time (min)*	266 (131–543)	254 (99–521)	0.5961
Estimated blood loss (mL)*	874 (150–3400)	332 (10–2650)	**0.0012**
# pts pRBC transfusion	11 (50)‡	8 (18.2)	**0.0495**
Vascular resection	2 (7.2)	3 (6.8)	1.000
Multivisceral resection	9 (32.1)	17 (38.6)	0.6229
Conversion to ODP	–	5 (11.4)	
Pulmonary complication	6 (21.4)	4 (9.1)	0.1723
Pancreatic fistula	2 (7.1)	6 (13.6)	0.4705
A	0	1 (2.3)	–
B	2 (7.1)	5 (11.4)	–
C	0	0	–
Post-pancreatectomy hemorrhage	0	1 (2.3)	1.000
A	0	0	–
B	0	1 (2.3)	–
C	0	0	–
Wound infection	4 (14.3)	4 (9.1)	0.7028
Intra-abdominal abscess	2 (7.1)	3 (6.8)	1.000
Readmission	6 (21.4)	5 (11.4)	0.3187
Reoperation	1 (3.6)	1 (2.3)	1.000
Morbidity (Clavien grade III-V)	7 (25)	6 (13.6)	0.3460
Mortality (90 days)	0	1 (2.3)	1.000
Intensive care stay (days)*	1.1 (0–15)	0.3 (0–4)	0.0915
Length of stay (days)*	9.4 (4–36)	5.1 (2–17)	**0.0001**

p values <0.05 are in bold
pRBC Intraoperative packed red blood cell transfusions
* Values are mean (range)
‡ Data missing for 6 patients
[a] Values in parenthesis are percentages unless otherwise indicated

Table 2 Operative variables and postoperative outcomes (90 days) for 72 patients undergoing open distal pancreatectomy (ODP) or laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma

Pathological examination (Table 3) revealed that tumor size, T-stage, and N-stage were well matched between the two groups. LDP was associated with a significantly higher number of harvested lymph nodes compared with ODP [25.9 vs. 12.7, respectively (p = 0.0001)]. LDP was also associated with a smaller tumor size, higher rate of negative margins, and lower lymph node ratio (LNR), but these differences did not reach statistical significance. Neoadjuvant therapy was used prior to ODP and LDP in one and two patients,

respectively. Three-fourths of patients in both groups underwent treatment with adjuvant chemotherapy, but the LDP group was able to initiate treatment within a significantly shorter time period after surgery 69.4 vs. 95.6 days, respectively (p = 0.0441).

Table 3 Pathologic findings for 72 patients undergoing open distal pancreatectomy (ODP) or laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma

Variable	ODP $n = 28$ (%)[a]	LDP $n = 44$ (%)	p value
T-stage			0.7808
T1	4 (14.3)	3 (6.8)	–
T2	2 (7.1)	9 (20.5)	–
T3	22 (78.6)	32 (72.7)	–
N-stage			0.6250
N0	18 (64.3)	25 (56.8)	–
N1	10 (35.7)	19 (43.2)	–
Margin status			0.1012
RO	23 (82.1)	42 (95.5)	–
R1/2	5 (17.8)	2 (4.5)	–
Tumor size (cm)*	4.5 (0.2–15)	3.6 (0.5–7.5)	0.1383
Number of lymph nodes resected*	12.7 (1–45)	25.9 (5–48)	**0.0001**
Lymph node ratio (N1 only)*	0.285 (0.034–0.7)	0.168 (0.021–0.8)	0.1235
Receipt of adjuvant chemotherapy[b]	18 (75)	31 (75.6)	1.000
Time to start adjuvant therapy (days)[c]	95.6 (26–198)	69.4 (38–143)	**0.0441**

p values <0.05 are in bold
* Values are mean (range)
[a] Values in parenthesis are percentages unless otherwise indicated
[b] Data available for ODP ($n = 24$) and LDP ($n = 41$)
[c] Data available for ODP ($n = 14$) and LDP ($n = 27$)

Table 3 Pathologic findings for 72 patients undergoing open distal pancreatectomy (ODP) or laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma

Survival

Kaplan–Meier estimated survival analysis (Table 4) revealed similar overall and stage matched survival for the two groups at all points through 5 years. There was no statistical difference in overall survival between LDP and ODP for all stages (p = 0.851) or when matched by stage (Fig. 1). The estimated median overall survival is 26.4 and 26.6 months for ODP and LDP, respectively. Only 1 patient in the ODP group was lost to follow-up.

Years	ODP[a] Estimated survival (95 % CI)			LDP Estimated survival (95 % CI)		
	All $n = 27$	Stage I $n = 6$	Stage II $n = 21$	All $n = 44$	Stage I $n = 10$	Stage II $n = 34$
1	78 % (64–95 %)	83 % (58–100 %)	76 % (60–97 %)	69 % (56–84 %)	80 % (59–100 %)	65 % (51–84 %)
2	52 % (26–75 %)	83 % (58–100 %)	43 % (26–70 %)	60 % (47–78 %)	69 % (45–100 %)	59 % (43–79 %)
3	44 % (29–68 %)	83 % (58–100 %)	33 % (18–61 %)	41 % (27–63 %)	69 % (45–100 %)	31 % (17–59 %)
4	32 % (19–56 %)	67 % (38–100 %)	22 % (10–51 %)	41 % (27–63 %)	69 % (45–100 %)	31 % (17–59 %)
5	32 % (19–56 %)	67 % (38–100 %)	22 % (10–51 %)	41 % (27–63 %)	69 % (45–100 %)	31 % (17–59 %)

[a] Data missing for 1 patient

Table 4 Kaplan–Meier overall survival estimates for 72 patients undergoing open distal pancreatectomy (ODP) or laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma by stage I and stage II pancreatic adenocarcinoma

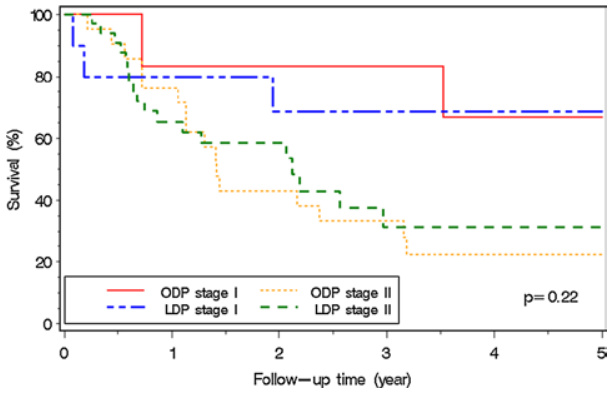

Fig. 1 Kaplan–Meier OS curves for 28 patients undergoing open distal pancreatectomy (ODP) and 44 patients undergoing laparoscopic distal pancreatectomy (LDP) for pancreatic ductal adenocarcinoma

