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TITOLO DELLA TESI DI DOTTORATO
MUC Expression and their prognostic value in Cholangiocarcinoma

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2 SUMMARY

Cholangiocarcinoma (CC) is a malignant tumour composed of cells resembling those of the bile ducts and the second most common primary hepatic tumor after hepatocellular carcinoma, comprising 5-10% of primary liver neoplasms.

Worldwide, cholangiocarcinoma accounts for 3% of all gastrointestinal cancers. Several studies have shown that the incidence and mortality rates of intrahepatic CC (IHCC) are rising, and those of extrahepatic cholangiocarcinoma (EHCC) are declining worldwide.

To date, radical surgery is the only therapy offering a potential cure for CC patients, whose prognosis is generally poor with survival limited to few months.

At present, the lack of a sensitive and specific early diagnostic marker is one of the reasons why CC has a fairly late presentation.

Our aim was to find out a sensitive and specific marker which could be detected in patient serum and be correlated with tumor type and tumor burden since the potential survival benefit from early detection is shown by 70-80% survival for patients with early cholangiocarcinoma that was discovered incidentally on transplantation for primary sclerosing cholangitis.

Mucins are heavily glycosylated glycoproteins and play a protective role in cells, in part serving as a barrier to the epithelial surface and to tumor cells.

Boonla C. et al. recently showed that MUC5AC mucin is present in significant concentrations in serum from patients with CC. In a recent
report, MUC5AC significantly correlated with neural invasion and advanced CC stage.

Only a few studies have been carried out about mucins expression, in particular the gastric type and their relationship with CC morphology and prognosis.

We stained all tissue for MUC1, MUC2, MUC6 and MUC5AC. The only interesting results were with MUC5AC.

Recently the Liver Cancer Study Group of Japan divided IHCC into three morphological types: mass-forming (MF), periductal infiltrating (PI) and intraductal growth (IG).

MF type is characterized by the presence of a spherical mass with a distinct border in the liver parenchyma, PI type presents tumor infiltration along the bile duct, occasionally involving the surrounding blood vessels and/or hepatic parenchyma, IG is characterized by papillary and/or granular growth into the bile duct lumen.

PI type of CC present a significantly higher frequency of perineural invasion, lymph node metastasis and extrahepatic recurrence than MF type.

The 5-year survival rates of patients with IG tumors or MF tumors is significantly better than those of patients with MF plus PI tumors or PI type alone.

There is increasing evidence that intrahepatic cholangiocarcinoma should be divided in peripheral CC and perihilar CC based on etiopathogenesis, biological behaviour and clinical features. Perihilar CC may evolve from the lining epithelia of the major branches of the right and left hepatic bile duct and also from peribiliary glands around them.
and histologically is an adenocarcinoma resembling many of the features of hilar or extrahepatic CC. Peripheral CC presumably develop from small bile duct, ductules or canals of Hering. Hepatic progenitor cell may be involved in the tumorigenesis of peripheral CC. Distinguishing perihilar from hilar CC is often difficult, especially in advanced cases.

In this study together with the surgeons we propose a different classification: **peripheral** CC for tumors that growth inside the liver parenchima, **perihilar** for tumors located in the liver but involving the hilum and for Klatskin tumors and **extrahepatic** for tumors of the distal biliary tract.

This classification correlates well with morphology. Most (30/35) peripheral CC are of the MF type with only 5 cases MF+Pl. Perihilar CC and EHCC are mostly Pl or MF+Pl reflecting a different growth pattern between the two. Beside a different growth pattern there is a statistical difference between the tumor types when comparing MUC5AC expression.

30 out of 35 (85,7%) peripheral CC were MUC5AC negative. 26 out of 39 (66,6%) perihilar CC were MUC5AC positive with different intensities but positive. 13 cases were negative. Of these 13 cases in 8 cases there were same positive cells but not enough to reach 5% of the total (our cut-off value), we don’t know if this positivity is of any significance, but it is something different compared to the true negativity that we see in MF. In our study MUC5AC seems to be a good immunohistochemical marker that can distinguish peripheral from perihilar CC, that correlates well with morphology and has a prognostic significance as well. This marker can be measured in the serum and can be used in the panel of tumor markers to search for in CC and could be useful in the follow-up.
3 GENERAL CONCEPTS

Cholangiocarcinoma (CC) is an epithelial cancer originating from the bile ducts with features of cholangiocyte differentiation. CC is the second most common primary hepatic malignancy and epidemiologic studies suggest its incidence is increasing in Western countries (Welzel TM, 2006). Advanced CC has a poor prognosis with a median survival of less than 24 months (Farley DR, 1995). The only curative therapy is surgical eradication (R0) or in selected cases liver transplantation, but unfortunately the majority of patients present with advanced stage disease not amenable of surgical resection.

Anatomically CC is classified into intrahepatic (IHCC) and extrahepatic (EHCC) forms of the disease. The extrahepatic form is more common, accounting for 80-90% of cases. Its is further divided into proximal or perihilar and distal depending on the location along the biliary tree (Bismuth-Corlette Classification). Three different growth patterns have been described: periductal infiltrating (PI), papillary or intraductal (IG) and mass forming (MF). Intrahepatic CC typically presents as an intrahepatic mass forming tumor while the extrahepatic type has commonly a PI or sclerosing pattern.

Intra and extrahepatic CC differ in term of morphology and clinical presentation but also in etiopathogenesis, molecular signatures and management. In the last years there have been significant new insights into the molecular pathogenesis of CC. A goal is to stimulate further
interest in this disease with the hope of improving outcomes for this highly lethal malignancy.
4 EPIDEMIOLOGY

Hepatobiliary malignancies account for 13% of the 7.6 million annual cancer-related deaths worldwide.

Although the entire biliary tree is at risk, tumor involving the bifurcation, of the hepatic duct (Klatskin tumor) are the most common accounting for 40-60% of cases (Shaib YH, 2004), followed by the distal type 20-30% and the intrahepatic form 5-15%.

The prevalence of CC shows a wide geographic variability, with the highest rates in Asia (Thailand, Laos, Cambodia), where there is a higher prevalence of risk factors and the lowest in Australia. Its prevalence in different racial and ethnic groups is heterogeneously distributed, with the highest age-adjusted prevalence in Hispanics and the lowest in African Americans.

