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Perspectives on the clinical application of Lewis antigens in pancreatic cancer: towards a personalized tumor marker strategy

Rossella Indellicato

R12186

Supervisor: Prof. ssa Anna Caretti

Co-supervisor: prof. Marco Trinchera

PhD Coordinator: Prof. ssa Chiarella Sforza

ABSTRACT

Background. Carbohydrate antigen 19.9 (CA19.9) is used as a tumor marker in gastrointestinal cancers, assuming that it is produced by cancer cells. Recent data has suggested that, in pancreatic ductal adenocarcinoma (PDAC), elevation of CA19.9 could depend on its reabsorption in the bloodstream. No specific association exists between cancer and CA19.9 with respect to the other two type 1 chain Lewis antigens, namely Lewis a (Lea) or Lewis b (Leb), because each one can be reabsorbed in the blood, according to their specific and individual expression. This thesis aims to verify if Lea and Leb can support CA19.9 in the management of pancreatic cancer, and to assess the relationship between the amounts and pattern of Lewis antigens detected in the pancreatic tissue (both normal and neoplastic) and circulating in the blood of patients.

Methods. Serum and fragments of surgical resection of PDAC patients are being collected. ELISA was performed on sera of 118 patients and 52 healthy controls, using anti-CA19.9, anti-Lea and anti-Leb antibodies. Real Time PCR (qPCR) was performed on cDNA of 14 tissue specimens (normal and cancer counterpart) to assess the level of each glycosyltransferase involved in the synthesis of Lewis antigens. PCR on genomic DNA evaluated the presence of *FUT2*/*FUT3* null alleles on 18 tissue's patients. Immunofluorescence (IF) was performed on 18 normal and cancer counterpart tissue sections using anti-CA19.9, anti-Lea and anti-Leb antibodies.

Results. In 14 out of 118 patients (12%), ELISA on sera showed an increase in CA19.9 and/or Lea with respect to healthy controls. In all the patients, no significant statistical difference in glycosyltransferases expression was assessed by qPCR between normal and cancer tissue. 11 out of 18 patients (61%) patients were found to be *FUT2*-/+ and/or *FUT3*-/+ but this does not always correlate with a reduction of Lewis antigen

expression. IF did not show a correlation between serum and tissue levels of Lewis antigens.

Conclusions. These results show that Lea could be used as a management marker in 12% of PDAC patients that underwent surgical resection. It could be an alternative marker in patients that are CA19.9 negative, and a complementary marker in those CA19.9 borderline positive.

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LIST OF ABBREVIATIONS

B3GALT5 β1,3 galactosyltransferase enzyme 5

BB blocking buffer

CA19.9 carbohydrate antigen 19.9

CT computer tomography

DAPI 4,6-diamidino-2- phenylindole

dsLea disialyl Lewis a

ELISA enzyme linked immunosorbent assay

EUS endoscopic ultrasonography

FDA Food and Drug Administration

FITC fluorescein isothiocyanate

FNA fine needle aspiration

FNB fine needle biopsy

Fuc fucose

FUT1 α1,2 fucosyltransferase enzyme 1

FUT2 α1,2 fucosyltransferase enzyme 2

FUT3 α1,3/4 fucosyltransferase enzyme 3

Gal galactose

GalNAc N-acetylgalactosamine

GAPDH glyceraldehyde-3-phosphate dehydrogenase

gDNA genomic DNA

GlcNAc N-acetylglucosamine

H&E hematoxylin/eosin

IF immunofluorescence

IHC immunohistochemistry

Lea Lewis a

Leb Lewis b

MRI magnetic resonance imaging

PBS-T phosphate buffered saline-Tween 20

PC pancreatic cancer

PCR polymerase chain reaction

PDAC pancreatic ductal adenocarcinoma

qPCR quantitative Real Time polymerase chain reaction

RIA radio immuno assay

sLea sialyl Lewis a

sLex sialyl Lewis x

ST3GAL3 galactose α2,3 sialyltransferase enzyme 3

ST3GAL4 galactose α2,3 sialyltransferase enzyme 4

ST3GAL6 galactose α2,3 sialyltransferase enzyme 6

ST6GALNAC6 N-acetylgalactosamine α2,6 sialyltransferase enzyme

TNM tumor node metastasis

WT wild type

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1. INTRODUCTION

The human pancreas is an about 15-cm-long retroperitoneal organ of the upper abdomen. It is a heterocrine gland, with both an exocrine and an endocrine component. The exocrine gland is made up of pancreatic acinar cells and duct cells that produce digestive enzymes and sodium bicarbonate, respectively, responsible for normal digestion. The endocrine gland is made up of secretory islet cells and secretes peptide hormones for the maintenance of glucose homeostasis [1].