DISCUSSION

Over the past decade, the safety and feasibility of laparoscopic pancreatic surgery has been proven by many surgeons and institutions around the world. Furthermore, advantages of the laparoscopic approach have been clearly reported for distal pancreatectomy [1–8, 15]. Gagner et al. reported the first LDP for malignancy in 1996 [22], and since then, multiple series have been published that included small subsets of patients undergoing LDP for PDAC, with data on short-term oncologic outcomes [23]. In a series of distal pancreatectomies for PDAC reported in 2013, the authors compared 28 robotic and LDP to 34 ODP and found less blood loss and shorter length of

stay but similar oncologic outcomes for the minimally invasive group [12]. Just recently, a group from Korea reported a propensity score matched comparative analysis of two groups of 51 patients each undergoing LDP or ODP for PDAC and found a shorter hospital stay for LDP but similar postoperative outcomes and overall survival between the two groups [13]. LDP has become an accepted method for dealing with all pathologies of the neck, body, and tail of the pancreas. In the past, the adoption of LDP in the United States had been relatively low, but the rate appears to be increasing [24, 25]. Despite mounting evidence and increasing popularity, some authors still question the appropriateness of LDP for the treatment of malignancy and call for randomized studies to evaluate its effectiveness [14]. This series represents one of the largest experiences regarding LDP for PDAC published to date and demonstrates that there is no oncologic rationale for withholding the possible benefits of LDP from patients who require operative resection for PDAC in the distal pancreas. Our experience is unique in that there was a clear and definitive change in the approach to PDAC of the distal pancreas from an open fashion to a minimally invasive fashion with the arrival of the senior author in August 2008. Prior to this, all but one patient underwent ODP. After 2008, all distal pancreatectomies were preferentially approached by a minimally invasive fashion, and only 2 patients underwent ODP after 2008. The first patient underwent ODP due to the need for portal vein resection, and the second patient underwent ODP by a low volume non-pancreatic surgeon. This provided a relatively nonbiased comparison between the ODP and LDP groups, which were free of selection bias. The

patients were very well matched with regard to demographics, comorbidities, and pathologic staging; similar to the group who utilized a propensity score-matching method [13]. Patients undergoing LDP experienced the expected advantages over ODP and had decreased blood loss and less need for intraoperative blood transfusions, and these results were similar to those that we and others have previously published regarding LDP versus ODP [9, 12, 26]. A review of recent literature clearly indicates that perioperative blood transfusions have been repeatedly associated with a poor long-term prognosis after resection of pancreatic cancer [27]. Unfortunately despite this improvement with LDP, long-term survival was not significantly impacted in this study. Perhaps future studies with sufficient patients will allow for this attribute to positively impact survival after LDP for PDAC, but there are numerous other factors that also affect survival. We were also able to demonstrate significantly increased lymph node retrieval with the use of LDP compared with ODP in our series. Previously, we reported a non-significant increase in lymph node retrieval for patients undergoing LDP for PDAC and pancreatic neuroendocrine tumors [9]. While this translated into a lower LNR for the patients undergoing LDP in this current study, this did not reach statistical significance. A decreased LNR has been shown to have prognostic significance for patients undergoing pancreatic resection for PDAC in a single institutional series of 905 patients [28], and it was seen in a Surveillance, Epidemiology, and End Results (SEER) database study [29]. It is unlikely that resection of additional peripancreatic lymph nodes will truly impact the prognosis of a patient undergoing pancreatic resection for PDAC.

However, we believe this data demonstrates the ability to ensure a wide en bloc resection of the body and tail of the pancreas afforded and even facilitated by the laparoscopic approach. Correspondingly, a negative margin (RO) was obtained in a higher percentage of patients undergoing LDP than ODP (95.5 vs. 82.1 %, p = 0.1012). While this did not reach statistical significance, similar findings were identified by a recent National Cancer Data Base (NCDB) study that found that patients undergoing LDP had an increased rate of margin negative resections over ODP (87 vs. 78 %, p = 0.042) [16]. The lack of selection bias in our data seems to indicate that a ''cleaner'' and wider en bloc resection can be performed using a minimally invasive method. RO resection is known to have a significant impact on survival, and although it was not born out in our survival results possibly due to the small numbers of patients in our study, this higher rate of RO resection with LDP could certainly contribute to improved overall and disease-free survival in patients undergoing PDAC resection. Our findings are similar to those reported in 2014 by Lee et al. This group described twelve patients undergoing minimally invasive, radical antegrade modular pancreatosplenectomy (RAMPS) for distal PDAC. Operative time, blood loss, transfusion rate, and overall complications reported were well within acceptable margins, and they were able to obtain a margin negative resection in all patients with an impressive 5-year disease-specific survival of 55.6 % [30]. While their series did not include patients undergoing en bloc gastric or colon resection, other groups have also demonstrated the ability of experienced surgeons to modify dissection planes to accomplish an oncologically appropriate resection even

with contiguous organ involvement [13, 31]. The minimally invasive approach can also be advantageous for patients in whom a need for conversion to open resection will be very likely. In this "hybrid" approach, a laparoscopic mobilization of the tail of the pancreas and spleen allows for a smaller open incision, oftentimes by a midline incision which spares a large left subcostal incision for the patient. This is particularly true for larger and more central lesions or those that require a multivisceral or vascular resection. Overall survival was very similar between those patients undergoing LDP and ODP and comparable to results previously published for LDP for PDAC [23]. Estimated three-year overall survival for LDP and ODP was 41 and 44 %, respectively. Currently, there are eight 5-year survivors from the ODP group and two 5-year survivors from the LDP group. The majority of patients in the LDP group have not reached this post-surgical time point yet because of the date of their initial surgery. The limitations of this study included a relatively small sample size, but our study can be considered large in comparison to what is currently available in literature. Additionally, data regarding the use of adjuvant therapy (chemotherapy and radiotherapy) including the time periods, regiments, completion rate, tolerance, and reason for withholding adjuvant treatment was often absent due to the nature of referrals to our institution. A large majority of patients are from afar and do not receive adjuvant treatments within our institution since they are treated closer to their home, which makes long-term follow-up of these parameters more challenging. As these data were collected over the course of a 20-year time period, there were clearly changes and

developments in technique and perioperative care that can account for some of the short-term and postoperative outcome improvements seen in this study. Surgical technique, perioperative anesthesia management, pathologic analysis, postoperative protocols, and comprehensive cancer management systems have undergone continuous progressive evolution in this time period. The overall treatment of PDAC is multifactorial and minimally invasive surgery is just one of the many tools that surgeons may utilize to lessen the impact of this disease on our patients.