In the last 4 decades, United States incidence rates of intrahepatic CC have increased by 165%, whereas the extrahepatic CC incidence has remained stable (Shaib YH, 2004a). The significant increase of IHCC was confirmed after correction for a prior misclassification of hilar CC as intrahepatic (Welzel TM, 2006). Similarly, increasing incidence rates of IHCC have also been reported in Western Europe and Japan.

The neoplasia is 1.5 times more common in men than women with a peak age in the seventh decade (Shaib YH, 2004).

The heterogeneity in rates between different regions, sexes and ethnic groups suggests increase is genuine. The cause for the increasing incidence has not been identified but is not explained by any observed change in the incidence of known risk factors since most cases are
sporadic. It is not associated with a significant increase in the proportion of patients with early disease, making it unlikely that it reflects improved diagnosis (Shaib YH, 2004). Given all these and that CC has risen in a relatively short period of time, an environmental factor is likely to play a role in carcinogenesis.

Higher rates of HCV infection, chronic non-alcoholic liver disease and obesity have been associated with intrahepatic CC. Some speculate that increased lipid mediators such as oxysterols may contribute to the current increased incidence in western societies (Khan SA, 2008).
5 ETIOLOGY

In the majority of cases, the etiology remains obscure. However, several conditions associated with inflammation and cholestasis have been identified as risk factors for CC (Byung IC, 2004).

Risk factors have different distribution based on geographic areas and can be different between extra and intrahepatic forms.

Primary sclerosing cholangitis is the commonest known predisposing factor for CC in the West. The prevalence of CC in this condition is 5-15%. The majority of PSC patients who develop CC do so within the first 2.5 years following the diagnosis so they should be carefully screened for it. (Shaib YH, 2004).

In East Asia, where the disease is common, it has been pathogenically associated with liver flukes infestation, particularly by the endemic *Opisthorchis viverrini* and *Clonorchis sinensis* (Matthew, 2004). They are endemic in portions of East Asia (north-east of Thailand) where ingesting undercooked fish is common. Several case-control studies as well as animal models have confirmed the correlation between liver fluke infection and CC. Another risk factor more commonly found in Asia than in Western countries is hepatolithiasis, for which an incidence rate of 10% CC has been described. A Taiwanese study found that up to 70% of patients undergoing resection for CC had hepatolithiasis (Okuda, 2002).

Biliary malformations such as Caroli’s disease and choledochal cysts carry a 10 to 15% risk of developing CC. Hepatitis C, chronic non-alcoholic liver disease and cirrhosis have also been reported as possible risk factors for CC especially the intrahepatic form (Shaib YH, 2007). Biliary-enteric
drainage procedures are associated with CC in the presence of recurrent cholangitis. Finally various chemicals such as the banned contrast agent thorotrast and industrial toxins (dioxins and nitrosamines) have been correlated with an increased risk for CC. Although most patients have no identifiable overt risk factors it remains possible that subclinical biliary tract inflammation underlies the pathogenesis of CC in most patients.
6 PATHOGENESIS

CC likely results from malignant transformation of cholangiocytes, although transformation of epithelial cells within peribiliary glands and/or biliary stem cell may also contribute to its development. There is evidence that a subset of CC and mixed hepatocellular carcinoma /CC originate from hepatic stem/progenitor cells (Nomoto K, 2006). Etiologic and experimental evidence implicates inflammation and cholestasis as key factors in the pathogenesis of CC. They create an environment that promotes damage in DNA mismatch repair genes/proteins, proto-oncogenes and tumor suppressor genes (Jaiswal M, 2001). Cytokines, growth factors and bile acids found in increased concentrations in inflammation and cholestasis, contribute to these molecular changes and augment the growth and survival of altered cells. Cytokines stimulate expression of inducible nitric oxide synthase (iNOS) in epithelial cells, and iNOS up-regulation is present in inflammatory cholangiopathies and CC (Jaiswal M, 2000). Increased iNOS activity results in generation of nitric oxide and reactive nitrogen oxide species (RNOS) that interact with the genome resulting in mutations and DNA strand breaks. Mutagenesis is further promoted by interaction between nitric oxide and RNOS with DNA repair enzymes. A variety of oncogenic mutations have been identified in human CC tissues. Their frequency depends on tumor type, stage, anatomical location, etiology and ethnic population. In sharp distinction with pancreatic ductal adenocarcinoma where it is present in more than 90% of cases, mutations of k-ras have only been described in 20% to 54% of cholangiocarcinomas (Dergham ST, 1997). Thus, despite shared
developmental ontology between pancreatic ducts and biliary tree, their adult cancers are different. Nuclear accumulation of p53 has been reported in 21.7% to 76% of cases of CC (Kang YK, 1999). Other inactivated tumor suppressor genes include p16, DPC4/Smad4 and APC. Correlation between these markers and prognosis varies among studies. The majority of these genetic changes were described in intrahepatic cholangiocarcinomas due to its larger cellularity.

Interleukin 6 (IL-6) appears to be a critical signaling molecule in the pathogenesis of human cancers (Hodge DR, 2005). A role has been described in breast and lung cancer. IL-6 is also a key cytokine in the pathogenesis of CC. It is produced at high levels by CC cells and elevated IL-6 serum concentrations have been reported in CC patients (Goydos JS, 1998). In addition to autocrine and paracrine IL-6 stimulation, CC cells overexpress the IL-6 receptor subunit gp 130 (Yokomuro S, 2000). The usual negative feedback regulation of IL-6 signaling is blocked by epigenetic silencing of suppressors of cytokine signaling 3 (SOCS-3). Uninhibited IL-6 stimulation results in up-regulation of the anti-apoptotic Bcl-2 protein Mcl-1, rendering CC resistant to cytotoxic therapies (Isomoto H, 2007). IL-6 has also been shown to increase telomerase activity resulting in inhibition of telomere shortening and thereby evasion of cell senescence (Yamagiwa Y, 2006). In CC cells, IL-6 activates p44/42 and p38 mitogen-activated protein kinases (MAPKs) that are critical for CC cell proliferation (Park J, 1999). Activated p38 MAPK decreases cyclin-dependent kinase inhibitor p21, a known negative cell cycle regulator (Tadlock L, 2001). There is also cross-communication between IL-6 and
other pathway; mechanism of IL-6 signaling in human CC are depicted in Figure 1.