Pancreas carcinoma is a rare and aggressive neoplasm, representing the seventh leading cause of global cancer deaths in industrialized countries [2], and, in particular, it was estimated to be the fourth leading cause of cancer deaths for both sexes in Europe [3]. Pancreatic cancer (PC) is a general term that refers mainly to pancreatic ductal adenocarcinoma (PDAC), since it accounts for more than 90% of pancreatic cancers. The remaining is represented by exocrine pancreatic cancers (adenosquamous carcinoma, squamous cell carcinoma, giant cell carcinoma, acinar cell carcinoma, and small cell carcinoma) [4].

1.1 DIAGNOSIS

Symptoms of PC are often nonspecific, and this leads to a delay in diagnosis. In fact, patients with PC usually present with either locally advanced mostly unresectable (30-35% of patients) or metastatic disease (50% of patients), unsuitable for curative surgical resection [5]–[7]. Most tumors (approximately 70%) arise at the head of the pancreas and often present with biliary obstruction leading to dark urine, jaundice, appetite loss, fatigue, weight loss, and exocrine pancreatic insufficiency. PC that arises from body and tail of the pancreas displays pain-related symptoms (abdominal and back pain), and cachexia-related symptoms (appetite loss, weight loss, fatigue) [5], [8].

Suspected PC is first evaluated with computer tomography (CT), as recommended by the Society of Abdominal Radiology and American Pancreatic Association consensus statement [9]. CT is preferred over magnetic resonance imaging (MRI) due to its lower cost and widespread availability, but MRI is used either when contrast-enhanced CT is contraindicated (due to renal insufficiency or severe contrast allergy), or when CT findings are equivocal [10]. These two non-invasive imaging techniques can identify a pancreatic mass and strongly suggest an underlying malignancy [11], but mainly they are able to assess the resectability of PC and the vascular invasion [12]. At this point, the diagnostic algorithm for PC patients proceeds in different directions: 1. patients with resectable tumor undergo surgery and receive appropriate neoadjuvant therapy (no need for a biopsy, when cross-sectional imaging exhibits typical features) [6], [13]; 2. patients with probable resectable tumor and with unresectable tumor need a cytological diagnostic confirmation by endoscopic ultrasonography (EUS) with fine needle aspiration (FNA), or a histological diagnosis by EUS with fine needle biopsy (FNB), that is also helpful for molecular evaluation and necessary before treatment [6], [11], [14].

1.2 STAGING AND PROGNOSIS

PDAC staging is based on the tumor node metastasis (TNM) system, described by the eighth edition of the American Joint Committee on Cancer Staging Manual [15]. Tumor size, location within the pancreas, involvement of surrounding vessels, and presence of metastatic disease are used to provide prognostic information regarding patient outcomes and to define if the PC is resectable, borderline resectable, locally advanced, and metastatic. Stages I and II are classified as clearly resectable, with the absence of tumor contact with the adjacent celiac trunk, hepatic artery, superior mesenteric artery, superior mesenteric vein, and portal vein. Stage III is defined as a

localized tumor with major vessel involvement. Stage IV is defined by the presence of distant metastatic disease [10]. See **table 1** for details.

	Primary tumor (T)		
Tx	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
Tis	Carcinoma in situ		
T1	Tumor limited to the pancreas, 2 cm or less in greatest dimension		
T2	Tumor limited to the pancreas, more than 2 cm in greatest dimension		
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or superior mesenteric artery		
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)		
Regional lymph nodes (N)			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Regional lymph node metastasis		
Distant metastasis (M)			
M0	No distant metastasis		
M1	Distant metastasis		
Anatomical stage/prognosis groups			
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage III	T4	Any N	M0
Stage IV	Any T	Any N	M1

Table 1. TNM staging of PDAC [15].