CONCLUSION

This study is currently one of the largest single institutional series to report LDP for PDAC and shows that this approach does not compromise survival. In fact, LDP improves RO resection rate and LNR and leads to a more immediate initiation of adjuvant chemotherapy after surgery, which may impact survival if analyzed in a larger number of patients. One of the advantages of this study is the lack of selection bias, a clear change in practice from open to laparoscopic at a particular time period, and a standardized laparoscopic technique by two experienced MIS pancreas surgeons. Ultimately, overall survival was not affected by the laparoscopic approach in this study despite the improvement in surrogate oncologic markers and may need larger studies to detect any differences. Nevertheless, LDP has been shown to have clear perioperative and short-term advantages over OPD and does not appear to pose any oncologic disadvantages. The outcomes of patients treated at our institution in the study time period provides evidence that the

laparoscopic approach is safe and may be responsible for improved short-term outcomes. However, a multi-institutional study comparing the MIS and open approaches in a contemporaneous group of patients may be of benefit to validate these outcomes.

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THIRD PAPER

Laparoscopic pancreatoduodenectomy: current status and future directions

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Abstract

In recent years, laparoscopic pancreatoduodenectomy (LPD) has been gaining a favorable position in the field of pancreatic surgery. However, its role still remains unclear. This review investigates the current status of LPD in high-volume centers. A literature search was conducted in PubMed, and only papers written in English containing more than 30 cases of LPD were selected. Papers with ''hybrid'' or robotic technique were not included in the analysis. Out of a total of 728 LPD publications, 7 publications matched the review criteria. The total number of patients analyzed was 516, and the largest series included 130 patients. Four of these studies come from the United States, 1 from France, 1 from South Korea, and 1 from India. In 6 reports, LPDs were performed only for malignant disease. The overall pancreatic fistula rate grades B–C were 12.7%. The overall conversion rate was 6.9%. LPD seems to be a valid alternative to the standard open approach with similar technical and oncological results. However, the lack of many large series, multi institutional data, and randomized trials does not allow the clarification of the exact role of LPD.

INTRODUCTION

Since widespread adoption of minimally invasive surgery (MIS) in the late 1980s, surgical technique has undergone a major transformation and there are very few abdominal disease processes and operations that have not been impacted. MIS techniques have been demonstrated to be safe and effective in the treatment of benign and malignant disease [1–3]. Today, the role of laparoscopic approach in pancreatic surgery is still debated. In 1994, Cuschieri [4] reported the first laparoscopic distal resection and, in the same year, Gagner and Pomp [5] reported the first laparoscopic pancreatoduodenectomy (LPD). Two decades later, laparoscopic distal resection has been widely adopted and becomes the technique of choice to treat pancreatic pathology of the body and tail by most experienced pancreatic surgeons. By contrast, the diffusion of LPD remains confined to a very few high-volume centers with no clear advantages over open pancreaticoduodenectomy (OPD). The slow progression of this technique is likely related to two major aspects. First, the retroperitoneal position of the pancreas and the close relation to major central mesenteric vasculature make dissection difficult and potentially hazardous. Second, biliary and pancreatic reconstruction is particularly challenging, requiring advanced surgical skills and additional time. In the recent years, several series of LPD have been published. However, many reports include only a small amount of patients, are retrospective in nature, and often include a variety of LPD procedures, such as hybrid or combined approaches. In this review, we summarize what is currently known about LPD from the published literature with respect to

the patients selected to undergo the procedure, the operative and postoperative outcomes, and the pathologies treated.

MATERIAL and METHODS

A literature review was performed using PubMed Central with the search terms ''laparoscopic pancreatoduodenectomy'', ''laparoscopic Whipple'', ''minimally invasive pancreatoduodenectomy'', and ''minimally invasive Whipple''. In addition, cross-referencing was performed, and the relevant articles were reviewed. The final search was made on May 5, 2016. Only articles written in English were included. The minimum number of LPD reported was 30. In the case of multiple articles from the same institution, only the most recent article with the most complete data was included to avoid any overlap in the data analysis. Articles with ''hybrid'' technique or robotic-assisted pancreatoduodenectomy (PD) were excluded from our review. The following variables were collected in all the studies: patient demographics, including number of LPD, inclusion period, institution, country, age, sex, body mass index (BMI), and malignant indication; operative variables, including operative time (OT), intraoperative estimated blood loss (EBL), vascular resection, conversion rate, and conversion to hybrid or robotic technique; pathological reports, including tumor size, pathology, benign indications, margin status (R0), and number of lymph nodes resected; and postoperative outcomes, including overall complication, major complication, postoperative pancreatic fistula (POPF), delay gastric empting (DGE), postoperative pancreatic hemorrhage (PPH), intraabdominal abscess, hospital length of stay (LOS), re-intervention, and mortality. Our previous

report [6] assessed postoperative morbidity using the expanded Accordion Severity Grading System described by Strasberg et al. [7]. In five studies [8–12], the postoperative complications were addressed using the Clavien–Dindo classification system [13]; in one article [8], only grades C3 are reported, in two [9, 12], grades 3–4 are reported, in one [11], grades ≥3, and in one [10], all the grades are reported. To determine major complications, we used grades C3 in the Clavien–Dindo classification and>3 in the Strasberg classification. The paper from Paniccia et al. [14] did not use the standard classification for postoperative complications. In addition, all studies used the International Study Group of Pancreatic Surgery (ISGPS) consensus definitions for POPF [15]. Regarding DGE [16], the definition from the ISGPS was used in four papers [6, 8, 10, 11], but not specified in the other papers [9, 12, 14]. PPH [17] under the ISGPS definition was used in three papers [6, 8, 11], and the other papers used different definitions of postoperative hemorrhage.