Tyrosine kinases receptors which can be targeted pharmaceutically, are over-expressed in many cancers. EGFR can directly be activated by bile acids and promote CC cell proliferation. EGFR activation is sustained in CC by failure to internalize the ligand-receptor complex, a homeostatic mechanism essential for receptor inactivation (Yoon JH, 2004). EGFR phosphorylation results in activation of the downstream MAPKs which in turn increase cyclooxygenase 2 (COX-2) expression in CC cells. COX-2 plays an important role in CC carcinogenesis through inhibition of apoptosis and growth stimulation (Han C, 2005). COX-2 is also stimulated by bile acids, oxysterols, iNOS and ErbB-2 (Lai GH, 2005).

Also hepatocyte growth factor and its receptor c-met are frequently over-expressed in CC and this represents an autocrine mechanism for sustained growth stimulation by CC (Aishima S, 2002).

In addition to the enhancement of these growth-promoting pathways, loss of growth inhibition has been demonstrated in CC. Response to transforming growth factor-β1 (TGF-β1) is aberrant resulting in increased proliferative rates.

Recent studies also support a relevant role of estrogens as modulators of cholangiocyte proliferation (Alvaro D, 2006a). Cell lines derived from intrahepatic CC (HuH-28) express ER-α and β. These cells also express IGF1 (insulin like growth factor 1) and its receptor and both IGF1 and estrogens stimulate HuH-28 cell proliferation with additive effects (Alvaro D, 2006b). In addition in estrogen-sensitive cancers it has been showed that estrogens promote neo-angiogenesis by acting on the vascular
endothelial growth factor (VEGF) (Hyder SM, 2000). A study demonstrated that HuH-28 cell express VEGF-A and VEGF-C and their receptors and estrogens markedly enhance their expression (Mancino MG, 2007). This may have a number of clinical implications. In summary there is a complex net of different factors and pathways involved in CC development, growth and propagation (Blechacz B, 2008).

Figure 1 Pathogenetic pathways
7 DIAGNOSIS

7.1 Signs and symptoms

In the majority of cases CC is clinically silent, with symptoms developing at an advanced stage of disease. Once symptomatic, the clinical presentation depends on tumor location and growth pattern. Intrahepatic mass-forming CC presents with symptoms typical for hepatic masses, including abdominal pain, malaise, night sweats and cachexia. Ninety percent of patients with extrahepatic CC present with painless jaundice, and 10% of patients with cholangitis (Khan SA, 2002). In case of unilobar biliary obstruction with ipsilateral vascular encasement results in atrophy of the affected lobe and hypertrophy of the unaffected lobe (Hann LE, 1996). Upon physical examination, this “atrophy-hypertrophy complex” phenomenon presents as palpable prominence of one hepatic lobe. Patients with PSC which develop cholangiocarcinoma experience worsening of their clinical conditions.

7.2 Tumor markers

Serum tumor markers are not useful for the diagnosis of CC. The markers CA-125 and CEA can be elevated, however they are non-specific and can be increased in other gastrointestinal or gynaecologic malignancies or cholangiopathies (Chen CY, 2002). CA 19-9 is the most commonly used tumor marker for CC (Nehls O, 2004). Its sensitivity and specificity are high (79% and 98%) in patients with PSC but low (53%) in patients without PSC. CA 19-9 can also be elevated in bacterial cholangitis and other
gastrointestinal and gynaecologic neoplasias and patients lacking the blood type Lewis antigen (10% of individuals) do not produce this tumor marker (Albert MB, 1988). However CA 19-9 can have a value in the follow-up post surgery. Other circulating factors with potential alone or in panels include RCAS-1, the tumor associated antigen receptor binding cancer antigen expressed on SiSo cells, the cytokeratine 19 fragment CYFRA 21-1 and MUC5AC. A soluble form of RCAS-1 in serum has been reported to have a sensitivity of 74% with a specificity of 96%, moreover, many cases negative for CA 19-9 showed RCAS-1 positiveness and the marker can have a potential role in monitoring treatment (Watanabe H, 2003). CYFRA 21-1 was found to be higher in CC compared to HCC and patients with benign liver disease, and increases with tumor stage, with a sensitivity of 87% and specificity of 95% (Uenishi T, 2003). A study showed that MUC5AC was identified in serum samples from 112 of 179 CC patients, whereas almost all healthy controls or those with other cancers were negative (Wongkham S, 2003).

Currently used tumor markers can’t be reliable by their self to make the diagnosis but have to be interpreted together with other clinical and radiological informations (Mali, 2006).

7.3 Imaging

Different imaging techniques are needed for the diagnosis of CC. Their main role is detection of bile duct obstruction, vascular compression or encasement, tumor staging and preoperative planning. Even though it does not have a high sensitivity and specificity, the first step is an abdominal ultrasound. It can visualize liver parenchyma and intrahepatic
masses but it can’t really differentiate between primitive or metastatic disease (Slattery J, 2006). Extrahepatic CC presents as biliary tract stricture and the first step is to differentiate a benign (iatrogenic, PSC, lithiasis) from a malignant stricture. US can show dilated ducts proximal to the stricture and sometimes can show an intraluminal mass (Foley WD, 2007).

Hepatic parenchyma, intrahepatic tumors, biliary dilatation and lymph-nodes can also be assessed via computed tomography (CT). Contrast enhanced CT shows a peripheral rim-like hyperenhancement in the portal and arterial phase in IHCC (Chen LD, 2008). CT angiography allows excellent visualization of the vasculature.

For evaluation of tumor location and intraductal extent, cholangiography is the most important diagnostic modality, especially for extrahepatic CC (Gores GJ, 2000). Endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP) or percutaneous transhepatic cholangiography (PTC) can be used for this purpose. ERCP and PTC can also allow therapeutic interventions (for example placement of biliary stents) as well as collection of tissue samples for pathologic and cytologic analysis. MRCP/magnetic resonance imaging (MRI) provides information about intrahepatic location and tumor dimensions of intrahepatic CC, ductal as well as periductal extent of extrahepatic CC, vascular involvement and metastasis (Manfredi M, 2004), (Figure 2).