The prognosis of PDAC is one of the worst among solid tumors, and the 5-year overall survival is 10% in 2020, compared to 5% in 2000 [5]. The modest increase of the survival rate can be attributed to the improvement of both multiagent cytotoxic therapies [5] and multimodality care. In fact, nowadays the clinical practice consists in the involvement of a multidisciplinary team, made up of physicians from surgical oncology, radiology, medical oncology, and radiation oncology disciplines [5], [6].

It could be useful to assess preoperative CA19.9, since its value is strongly associated with tumor stage. A decrease in CA19.9 level is the best index of improved prognosis.

On the contrary, patients with increased CA19.9 after resection have a significantly shorter median survival time [16].

1.3 RISK FACTORS

The risk of development of PDAC has been associated to many factors, both modifiable and non-modifiable. Tobacco smoking, obesity, dietary habits, alcohol consumption, chronic pancreatitis, belong to the first category [3], [12], [17], while age, gender, non-O blood group, diabetes mellitus, family inherited history of the disease and germline mutations in specific genes belong to the second category [4], [18]. Since the risk of developing PC is increased in patients with a family history of this disease, it is recommended to perform annual screening tests (MRI and/or EUS), while it is not recommended population-based screening as the lifetime risk of developing PC is considerably low (about 1%) in an unselected population [19].

1.4 TREATMENT

Treatment options for PC are strictly dependent on the type of tumor. The only curative treatment for PC is upfront surgery, but it is feasible just for patients with resectable PC (10-15% of cases) [5], [20]. Soon after resection, the risk of relapse is high. Accordingly, postoperative restaging may be useful in identifying patients with early recurrence, since they will benefit of palliative treatment instead of adjuvant chemotherapy [6].

For candidate patients, surgery is followed by adjuvant chemotherapy with FOLFIRINOX (fluorouracil, irinotecan, leucovorin, oxaliplatin), with a median overall survival of 54.4 months compared with 35 months for single-agent gemcitabine [5], [13]. For patients with locally advanced and unresectable disease, systemic therapy

followed by radiation is an option for definitive locoregional disease control. For patients with mutated *BRCA* and metastatic PDAC, olaparib, a poly (adenosine diphosphate [ADB]-ribose) polymerase inhibitor, is a maintenance option that improves progression-free survival following initial platinum-based therapy [5].

Further research on molecular and tumor microenvironmental changes during PC development is necessary to identify patient subpopulations that can benefit of target therapy, improving their treatment outcome [17].

On the contrary, immunotherapy for PC did not achieve any success, in fact immunotherapeutics that proved to be effective against other malignancies, have not been successful against pancreatic tumor cells, possibly due to the immune tolerance mechanism of PC [12].

1.5 BIOSYNTHESIS OF LEWIS ANTIGENS

Aberrant glycosylation is a distinctive trait of carcinogenesis, and it can derive from both genetic and epigenetic modification on glycogenes [21]. This leads to a different expression of cell surface components, including antigens of the Lewis histo-blood group. These antigens were first discovered on red blood cells, and later recognized in plasma, saliva, and other secretion [22], but they are also present in most epithelial tissues (stomach, colon, uterus, and salivary glands), biliary system, and ductal cells in the pancreas [23], [24]. They can be described as oligosaccharide determinants (3-5 sugar residues) constituting the end of various carbohydrate chains of glycoproteins (mainly of mucin type), and glycosphingolipids, located on the outer surface of the cell membranes [25]. The expression of Lewis histo-blood group antigens requires the action of several distinct glycosyltransferases (**figure 1**).