RESULTS

Patients' selection

In a total of 728 papers, 7 articles [6, 8–12, 14] fulfilled our inclusion criteria for this review and included 516 patients who underwent LPD. All studies were single center reports, and no multi-institutional or prospective reports were identified. The mean observational period of the studies was 6 years (range 1–15), with reports coming from four countries on three continents. The demographic information for review articles is listed in Table 1. At the

turn of the century, laparoscopy for the treatment of pancreatic disease was only for staging and palliation of pancreatic malignancies [18, 19]. Since then, the feasibility and safety of LPD have been clearly demonstrated. Palanivelu et al. presented the first large series of LPD in 2007 [20], and this group was the first to propose that not only was it possible and safe to perform LPD, but there may also be advantages in comparison with open resection. As expected, careful patient selection was performed during the exploration and early adoption phase of this technique. In 2009, the same group reported a larger series of 75 patients to demonstrate the oncological adequacy and safety of LPD [21]. Indeed, the vast majority of LPD in the included studies have been performed for patients with underlying malignant disease, and no large series containing treatment of benign disease by LPD in large numbers have been published. Regarding the age, sex, and BMI of those undergoing LPD, there were no differences in the recruitment of the patients. In fact, there were no common univocal selection or exclusion criteria for LPD amongst the studies. In our previous report [6], regarding our personal experience with LPD, the decision to perform LPD was not based only on clinical factors or preoperative diagnosis. In addition, the patients' preferences were taken into consideration when deciding the surgical approach. However, as common sense dictates, large lesions with major vascular involvement or clearly hostile abdomen were excluded from LPD. Similar criteria were adopted by Kendrik and Cusati in their report [22]. Song and colleagues [10] included in their series only patients with periampullary tumors without preoperative T4 or M1 staging, suspicion of vascular

involvement, high cardiopulmonary morbidity, severe obesity ([30 kg/m2 for men;[35 kg/m2 for women), or expected severe adhesion or inflammation. Delitto et al. [12] adopted similar criteria regarding BMI, with a cutoff of BMI ≥40. In addition to the previous exclusion criteria, Dokmak and colleagues [9] proposed that further contraindications to LPD included the need for multiple frozen sections, such as for intraductal papillary mucinous neoplasms or the division of a median arcuate ligament. Only in the reports published by Croome et al. [8], Mesleh et al. [23], and Senthilnathan et al. [11] was vascular involvement not exclusion criteria for LPD.

Table 1 Patient selection

	Asbun et al. [6]	Croome et al. [8]	Dokmak et al. [9]	Song et al. [10]	Paniccia et al. [14]	Senthilnathan et al. [11]	Delitto et al. [12]
Number of LPD	53	108	46	97	30	130	52
Period of inclusion	2005–2011	Jan 2008–July 2013	Apr 2011–Apr 2014	Jan 2007–Dec 2012	Jan 2013–Dec 2014	Mar 1999–Apr 2013	Nov 2006–Feb 2014
Institution	Mayo Clinic—Florida	Mayo Clinic—Rochester	Beaujon Hospital	Ulsan University College	University of Colorado	GEM Hospital and Research Center	University of Florida
Country	USA	USA	France	South Korea	USA	India	USA
Age (years)	62.9 ± 14.1	66.6 ± 9.6	60 (27–85)	48.6 ± 14.1	63.1 (53.8–70.8)	54 (28–76)	65.3 ± 1.7
Male/female, *n* (%)	29 (54.7%)/24 (45.3%)	51 (47.2%)/57 (52.8%)	26 (57%)/20 (43%)	48 (49.4%)/47 (50.6%)	–	1:1.6	34 (65%)/18 (35%)
BMI (kg/m^2)	27.6 ± 7.1	27.4 ± 5.4	22.6 (17–30)	22.7 ± 2.8	–	27.9 (22.6–33.8)	26.3 ± 0.8
Malignant *n* (%)	41 (77.3%)	108 (100%)	46 (100%)	97 (100%)	–	130 (100%)	52 (100%)

BMI body mass index (kg/m^2), *LPD* laparoscopic pancreatoduodenectomy

Operative details

Operative variables are summarized in Table 2. The reported OT was between 4 and 10 h. In the studies reported by Croome et al. [8] and Delitto et al. [12], there were no differences in OT comparing LPD vs OPD. On the contrary, Asbun and Stauffer [6], Dokmak et al. [9], and Song et al. [10] report that OT in LPD is greater than in OPD. This difference is significant in all three

series with longer median OT of 139, 78, and 129 min, respectively. However, all the series described a reduction in the OT with increased experience. Song et al. described that, after the initial 50 cases of LPD, OT were decreased, becoming similar to that of OPD (15). Kendrick and Cusati reported a mean OT of 7.7 h for the first 10 patients, which improved to 5.3 h for the last 10 patients in their initial series of 62 patients [22]. It should also be noted that there was a learning curve not only for the surgeon, but also for all those involved in the procedure, including operating room staff, anesthesia team, and surgical assistants. Increased OT is the necessity of a meticulous dissection and precise reconstruction in LPD and may also be related to training new surgeons in the performance of the procedure. The majority of the studies report a significant decrease in EBL when compared to OPD. This appears to be inherent to the laparoscopic technique, where any significant bleeding would obscure that the operative field and decreased blood loss may be one of the greatest advantages to LPD. EBL is directly related to a need for intraoperative blood transfusions, and therefore, many series also show a decreased use of blood transfusions for LPD. For patients with malignancy, minimizing blood transfusion has been reported to have a positive effect on the long-term survival [24–30]. Despite many who would consider vascular involvement requiring reconstruction as exclusion criteria for LPD, 2 series reports experience with vascular resections during LPD for 22 patients [8] and 1 patient [11]. In the author's series comparing cost between 75 LPD and 48 OPD, there were no differences in the rate of vein resection between the laparoscopic (17%) and open (15%) procedures [23].

Croome et al. [31] and Palanisamy et al. [32] more recently reported their experience demonstrating the feasibility of major vascular resection during LPD. The description from Palamisamy et al. [32] is a report of a single case of portal resection with type IV vascular reconstruction. The aim of this paper was to show the technical feasibility of this procedure. Croome et al. [31] reported a large experience in vascular resections (31 patients) with different types of vascular involvement and reconstruction with a comparison between laparoscopic and open vascular resections. Laparoscopic resection had less EBL than open approach, but with longer clamping time (LPD 46.8 ± 30.8 min vs OPD 25.1 ± 16.2 min, p\0.001). No differences were found between the two groups in terms of postoperative outcomes. The conversion rate is lower than 10% in all the series. A common cause of conversion is reported to be the unexpected major vascular infiltration. Several groups report conversion to a hybrid technique [6, 8, 9, 22]. Croome et al. [8] report the experience of five patients undergoing LPD using robot-assisted methods as an adjunct for reconstructions after total LPD. Some authors suggest adopting the hybrid technique during the learning curve to improve the MIS technique at the beginning of the center's experience. In addition, conversion to hybrid or OPD should not be considered as a failure as long as is it not at the expense of increased postoperative morbidity [6]. It is important to make the conversion before massive bleeding occurs, or when persistent failure to progress will result in an unreasonable and excessive increase in OT. This concept is stressed by the authors of all the series.