In indeterminate cases, establishment of a diagnosis can be attempted with positron emission tomography (PET) with 18F-2-deoxy-glucose. Sensitivity and specificity of integrated PET/CT in the identification of primary lesions has been reported as 93% and 80% for intrahepatic CC
and 55% and 33% for extrahepatic CC. For regional lymph node metastases, the sensitivity of PET/CT was 12% and the specificity 96% (Petrowsky H, 2006). False positive PET scans have been reported in the setting of chronic inflammation. A recent report suggested that PET scanning in non-PSC patients can change management and therefore is useful in staging (Corvera CU, 2008).

Figure 2 A gadolinium-enhanced MRI scan

7.4 Pathological Aspects

Cholangiocarcinoma is a malignant tumor composed of cells resembling those of bile ducts. 90% of cases are adenocarcinomas but rare types like squamous and sarcomatous might be present. According to WHO classification (Sobin WHO) the term cholangiocarcinoma is reserved for carcinomas arising in the intrahepatic bile ducts. Tumors arising from extrahepatic ducts should be designated as extrahepatic bile duct
carcinomas. However clinical and pathological differentiation of the two entities can be difficult. Cancers arising from the left and right hepatic ducts and at the bifurcation are called “hilar” and they are considered extrahepatic carcinomas.

Intrahepatic CC are classified according to the Liver Cancer Study Group of Japan guidelines (LCSGJ 2003) into three groups:

- **Mass forming (MF):** nodular type of growth, well demarcated but not encapsulated greyish-white lesion. A single nodule, but often small metastatic nodule around the principal tumor are found. (Figure 3 and 6)

- **Periductal infiltrating (PI):** tumor infiltrates and proliferates along the bile duct, which is usually thickened. There is minimal mass formation and thickening and enlargement of the portal region. The infiltration in the liver has an arborescent appearance. In most cases there is also extensive parenchymal infiltration. (Figure 4 and 7)

- **Intraductal polypoid growth (IG):** tumor have a polypoid growth inside the bile ducts which are dilated. (Figure 8)

Hilar cholangiocarcinomas can also have three different patterns described as **sclerosing** (70%), (figure 5), **nodular** and **papillary**. In the sclerosing type the tumor infiltrates and proliferates along the extrahepatic bile duct, which is thickened in most cases. Mass formation may be minimal and there could be thickening and enlargement of portal region. The infiltration in the liver has an arborescent appearance. Extensive parenchymal infiltration is also observed in many cases. Bile ducts are dilated and there is frequently cholestasis, biliary fibrosis and
cholangitis with abscess formation. For extrahepatic CC it is important to define the extent of involvement along the bile duct. There are a few classifications but the most used is the topographic Bismuth-Corlette classification (Figure 9). Differentiation of intrahepatic from extrahepatic bile duct cancer may be difficult in cases with massive tumor at the hilum of the liver. Maybe the pathological differentiation of intra and extrahepatic bile duct carcinoma will become easier thanks to morphological, immunohistochemical and molecular studies.

Microscopic parameters are the degree of differentiation, vascular and neural infiltration and lymph node involvement. 90% of cases are adenocarcinomas but rare types like squamous and sarcomatous might be present. The grades, well, moderately and poorly differentiated are based on architectural and cytologic features. Well-differentiated CC form relatively uniform tubular or papillary structures; moderately-differentiated CC have moderately distorted tubular patterns with cribriform formations and/or a cord-like pattern; poorly-differentiated CC show severely distorted tubular structures or single cells with marked cellular pleomorphism.
Figure 3 Mass forming type tumor with a satellite nodule

Figure 4 Periductal infiltrating type tumor
Figure 5 Hilar tumor, sclerosing type

Figure 6 Mass forming tumor composed of glands in a fibrous stroma
Figure 7 Small glands infiltrating along the duct

Figure 8 Intraductal growth type
Figure 9 Bismuth Corlette Classification
8 MUCIN

Mucins are high-molecular weight glycoproteins synthesized by a broad range of epithelial tissues, mainly ductular and glandular. Mucins have an important role in growth; fetal development; epithelial renewal, differentiation and integrity; carcinogenesis; and metastasis. Structurally mucin glycoproteins consist of a protein backbone with a large number of O-linked carbohydrate side chains. The protein backbone is composed of a variable number of tandem repeat regions rich in threonine and serine amino acids residues, the sequence and length of which are unique for each particular mucin (Seregni, 1997). Genes coding for the protein component are designated as MUCs. At present 19 mucin glycoproteins have been assigned to the MUC gene family (Boonla, 2005). They can be subdivided into membrane-associated and secreted forms (Baldus, 2000).

Secreted (or gel-forming) mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7) play an important role in lubrication, protection and formation of a selective barrier of epithelial surfaces. They are produced throughout the entire gastrointestinal tract, mammary and salivary glands, the pancreas and gallbladder, the respiratory tract and reproductive organs. The membrane-bound mucins (MUC1, MUC3, MUC4, MUC13, MUC15, MUC 16 and 17) apparently serve a protective function, protecting the glycoproteins from cell surface proteolysis and protecting the cells from attack by others. However the exact function of this group of mucins is not understood fully (Boonla, C 2003).

In normal tissues, mucins seem to be expressed in a relatively organ and cell-specific manner (Ho SB, 1995). Some mucins can be observed in
several type of tissues, whereas others exhibit a more limited pattern of expression. For example, MUC1 is expressed on the apical surfaces of most epithelial cells, including those of the breast and the digestive, respiratory and genitourinary tracts (Audie JP, 1993). In contrast, the distribution of MUC2 and MUC5AC seems to be more restricted, with MUC2 specifically expressed in goblet cells of the small intestine and colon and MUC5AC preferentially expressed in the stomach and respiratory tracts (Copin MC, 2000). In many human carcinomas, the expression profile of mucins is altered; certain mucins are up-regulated whereas others are down-regulated (Lau SK, 2004). Some mucins are correlated with a poor prognosis and with increased metastatic potential in certain malignancies.