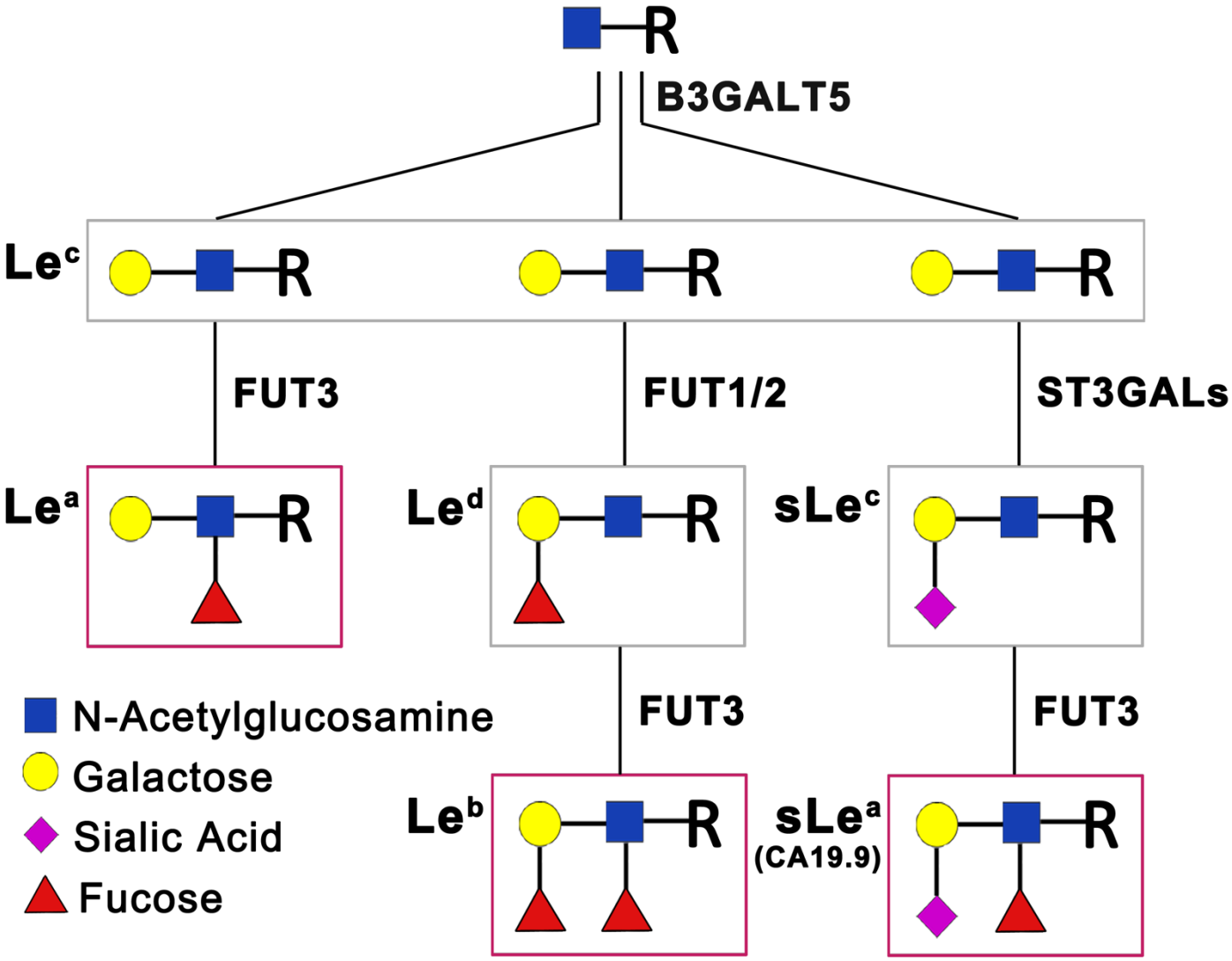


Figure 1. Synthetic pathway of type 1 chain Lewis antigens. Enzyme symbols are according to the HUGO nomenclature: B3GALT, β1,3-galactosyltransferase; FUT3, α1,3/4-fucosyltransferase; FUT1/2, α1,2-fucosyltransferase; ST3GAL, galactose α2,3-sialyltransferase.

The ABO, H and Lewis blood group antigens are all synthesized from the following precursor structures [23], [26], [27]:

Type 1: Galβ1-3GlcNAcβ1-R;

Type 2: Galβ1-4GlcNAcβ1-R;

Type 3**:** Galβ1-3GalNAcα1-R;

Type 4: Galβ1-3GalNAcβ1-R.