Table 2 Operative details

	Asbun et al. [6]	Croome et al. [8]	Dokmak et al. [9]	Song et al. [10]	Paniccia et al. [14]	Senthilnathan et al. [11]	Delitto et al. [12]
Operative time (min)	541 ± 88	379.4 ± 93.5	342 (240–540)	480.4 ± 116.4	340 (308–377)	310 ± 34	361 ± 7
EBL (ml)	195 ± 136	492 ± 519.3	368 (50–1200)	592 ± 376	300 (200–400)	110 ± 22	260 ± 36
Vascular resection, *n* (%)	–	22 (20.4%)	–	–	–	1 (0.7%)	–
Conversion, *n* (%)	9 (15%)	7 (6.4%)	3 (6.5%)	–	2 (6%)	1 (0.7%)	7 (9%)
Conversion to hybrid, *n* (%)	3 (5.6%)	5 (4.6%)	1 (2.2%)	–	–	–	–

EBL estimated blood loss

Postoperative outcomes

Postoperative outcomes are summarized in Table 3. The overall postoperative complication rate was 26.8–74%. However, major complication rates reported for Clavien–Dindo grades ≥3 [9–12] were 8.2–28%. Croome et al. [8] only reported those patients with Clavien–Dindo grades ≥3 b, accounting for a major complication rate 5.6%. In our previous report [6], using the expanded Accordion Severity Grading System described by Strasberg et al. [7], major complications (Clavien–Dindo grades ≥3) occured in 13 patients (24.5%). In general, complication rates reported in these series are comparable to most other high-volume centers reporting open pancreatoduodenectomy outcomes. While it is difficult to compare the data for specific complications, such as POPF, DGE, and PPH, due to incomplete data information, the reported rates are similar to those reported in the previous literature, such as for the overall morbidity. Reported rates of POPF grades B–C were 6.3–44% in the different series. Dokmak et al. [9] reported the incidence of the overall POPF as 48% (grade A: 4%; grade B: 20%; grade C: 24%). In the same paper, they report the overall POPF rate

after OPD as 41% (grade A: 9%; grade B: 26%; grade C: 6%). While there were no significant differences between LPD and OPD in the overall POPF rate, the rate of POPF grade C was higher in the LPD group. The authors suggested that this resulted from the difficult pancreatic anastomosis required for LPD in some periampullary tumors with a soft pancreas and a non-dilated small main pancreatic duct. However, besides this single experience, the other series report a lower POPF grades B and C rates for LPD ranging from 8.4 to 24% (Table 3). Most series report a decreased LOS for LPD except for Dokmak et al. [9]. For PD, however, LOS is generally predicated on the presence or absence of major complications. As major complications are generally driven by POPF, it is worth noting that decreased LOS will be accomplished by minimizing the POPF and major complication rates. The difference in LOS seen in the included studies may also be due to a more aggressive postoperative management of the patients undergoing LPD in comparison with OPD rather than the laparoscopic approach itself. Several series show that patients treated by LPD had a faster recovery, leading to an earlier start of adjuvant therapy. Croome et al. [8] reported a median time between surgery and adjuvant treatment of 48 (17–116) days for LPD vs 59 (25–302) days for OPD ($p = 0.001$). Song et al. [10] reported that 81.8% of the patients resected with LPD received adjuvant treatment compared to 69.7% of patients resected with OPD. Conversely, Nussbaum et al. [33] reported that LPD was not associated with an earlier initiation of adjuvant chemotherapy and that the interval time was more due to the major postoperative complications, not the surgical approach. Similar to this study,

we have recently reported our long-term outcomes for pancreatic ductal adenocarcinoma after LPD and note that there was no advantage to the initiation of adjuvant therapy for LPD (Stauffer et al. 2016 Surg Endoscopy). In a direct cost analysis of LPD vs OPD, LPD was found to be associated with increased costs within the operating room due to operating time and supply costs [23]. However, the overall cost between the two techniques was noted to be similar due to decreased postoperative admission costs for LPD.

Table 3 Postoperative outcomes

	Asbun et al. [6]	Croome et al. [8]	Dokmak et al. [9]	Song et al. [10]	Paniccia et al. [14]	Senthilnathan et al. [11]	Delitto et al. [12]
Overall complications	25 (47.2%)	–	34 (74%)	26 (26.8%)	–	29.7%	–
Major complications	13 (24.5%)[a]	6 (5.6%)[b]	13 (28%)	8 (8.2%)	–	14 (10.7%)	13 (25%)
POPF (%)							
A	3 (7.1%)	–	2 (4%)	23 (24.2%)	8 (27%)[d]	–	3 (6%)
B	1 (2.4%)	12 (11%)	9 (20%)	6 (6.3%)	5 (17%)[d]	6 (4.6%)	6 (12%)
C	3 (7.1%)		11 (24%)	0 (0%)	2 (7%)[d]	5 (3.8%)	
DGE (%)	6 (11.3%)	10 (9%)	8 (17%)	15 (15.4%)	10 (33%)[d]	14 (10.7%)	–
PPH (%)	5 (9.4%)	8 (7%)	11 (24%)[c]	–	3 (10%)[d]	6 (4.6%)	5 (10%)[c]
Intraabdominal abscess	10 (18.9%)	–	2 (4%)	–	6 (20%)	2 (1.5%)	–
LOS	8 ± 3.2	6 (4–118)	25 (6–104)	14.1 ± 7.7	11 (8–15)	8.1 ± 8.6	9 ± 0.7
Re-operation	2 (3.8%)	–	2 (4.3%)	–	–	5 (3.8%)	–
Mortality	3 (5.7%)	1 (1%)	1 (2%)	1 (1%)	0	2 (1.5%)	1 (2%)

DGE delay gastric emptying, *LOS* length of stay, *POPF* postoperative pancreatic fistula, *PPH* postpancreatectomy hemorrhage, *Major complications* Clavien–Dindo classification grades ≥3

[a] Accordion Severity Grading System described by Strasberg grades 3–5

[b] Clavien–Dindo classification grade ≥3b

[c] Non-International Study Group of Pancreatic Surgery definition of PPH

[d] Nonspecified criteria of POPF, DGE, and PPH

Pathology

Extended pathological reports are listed in Table 4. The tumor dimensions were similar for all series, ranging from 1.2 to 4 cm. In general, LPD was associated with small-tomoderate dimension tumors and with smaller tumor size than OPD in most comparative series. As noted previously, a large proportion of patients in all series underwent LPD for malignant indications. Periampullary adenocarcinoma is the most frequent indication for LPD, with

pancreatic ductal adenocarcinoma being the most common. Negative margins were achieved in 60–100%, with four series reaching C90% R0 margin rate. Delitto et al. [12] reported higher negative margins for LPD compared to OPD (90.4 vs 74%, p = 0.03), and there may be a potential advantage with a precise dissection performed under direct magnified vision. The mean lymph node retrieval after LPD was 15–23 lymph nodes. Our series for patients with pancreatic adenocarcinoma showed higher lymph node retrieval for patients undergoing LPD compared to OPD, although this was related to the time period in which these operations were performed [34]. The lack of prospective or randomized trials and the relatively short follow-up prevents definitive conclusions regarding the overall long-term survival of patients with malignant disease treated with LPD. The results presented by Senthilnathan et al. [11] represent the largest series with the longest follow-up of patients undergoing LPD for periampullary malignancy. They describe 130 LPD from 1999 to 2013 with a median survival rate of 33 months. This survival rate was lower than that reported in their previous experience (24). The authors justify this decreased survival rate with the widened criteria of patient selection for LPD with growing expertise, as compared with highly selective cases in earlier series. The 5-year survival rate was 29.42% [11]. Our group recently reported our longterm survival for patients undergoing LPD for pancreatic ductal adenocarcinoma. Five-year survival was noted to be 32%, comparing favorably, but statistically similar, to OPD (15%) [34].