Biliary epithelial cells in the intrahepatic large bile ducts constantly express MUC3, MUC6 and MUC5B apomucin; whereas MUC5AC is expressed rarely (Sasaki M, 1995). Many aetiological factors associated with CC comprise an inflammatory background. It has been demonstrated that cytokines produced in inflammatory conditions from neutrophils lead to mucins synthesis via the transactivation of the epidermal growth factor receptor (Takeyama K, 2000). Altered gastric-mucins expression has been reported in biliary pre-invasive neoplastic lesions and CC (Lee KT, 2001) suggesting that biliary epithelial cells exhibit a gastric mucin phenotype during carcinogenesis. Extensively expressed MUC1 apomucin, focally expressed MUC2 and frequently expressed MUC5AC have been documented by others (Sasaki M, 1998). A study, including intrahepatic CC only, showed that MUC5AC is expressed in 40% of cases, at higher levels in the hilar type than in the peripheral type and that it is highly
correlated with lymph node metastasis reflecting tumor invasiveness. They also showed a correlation with survival and MUC5AC, with positive tumors having a 5 year survival of 10% compared to 35% of MUC5AC negative tumors (Aishima S, 2006). MUC2 and MUC5AC were frequently expressed in IHCC from the hilar portion of the liver (tumors involving the second branch of the bile duct). These results suggest that aberrant mucins in IHCC are differentially expressed according to different regions of the liver from which the tumor originate and these regions are in turn differentially associated with the size of the biliary tree. Another paper from the same author highlights that the frequency of perineural invasion, lymph node metastasis and extrahepatic recurrence of hilar IHCC is significantly higher than that of the peripheral type (Aishima S, 2007). A study also showed that MUC5AC originating from CC tissues can be detected in patient serum with high sensitivity and specificity and detection of MUC5AC from individual serum corresponded well with the degree of expression of the mucin in the tumor tissue (Wongkham S, 2003). The pyloric gland type (MUC6) is instead associated with a better survival. MUC1 is frequently found in the developing intrahepatic bile ducts of fetal liver but not in the normal adult epithelium. It is found to be expressed in a large number of IH and EH CC and has been significantly correlated with poor prognosis and vascular invasion (Boonla, C 2005). MUC1 is not expressed by hepatocarcinomas. The presence of MUC1 is quite interesting because attempts to eliminate or control metastasis of tumor cells via anti-MUC1 and phase I/II trials of cancer vaccines using antigen MUC1 are now underway (Musselli, C 2001).
9 STAGING and TREATMENT

CC has been classified using the UICC/AJCC TNM (tumor-node-metastasis) system. This classification is the same for intrahepatic CC and hepatocarcinoma.

AJCC/UICC staging for intrahepatic CC

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* T1 = solitary tumor no vascular invasion; T2 = solitary tumor with vascular invasion or multiple lesions none bigger than 5 cm; T3 = multiple lesions > 5 cm or lesion involving major branch of the portal or hepatic veins; T4 = tumor with invasion of adjacent organs other than gallbladder or with perforation of visceral peritoneum.

(American Joint Committee on Cancer 2002: AJCC, 6th edition)

TNM classification and UICC/AJCC staging for hilar CC is the same as for extrahepatic CC.

AJCC/UICC staging for extrahepatic and hilar CC

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This classification is a pathologic staging system and therefore requires the surgical specimen. An optimal system should provide information about disease extent, vascular involvement and metastasis without subjecting the patient to surgical treatment. It should also take into account treatment options, performance status and age and correlate with clinical outcomes. There is urgent need for such a validated staging system in hilar CC. Without a staging system, stratification of patients for clinical trials is currently hampered. A staging system taking into account parameters of resectability such as biliary extent, vascular encasement and hepatic lobar atrophy has been proposed by Memorial Sloan-Kettering Cancer Center. Resectability, likelihood of R0 resection, metastatic spread to N2 level lymph nodes and survival correlated with tumor stage of this modified classification (Jarnagin WR, 2001).
Memorial Sloan Kettering Cancer Staging System for Hilar CC

Surgical treatment is the only curative therapy for CC and is therefore the treatment of choice when feasible.

Resectability (R0 resection) is low, around 10% both for intra and extra hepatic CC.

Criteria of non resectability are:

1) patient comorbidities, like cirrhosis and portal hypertension;
2) bilateral involvement of bile duct distal to II biliary radicles;
3) atrophy of one lobe with encasement of contralateral portal vein or II biliary radicles;
4) encasement of portal vein or hepatic artery close to bifurcation;
5) distant lymph node metastasis (paraaortic, retropancreatic) and distant metastasis
Intrahepatic CC need major hepatic resections that have a low morbidity and mortality due to the fact that they origin in non cirrhotic livers.

Extrahepatic CC requires resection of the biliary tree associated with hepatic resection. The association of resection of the caudate lobe increases surgical resectability because this lobe is often infiltrated. (Seyama, 2007) Several techniques have been evaluated for their potential to increase resectability, including preoperative portal vein embolization plus extended hepatectomy (Abdalla EK, 2002). The goal of portal vein embolization is to induce hyperplasia of the non-embolized lobe increasing the volume of the remnant liver following an extended hepatectomy (Nagino M, 2006). This strategy achieved increased surgical radicality in patients with hilar CC and marginal remnant liver volumes.

Five year survival rates after R0 resections are 22% to 44% for intrahepatic CC, 11% to 41% for hilar CC and 27% to 37% for distal extrahepatic CC (Nagorney DM, 2006).

Recurrence of disease is frequent and present in more than 50% of resected patients. Recurrence occurs on average after two years from surgery. They are more common in the liver (74%), peritoneum (22%), lymph nodes and bone (11%) for intrahepatic CC and in the peritoneum for extrahepatic forms.

Adjuvant and neoadjuvant treatments for CC, including chemotherapy, radiation therapy and photodynamic therapy, cannot be recommended. The most studied chemotherapeutic drugs are 5-FU and gemcitabine; the latter was approved in 2002 by FDA (Alberts SR, 2007). However studies either failed to show significant effects or were statistically underpowered, non-randomized or restricted to short term follow-up.
Results of liver transplantation for CC are discouraging. However recently some promising results come from the Mayo Clinic where they developed a new liver transplantation protocol for extrahepatic CC with very strict selection criteria (Rea DJ, 2005).

Palliative therapies are important in the management of the disease because it causes significant morbidity related to cholestasis and its complications, abdominal pain, cachexia and bacterial cholangitis. Biliary drainage can be performed endoscopically or when not feasible with PTC.