Table 4 Pathology

	Asbun et al. [6]	Croome et al. [8]	Dokmak et al. [9]	Song et al. [10]	Paniccia et al. [14]	Senthilnathan et al. [11]	Delitto et al. [12]
Tumor size (cm)	2.74 ± 1.6	3.3 ± 1.0	2.82 (1.2–4.0)	3.1 ± 1.4	–	3.13 ± 1.21	25 ± 0.1
Ampullary adenocarcinoma, *n* (%)	8 (15.1%)	–	12 (26%)	–	–	41 (31.5%)	13 (25%)
PDAC, *n* (%)	22 (41.5%)	108 (100%)	15 (32%)	–	–	58 (44.6%)	28 (54%)
IPMN, *n* (%)	8 (15.1%)	–	6 (13%)	42 (43.3%)	–	–	–
Neuroendocrine, *n* (%)	6 (11.3%)	–	6 (13%)	18 (18.6%)	–	–	–
Miscellaneous malignant, *n* (%)	5 (%)	–	7 (16%)	24 (24.8%)	–	31 (23.8%)	11 (21%)
Benign, *n* (%)	4 (%)	–	–	13 (13.4%)	–	–	–
Margin negative rate (R0), *n* (%)	37 (94.9%)	84 (77.8%)	9 (60%)[a]	–	30 (100%)	90.8%	47 (90.4%)
Lymph nodes	23.4 ± 10.1	21.4 ± 8.1	20 (8–59)[a]	–	–	18.2 ± 4.7	23 ± 1.2

Data are reported with mean and standard deviation or mean and range

IPMN intraductal papillary mucinous neoplasms, *PDAC* pancreatic adenocarcinoma

[a] Data regarding only PDAC patients

DISCUSSION

Despite the increasing number of publications and interest regarding LPD, few large series are available and experience is still limited to a small number of highly specialized surgeons and institutions. In the majority of these centers and when performed by experienced surgeons, LPD has been proven to be feasible and safe with no inferiority to the open technique and in certain aspects, showing some benefits, such as EBL. This, however, may not be the case when analyzing results at lower volume centers. Sharpe and colleagues [35] analyzed the US National Cancer Database and found that LPD was performed for pancreatic ductal adenocarcinoma for 9% of patients (384 LPD vs 4037 OPD) in 2010 and 2011, 75.7% of hospitals performed OPD only, 23.3% performed \10 LPD, and 1% performed ≥10 LPD. Interestingly, the majority of LPD procedures (96.2%) were performed in low-volume centers (<10 LPD/year). Subgroup analysis revealed an alarming finding of a high mortality (7.5%) for low-volume centers performing LPD,

underscoring the potential risks of this operation possibly resulting from being performed without the appropriate training or experience [35]. The data compiled in this review were highly selected to avoid the inclusion of a heterogeneous group of patients. There are many publications with smaller numbers of patients, as well as series that include hybrid or a composite of MIS techniques. A systematic review on robotic PD written by Cirocchi and colleagues [36] demonstrated how in different studies, there were a variety of techniques reported in a single group of patients. The authors concluded that the paper reported no difference in outcomes and safety in patients undergoing standard open, laparoscopic, or robotic PD. A recent paper published by Boggi et al. [37] described the safety and feasibility of pancreatic robotic resection in 200 consecutive robotic resections performed in 7 years. Within this patient group, the results of 83 robotic PD were reported with similar outcomes between open and robotic approach [37]. Zureikat et al. have recently reported a large multi-institutional series of robotic PD vs OPD [38] and found that many of the advantages and disadvantages of LPD are similar to the robotic approach. They found no difference for mortality, POPF, and length of stay, but found less major complications for the robotic platform. In selected papers, case matched controlled analysis was used to better compare LPD to OPD [9, 10]. Other authors used national or administrative database registries to evaluate the current status of minimally invasive PD [35, 39]. These studies have a larger number of patients with statistical power, but they miss the granular details of the patient's actual surgical treatment and risk studying patients

undergoing operations at low-volume centers without appropriate pancreatic surgical experience. Even though the conclusions of these studies are valid, one should keep in mind that this can lead to drawing flawed conclusions from skewed data resulting from inexperienced surgeons. Unfortunately, no prospective or multicenter studies comparing LPD with ODP were available. In conclusion, experience with LPD is increasing worldwide, but more time is needed to draw a definite conclusion. Data available in the literature suggest that LPD is safe, feasible, and associated with similar postoperative and oncological outcomes to OPD. LPD should be implemented in high-volume centers with appropriately trained and experienced pancreatic surgeons to achieve the safest results. Randomized, controlled trials and multicenter studies are necessary to solidify the potential advantages of LPD. Even though it is clear that LPD is here to stay, its role still needs to be better defined. Time will shed more light on this issue, as more surgeons obtain experienced in the procedure, and improved studies are published. What is clear is that today, there is no role for a surgeon to embark in minimal access pancreatoduodenectomy, either laparoscopic or robotic assisted, without the appropriate training. There are now several centers in which these techniques are well established and the procedures are being done safely and efficiently.

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FOURTH PAPER

Laparoscopic Distal Pancreatectomy with En Bloc Splenectomy

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Minimally Invasive Surgery of the Pancreas – Updates in Surgery Series –Springer

In press

Introduction

In recent years, the widespread application of minimally invasive surgery has gradually expanded to several surgical fields, and has become the gold standard of care for many surgical diseases [1]. The advantages of minimally invasive surgery for diseases of the pancreas are still under evaluation. Nowadays, there is a widespread use of laparoscopic and robotic techniques also in pancreatic surgery, and several reports confirm the feasibility and safety of this approach. At the beginning of the laparoscopic era only patients with benign or functional diseases were considered candidates for a laparoscopic approach [2]. The oncological appropriateness of this approach compared to the open approach was debated for a long time. Technological and instrumental improvements as well as an increased experience have extended the feasibility of performing more complex surgical procedures such as distal pancreatectomy and pancreatoduodenectomy. In addition, recent advances in surgical techniques and perioperative management have facilitated safe and successful pancreatic resections, which represent the first

step for a cure for patients with pancreatic malignancies.

Pancreatoduodenectomy and distal pancreatectomy are the two common surgical procedures performed to treat pancreatic diseases. Laparoscopic pancreatoduodenectomy (LPD) requires a high level of laparoscopic skill, a clear understanding of the anatomy and a high level of expertise in open pancreatic surgery. For these reasons, the use of the laparoscopic approach for surgery of the head of the pancreas is still debatable.

For laparoscopic distal pancreatectomy (LDP), the complexity of the surgery is significantly less and the operation requires a lesser level of laparoscopic skills when compared to pancreatoduodenectomy. LDP does not require any pancreatic, biliary or gastrointestinal reconstruction, which is an important Achilles' heel for LPD. The first report of LDP was published in 1994 by Cuschieri [3]. From the beginning, LDP has shown equivalent postoperative outcomes to open distal pancreatectomy (ODP), with other advantages in regards to less intraoperative blood loss, less postoperative pain and a shorter hospitalization. Benign lesions, neuroendocrine tumors and borderline lesions were the first indications for LDP reported in the literature [2, 4]. Nowadays malignant diseases of the body and tail of the pancreas are also approached with the laparoscopic technique. Several reports have investigated the oncological safety of LDP compared with open operation, finding similar outcomes in terms of radical resection rate, lymph node retrieval and overall survival. However, most of these reports analyzed retrospective data with considerable differences in patient selection. A significant bias, especially at the beginning of the laparoscopic era,

was that advanced cancers were still treated with an open approach [5]. A recent paper reporting on 20 years' experience with the treatment of distal pancreatic adenocarcinoma at the Mayo Clinic (Jacksonville, FL, USA) confirmed less blood loss and need for blood transfusion, shorter hospital stay and a faster time to initiate chemotherapy in patients treated with LDP compared to ODP [6]. No significant difference was found in survival due to the fact that survival is influenced more by the biology of the pancreatic cancer than by the surgical technique adopted. However, strong evidence on the oncological results of LDP by means of randomized trials with long-term follow-up is still lacking. A useful guide for selecting patients for LDP was proposed by Lee et al. [7], who presented, in 2014, the so-called Yonsei criteria. The Yonsei criteria were developed to identify patients eligible for a laparoscopic approach. They advocate using the laparoscopic approach only if the cancer is confined into the pancreas with an intact fascia layer between the pancreas and the left adrenal gland and at least 1 cm clearance from the celiac axis. These criteria are used as recommendations but many surgeons do not always follow them strictly. In fact, several papers report multivisceral laparoscopic resections, in relation to the experience of the center.

Surgical Technique for Laparoscopic Distal Pancreatectomy

Historically, distal pancreatectomy included removal of the spleen due to the close relation between the body-tail of the pancreas and the splenic vessels. Preservation of the spleen is still largely debated in distal pancreatectomy. In avoid immunological deficit, leukocytosis, thrombocytosis and

postsplenectomy sepsis.

Benoist and colleagues et al. [8] reported that spleen preservation was associated with more surgical complications when compared to distal pancreatectomy with splenectomy.

A different conclusion was reported in a retrospective review from the Memorial Sloan-Kettering Cancer Center, comparing distal pancreatectomy with and without splenectomy. The authors concluded that preserving the spleen was associated with a reduction in perioperative infectious complications, severe complications, and length of hospital stay [9].

Spleen preservation during distal pancreatectomy can be performed following two different techniques. Kimura et al. [10] proposed a technique for preservation of the splenic vessels in open distal pancreatectomy; this technique was later modified for the laparoscopic approach. Warshaw [11] proposed another spleen preservation technique with ligation of the splenic vessels. Both techniques are not free from complications such as spleen infarction for the Warshaw technique or postoperative bleeding for the Kimura technique.

A Consensus Conference in Laparoscopic Surgery for Pancreatic Neoplasms was held in Amsterdam in June 2016, during the 24th International Congress of the European Association for Endoscopic Surgery (EAES) [12]. The conclusion was that LDP is a feasible and safe alternative to the open approach in the treatment of both benign and malignant pancreatic lesions, providing advantages in terms of reduced blood loss, enhanced postoperative recovery and shorter hospital stay. The spleen-preserving approach is

strongly recommended for benign tumors, but there is no agreement for this technique in invasive cancer. Kawaguchi et al. described his experience in a small number of cases of pancreatic adenocarcinoma treated with splenic vessel ligation and spleen preservation with extensive lymphadenectomy, reporting no difference in terms of 5-year survival compared to distal pancreatectomy with splenectomy [13]. According to the EAES consensus, in patients with adenocarcinoma of the pancreas, splenectomy is recommended to achieve an adequate oncologic margin and lymph node clearance. However, for patients with benign or low-grade malignant tumors in the body/tail of the pancreas, preservation of the spleen with its immune function reduces the risk of overwhelming postsplenectomy infection and other complications related to the splenectomy procedure itself. Moreover, some authors reported that splenectomy may have a negative influence on longterm survival along with an increased risk of other cancers [14]. According to Strasberg et al., the greatest advantage surgery can offer patients with pancreatic adenocarcinoma is radicality of the resection. To achieve this, it is mandatory to resect the pancreatic tail en bloc with the spleen and all the lymph nodes around the splenic vessels and splenic hilum. In 2003, Strasberg described his surgical approach, called radical antegrade modular pancreatosplenectomy (RAMPS), which aimed to improve radicality of resection for left-side pancreatic tumors [15]. With this technique, the horizontal dissection plane from right to left allows a radical resection of regional lymph nodes (Fig. 21.1).