The growing understanding of the molecular pathogenesis of CC opens new therapeutic options for molecular targeting. Major targets are antiapoptotic and growth-stimulating pathways. Methylation inhibitors, the multikinase inhibitor Sorafenib, IL-6 neutralizing antibodies, MAPK inhibitors, COX-2 inhibitors- achieved growth inhibition and/or induction or sensitisation to apoptosis in vitro (Wiedmann M, 2006). These studies are quite promising however better in vivo models of CC will be necessary to study targeted therapies.
10 MATERIALS AND METHODS

10.1 Patients

From 1983 to 2007 224 patients with cholangiocarcinoma have been evaluated based on clinical, laboratory and imaging data at Policlinico G.B. Rossi, University of Verona and Ospedale Civile Maggiore, Verona. Of these 154 have undergone a laparoscopic look. 57 were not resected because of disease stage. Of the 97 patients resected, 79 cases where selected for immunohistochemistry and of these for 55 patients we have a long term follow-up. 66.2% of the patients were men, and 33.8% were women.

Their ages ranged from 19 to 83 years, with a medium age at surgery of 58 years.

Patients selected had either a hilar or peripheral CC with no distant metastasis.

Surgical resection were as follow:

- hilar tumors had a biliary tract resection with a major or minor hepatic resection, with regional lymphadenectomy when needed followed by a bilio-digestive anastomosis;

- intrahepatic tumors had a major or minor heptectomy with regional lymphoadenectomy and resection of a biliary tract if involved.

Patients were followed every 6 months, with clinical, laboratory and imaging (US and CT) investigations.
**10.2 Tissue specimens and macroscopical classification**

Tumors were classified in origin as intrahepatic CC, involving only the periphery of the liver and extrahepatic CC if they were arising either from the bifurcation of the hepatic ducts or the distal extrahepatic bile ducts. We collected 44 intrahepatic cholangiocarcinoma (IHCC) and 35 extrahepatic cholangiocarcinoma (EHCC). The Extrahepatic CC (EHCC) were further subdivided into Hilar type or Klatskin if arising from the confluence of the bile ducts (n=30) or simply EHCC if involving the distal bile ducts (n=5). Tumour size was recorded as the largest diameter in the fresh samples.

The IHCC diameter was variable between 2 and 16 cm, with a median value of 5.25 cm, while the EHCC diameter varies from 0.5 to 4 cm, and the median value is 1.5 cm.

Intrahepatic CC (IHCC) were further classified morphologically into the three basic types: mass-forming (MF) type, periductal-infiltrating (PI) type, and intraductal growth (IG) according to the Classification of the Liver Japan study group (2003).

MF type forms a definite mass, located in the liver parenchyma.

PI type is defined as a tumor which extends mainly longitudinally along the bile duct, often resulting in dilatation of the peripheral bile ducts. The IG type proliferates toward the lumen of the bile duct papillarily or like a tumor thrombus.

For extrahepatic CC the pattern of growth was either nodular (MF), periductal sclerosing (PI type) or intraductal papillary growth (IG type).
After this first subdivision recently together with the surgeons we reclassified all cases according to recent papers (Nakanuma Y, 2008; Sano T, 2008; Aishima S, 2007) and their experience. Intrahepatic CC are largely divided now into peripheral and perihilar because etiopathogenesis, tumorigenesis, biological behaviour and clinical features seem to be different. Hepatic progenitor cells or stem cells may be involved in the tumorigenesis of peripheral CC. Perihilar CC may evolve from the lining epithelia of the major branches of the right and left hepatic duct and also from peribiliary glands around them (Nakanuma Y, 2008). Distinguishing perihilar CC from hilar CC is often difficult, especially in advanced cases, so perihilar/hilar seems to be an alternative and more practical term for these cases. For these reasons our group wants to propose the use of the term “PERIPHERAL” for tumors arising inside the liver parenchyma without connection with the hilum and the term “PERIHILAR” for tumor of the hilar region with involvement of the hepatic hilum with compression and distension of the biliary tract due to occlusion related to infiltration or compression when they growth in the confluence or compression or infiltration due to a mass, and “EXTRAHEPATIC” for tumors of the distal biliary tract (figure 10).
Applying this classification we had 35 peripheral CC, 39 perihilar tumors and 5 involving the distal bile duct. The morphological classification did not change.

Tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxilin and eosin for histological examination. We retrospectively reviewed all pathologic findings and the results of long-term follow up when present.

10.3 Immunohistochemical staining and histological evaluation

Immunohistochemical staining was performed on 79 serially cut 3-µm-thick paraffin sections applying the following antibodies: MUC1 (Novo-Castra, clone Ma695, dilution 1:100, Newcastle, UK), MUC2 (Novo-Castra, clone CCP58, dilution 1:200), MUC5AC (Novo-Castra, clone CLH2, dilution
MUC6 (Novo-Castra, clone CLH5, dilution 1:100). Antibody detection was performed with the BOND MAX instrument by adding the polymer and 3,3’-diaminobenzidine (Vectastatin ABC kit; Vector Laboratory, Burlingame, CA).

The antibodies were estimated semiquantitatively: negative (-) if there were less than 5% positive cells, grade 1 (+) if the percentage was between 5 and 20%, grade 2 (++) if it was between 20 and 50% and grade 3 (+++) if positive cells were more than 50%.

**Antibodies used**

<table>
<thead>
<tr>
<th>ANTIGENE</th>
<th>CLONE</th>
<th>COMPANY</th>
<th>DILUITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>Ma695</td>
<td>NOVOCASTRA</td>
<td>1:100</td>
</tr>
<tr>
<td>MUC2</td>
<td>CCP58</td>
<td>NOVOCASTRA</td>
<td>1:200</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>CLH2</td>
<td>NOVOCASTRA</td>
<td>1:100</td>
</tr>
<tr>
<td>MUC6</td>
<td>CLH5</td>
<td>NOVOCASTRA</td>
<td>1:100</td>
</tr>
</tbody>
</table>

MUC1 is characterized by staining along the apical membranes of the tumor cells in better differentiated tumors, in less-differentiated tumors, membranous and/or cytoplasmic staining was present (Figure 11).

MUC2 and MUC6 have a cytoplasmic staining pattern.

MUC5AC has a cytoplasmic and luminal pattern of reactivity (Figure 12,13,14).
Figure 11 MUC1 positive MF tumor

Figure 12 MF tumor negative for MUC5AC
Figure 13 MUC5AC positive PI type CC and dysplastic epithelium

Figure 14 MUC5AC positive, poorly differentiated PI type CC
10.4 Statistical analysis

Patients were analysed retrospectively, data collected and the statistical analyses performed using a statistical analysis software package (SPSS version 13, SPSS Inc. Chicago, IL).

The correlation between the immunohistochemical results and the pathological and prognostic parameters was assessed by means of the $\chi^2$ test.

Disease-specific survival was considered as the period of survival between surgery and the date of the last follow-up or death by disease.

Survival curves were calculated by the Kaplan-Meier method and the differences between the curves were analysed by the log rank test. The result were considered significant if the P-value was < 0.05.
11 RESULTS

Peripheral cholangiocarcinoma were in the majority of cases of the mass forming type (30 out of 35). The remaining 5 cases were classified as MF+PI. Perihilar CC were classified as PI (sclerosing) in 27 cases (27/39), as MF+PI in 8 cases and MF alone in 4 cases.

Extrahepatic CC were PI in all cases.

We did not have any "pure" intraductal growth (IG) case (Figure 15).

![Figure 15 Distribution of morphologic types between subcategories](image)

<table>
<thead>
<tr>
<th>Type</th>
<th>MF</th>
<th>MF+PI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PER</td>
<td>4</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>P</td>
<td>30</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

MUC expression has been correlated to prognostic factors such as vascular invasion, perineural invasion and lymph-node metastasis.

MUC1 expression didn’t show significant differences between tumors types (Table 1).
Table 1. MUC1 expression.

<table>
<thead>
<tr>
<th>MUC1 MORPHOLOGY</th>
<th>TYPE</th>
<th>MUC1</th>
<th>P</th>
<th>PER</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>-</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>7</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>12</td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>MF # cases</td>
<td></td>
<td>30</td>
<td>4</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>MF+PI</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MF+PI # cases</td>
<td></td>
<td>5</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>-</td>
<td>2</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>2</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>PI # cases</td>
<td></td>
<td>5</td>
<td>27</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td># cases</td>
<td></td>
<td>5</td>
<td>35</td>
<td>39</td>
<td>79</td>
</tr>
</tbody>
</table>

P = peripheral CC; PER = perihilar CC; EE = extrahepatic CC

MUC2 was negative or only slightly positive in the majority of cases (Table 2).

Table 2. MUC2 expression

<table>
<thead>
<tr>
<th>MUC2 MORPHOLOGY</th>
<th>TYPE</th>
<th>MUC2</th>
<th>P</th>
<th>PER</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>-</td>
<td>29</td>
<td>4</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MF # cases</td>
<td></td>
<td>30</td>
<td>4</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>MF+PI</td>
<td>-</td>
<td>5</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>MF+PI # cases</td>
<td></td>
<td>5</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>-</td>
<td>5</td>
<td>27</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>PI # cases</td>
<td></td>
<td>5</td>
<td>27</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td># cases</td>
<td></td>
<td>5</td>
<td>35</td>
<td>39</td>
<td>79</td>
</tr>
</tbody>
</table>

Also MUC6 was negative in the majority of tumors and showed, sometimes a positivity in normal glands (Table 3).
Table 3. MUC6 expression

<table>
<thead>
<tr>
<th>MUC6 MORPHOLOGY</th>
<th>MUC6</th>
<th>TYPE</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>-</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MF # cases</td>
<td>30</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>MF+PI</td>
<td>-</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MF+PI # cases</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>PI</td>
<td>-</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PI # cases</td>
<td>5</td>
<td>27</td>
<td>32</td>
</tr>
</tbody>
</table>

# cases 5 35 39 79

MUC5AC is the only one that shows interesting results statistically significant. The intrahepatic group showed significant differences between MUC5AC expression, morphology and prognostic parameters (Table 4).

Table 4. MUC5AC expression

<table>
<thead>
<tr>
<th>MUC5 MORPHOLOGY</th>
<th>MUC5</th>
<th>TYPE</th>
<th># cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>-</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MF # cases</td>
<td>30</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>MF+PI</td>
<td>-</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MF+PI # cases</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>PI</td>
<td>-</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>PI # cases</td>
<td>5</td>
<td>27</td>
<td>32</td>
</tr>
</tbody>
</table>

# cases 5 35 39 79
Almost all cases (27/35) of the MF type were completely negative for MUC5AC. 3 MF cases were positive and in 2 of them the tumor showed a growth inside the dilated ducts. This intraductal growth does not mean IG type because a pure IG is just inside the duct and does not have an infiltrating component but it is a peculiar growth pattern that we don’t see often and maybe these tumors are something different from the classic MF. MF+PI types were positive in 2 cases and negative in 3. Perihilar CC were positive in 26 (26/39) cases with 10 cases +, 7 cases ++, and 9 cases +++.

EHCC were negative in 2 cases, + in 2 cases and +++ in 1 case.

We have correlated MUC expression with different prognostic factors. The perihilar group did not show significant correlation with the parameters evaluated (lymph-node metastasis, vascular invasion, perineural invasion, tumor dimension and surgical resectability).

Intrahepatic mass forming CC (MF) have a lesser degree of perineural infiltration, lymph-node metastasis and macroscopic vascular invasion and they are MUC5AC negative in the majority of cases.

MUC5AC positive tumors are associated with perineural infiltration (TAB 5).

**Table 5**

<table>
<thead>
<tr>
<th>MUC5AC</th>
<th>PERINEURAL INFILTRATION</th>
<th>NO</th>
<th>PRESENT</th>
<th># CASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEGATIVE</td>
<td>Count</td>
<td>20</td>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1</td>
<td>46,5%</td>
<td>53,5%</td>
<td>100%</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>Count</td>
<td>4</td>
<td>32</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1</td>
<td>11,1%</td>
<td>88,9%</td>
<td>100%</td>
</tr>
<tr>
<td># CASES</td>
<td>Count</td>
<td>24</td>
<td>55</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1</td>
<td>30,4%</td>
<td>69,6%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Significant differences merge between lymph-node positive and negative tumors and MUC5AC expression (TAB 6).