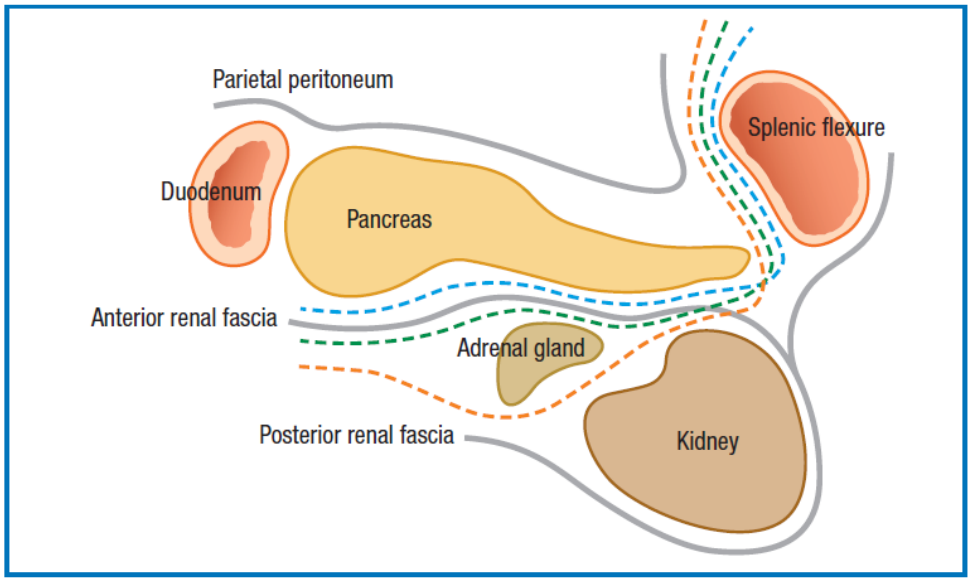

Fig. 21.1 Radical antegrade modular pancreatosplenectomy (RAMPS) planes: blue line, standard distal pancreatectomy plane of resection; green line, anterior RAMPS plane of resection; orange line, posterior RAMPS plane of resection

In LDP, right-to-left resection is very commonly performed, and application of the RAMPS approach to LDP seems to achieve good results [7]. In a recent paper, Kim et al. [16] described his laparoscopic RAMPS technique and reported favorable results in terms of negative posterior margin, lymph node retrieval and also better disease-free and overall survival. The magnified view obtained with laparoscopy allows a better visualization of the correct anatomical dissection plane, which improves the radicality of the resection. Another example of a useful alternative surgical technique is the "clockwise technique" described by Asbun et al. in 2011 [17]. For this technique, the patient is placed in a modified right lateral position. The surgeon stands to the right of the operating table. There are five steps to this technique, which is performed using four trocars (Fig. 21.2):

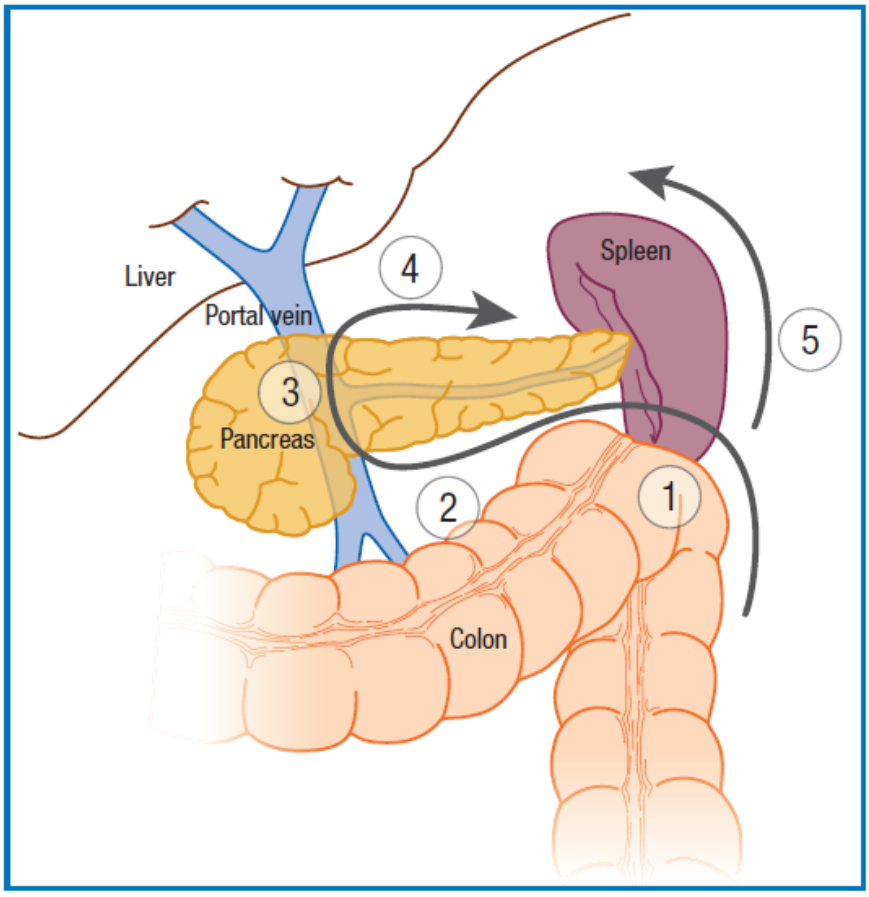

Fig. 21.2 The five steps of the Asbun clockwise technique

1. mobilization of the splenic flexure of the colon and exposure of the pancreas;

2. dissection along the inferior edge of the pancreas and choosing the site for pancreatic division;

3. pancreatic parenchymal division and ligation of the splenic vein and artery;

4. dissection along the superior edge of the pancreas;

5. mobilization of the spleen and specimen removal.

Similar to the RAMPS technique also the "clockwise technique" affords wide exposure of the pancreas and the plane of dissection can be chosen to include

or exclude the left adrenal gland, the Gerota's fascia, or the superior leaflet of the transverse colon mesentery.

Postoperative Management and Complications

Many studies have reported that following an LDP the incidence of postoperative pancreatic fistula is the same as in open surgery, ranging between 0 to 34% [5]. No differences were reported when comparing the fistula rates in pancreatic resection with or without splenectomy. The correct management of the pancreatic stump is not supported by validated recommendations or guidelines [18]. Several methods for closure of the pancreatic stump are described in the literature, such as the duct ligation, ultrasonic and stapler closure, fibrin glue occlusion, meshes and pancreatoenteric anastomosis [19, 20]. The suture of the pancreatic stump can be reinforced with tissue sealants but also this technique is still debated [21]. In 2012 Montorsi et al. [22] published a multicenter Italian randomized controlled trial on the efficacy of an absorbable fibrin sealant patch after distal pancreatic resection. In this trial 20% of the resections were performed laparoscopically and no differences in terms of postoperative pancreatic fistula were found.

The use of surgical abdominal drains and the timing of their removal are controversial. In the majority of the surgical experiences the use of drains is strongly recommended, although some authors have proposed a selective use of the drains. The rationale for this second position is that an abdominal

drain can easily be itself the cause of infection of a postoperative fluid collection close to the pancreatic stump.

In conclusion, laparoscopic distal pancreatectomy with en bloc splenectomy is a safe and feasible technique that the surgeon can adopt also in case of malignancies of the body and tail of the pancreas. Splenectomy is not strictly recommended but is still indicated in the treatment of borderline tumors or premalignant diseases such as intraductal papillary mucinous neoplasms.

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