### Table 6

<table>
<thead>
<tr>
<th>MUC5AC</th>
<th>LYMPH-NODE METASTASIS</th>
<th># CASES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO</td>
<td>PRESENT</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>Count: 33</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1: 80,5%</td>
<td>19,5%</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>Count: 21</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1: 55,3%</td>
<td>44,7%</td>
</tr>
<tr>
<td># CASES</td>
<td>Count: 44</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>% within muc5cod1: 69,2%</td>
<td>30,8%</td>
</tr>
</tbody>
</table>

Median survival of R0 resected patients was 31 months with an overall 3, 5 years survival rates of 46% and 23%, while median survival was 14 months for R+ resected patients with an overall 3, 5 year survival rates of 24% and 0%. Survival is also correlated with morphology: MF+PI and PI types have significant lower survival rates than MF type alone (TAB 7).

### Table 7

<table>
<thead>
<tr>
<th>PERIPHERAL CC</th>
<th># CASES</th>
<th>SURVIVAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>24</td>
<td>Median (95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(24-76)</td>
</tr>
<tr>
<td>MF+PI</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3-35)</td>
</tr>
<tr>
<td>PERIHILAR CC</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21-27)</td>
</tr>
</tbody>
</table>
Cholangiocarcinomas are rare tumors, comprising 3% of gastrointestinal tumors. Several studies have shown that the incidence and mortality rates of intrahepatic CC (IHCC) are rising, and those of extrahepatic cholangiocarcinoma (EHCC) are declining worldwide.

To date, radical surgery is the only therapy offering a potential cure for CC patients, whose prognosis is generally poor with survival limited to few months.

At present, the lack of a sensitive and specific early diagnostic marker is one of the reasons why CC has a fairly late presentation.

Many papers so far have highlighted the fact that intrahepatic and hilar CC have different biologic and pathologic characteristics, and some are trying to demonstrate it at the molecular level showing different frequency of mutations.

They seem to be different at a morphological level as well and they have been subdivided in MF, PI and MF+PI for the intrahepatic forms and this has a counterpart in the hilar type as well (sclerosing or/and nodular).

Many of these studies are quite interesting but due to the relatively low incidence of CC, many studies have grouped these tumors together; though in studies where sub-group analyses have been performed clear differences are apparent. We feel that there is a need for a better definition of what we are dealing with because it is the only way to compare studies and try to understand more of this rare disease.
There is increasing evidence that intrahepatic cholangiocarcinoma should be divided in peripheral CC and perihilar CC based on etiopathogenesis, biological behaviour and clinical features. Perihilar CC may evolve from the lining epithelia of the major branches of the right and left hepatic bile duct and also from peribiliary glands around them and histologically show an adenocarcinoma resembling many of the features of hilar or extrahepatic CC. Peripheral CC presumably develop from small bile duct, ductules or canals of Hering. Hepatic progenitor cell may be involved in the tumorigenesis of peripheral CC. Distinguishing perihilar from hilar CC is often difficult, especially in advanced case.

In this study together with the surgeons we propose to use the terms “PERIPHERAL” CC for tumors that growth inside the liver parenchyma, “PERIHILAR” for tumors of the hilar region with involvement of the hepatic hilum with compression and distention of the biliary tract due to occlusion related to infiltration or compression when they growth in the confluence or compression or infiltration due to a mass and “EXTRAHEPATIC” for tumors of the distal biliary tract.

This classification correlates well with morphology. Most (30/35) peripheral CC are of the MF type, so well delimited masses confined in the liver with blurred burden, with only 5 cases being MF+PI. Perihilar CC and EHCC are mostly PI or MF+PI reflecting a different growth pattern between the two. This is in part due to the different anatomical location and to the fact that tumors involving the bile ducts give signs of themselves before, but it could also be due to a different pathogenetic pathway.

Beside a different growth pattern there is a statistical difference between the tumor types when comparing MUC5AC expression.
30 out of 35 (85.7%) peripheral CC were MUC5AC negative. 26 out of 39 (66.6%) perihilar CC were MUC5AC positive with different intensities but positive. 13 cases were negative. Of these 13 cases in 8 cases there were same positive cells but not enough to reach 5% of the total (our cut-off value), we don’t know if this positivity is of any significance, but it is something different compared to the true negativity that we see in MF type.

For extrahepatic CC the results are almost half positive (3 cases) and half negative (2 cases).

These differences in MUC5AC expression support the hypothesis of a different origin of peripheral and perihilar CC, with perihilar tumors acquiring a gastric phenotype. This is sustained by the fact that in many cases (data not shown) we find MUC5AC positivity in the dysplastic duct epithelium. If MUC5AC can differentiate between peripheral and perihilar tumors it can have a value in choosing the therapeu tic options.

Extrahepatic CC have variable results, this can be related to the small number of cases but it can also be expression of a different pathogenesis and some author put them together with gallbladder and coledocus neoplasias.

MUC5AC seems to be a prognostic factor as well with negative tumors having a 5 year survival of 35% compared to 10% of positive cases. Boonla suggests that this mucin creates a barrier between tumor cells and the immune system, so tumor cells can proliferate and give metastasis. MUC5AC expression is also correlated with perineural invasion one of the prognostic factors for CC. So MUC5AC seems to be a prognostic factor that correlates well with morphology and differentiates two different
tumor types. This molecule is interesting also because it can be measured in the serum and in the bile, as Boonla highlighted in one of his papers and can be used as a marker at least for perihilar CC. Our group wrote a study project that includes MUC5AC serum and bile detection in every case of cholangiocarcinomas and we are waiting for it to start soon to see if it correlates with tumor types, tumor burden and prognosis.
13 CONCLUSIONS

Cholangiocarcinoma are rising in incidence, especially the intrahepatic ones. There is a need for a tumor marker with a better specificity and sensibility than those currently used.

There is also the need for a homogeneous classification in the different series.

We identified in MUC5AC a good immunohistochemical marker that can distinguish peripheral from perihilar CC, that correlates well with morphology and has a prognostic significance as well. This marker can be measured in the serum and can be used in the panel of tumor markers to search for in CC and could be useful in the follow-up.

We also propose a classification that comprise peripheral, perihilar and extrahepatic cholangiocarcinomas based on differences in morphology, etiology and behaviour.


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