These moieties start with a N-acetyl-glucosamine (GlcNAc) residue, followed by a galactose (Gal) residue. The Gal unit is usually the β anomer and can be attached to either the 3- or the 4- position of GlcNAc. When the sugar chain is composed by the Galβ1-3GlcNAc sequence, it belongs to the type 1 chain, whereas when the sugar chain is composed by the Galβ1-4GlcNAc sequence, it belongs to the type 2 chain. This passage can be mediated by different galactosyltranferases, depending on the position of GlcNAc: the β1-3 linkage is mediated by a β1-3 galactosyltransferase (usually the enzyme 5, named B3GALT5), the β1-4 linkage by one of four different β1-4 galactosyltransferases [28]. Further modifications can occur on either the Galβ1-3GlcNAc (type 1) or Galβ1-4GlcNAc (type 2) chains, but only the type 1 chain Lewis antigens will be taken into account in this thesis.

The Lewis antigen system is based on expression of genes members of the fucosyltransferase family, which catalyzes the addition of an α-fucose residue to the precursor oligosaccharides in the last step of Lewis antigen biosynthesis [29]. Such a residue is necessary for antigenicity.

A fucose (Fuc) residue can be added to the Galβ1-3GlcNAc moiety by an α1,4 fucosyltransferase (commonly the enzyme 3, FUT3, encoded by the *FUT3* gene, also called *Lewis* gene, or *Le* gene) [22], [30]. The new trisaccharide structure Galβ1-3[Fucα1-4]GlcNAc is called Lewis a (Lea). Once this fucose has been attached to the GlcNAc, it is not possible to add any other sugar. The other Lewis antigens are synthetized starting again from the previous disaccharide structure. Lewis b (Leb) is produced, firstly, by linking an α-fucose residue to the terminal β-galactose through 1-2 linkage by the α1-2 fucosyltransferase, encoded by *FUT2* (also called *Secretor*, or *Se* gene) or *FUT1* genes, and, secondly, by adding the other α-fucose, giving rise to the difucosylated tetrasaccharide structure Fucα1-2 Galβ1-3[Fucα1-4]GlcNAc [31],

[32]. Sialyl Lewis a (sLea), the epitope of CA19.9 antigen, follows the same scheme of Leb, but instead of adding the α-1,2-fucose residue to the terminal β-galactose, it is added a sialic acid in an α2-3 linkage by an α2-3 sialyltransferase (such as the enzyme 3, ST3GAL3) [33], [34]. This results in the synthesis of the sialylated precursor Sial-α2-3Galβ1-3GlcNAc (sialyl-lacto-N-biose), to which, successively, FUT3 will transfer the terminal fucose, forming the sLea structure (Sial-α2-3Galβ1-3[Fucα1-4]GlcNAc) [34].

Lewis phenotype is determined by the combination of the Lewis/Secretor genotype. In human, 2 alleles are present at the *Lewis* (*FUT3*) locus: *Le*, which encodes a functional fucosyltransferase, and *le*, which encodes a nonfunctional one [35]. Also the *Secretor* (*FUT2*) locus have 2 alleles, one functional and the other nonfunctional, that are *Se* and *se*. Secretors have at least one functional *Se* allele, while non-secretors are homozygous for nonfunctional *se* alleles (*se*/*se*) [22]. Combination of these functional and nonfunctional alleles gives rise to 4 different phenotypes:

1. Le(a+b+): enzymatic activity of FUT2 is more efficient than the enzymatic activity of FUT3. When *FUT2* presents the A385T missense mutation, its activity decreases (about 5% as compared with the wild type allele-coding enzyme) [32] and this is why individuals with this mutation express both Lea and sLea, on top of Leb;
2. Le(a-b+): they are called secretors and express Leb and sLea antigens [33];
3. Le(a+b-): they are called non-secretors (about 20% of Europeans and North Americans) and express Lea and sLea antigens [33].
4. Le(a-b-): individuals lacking a functional *FUT3* allele (*le*/*le*) are called Lewis negative and are characterized by the Le(a-b-) phenotype, meaning that they do not express neither Lea, nor Leb, nor sLea. They represent the 5-10% of Caucasian population [33], [36], [37].

Several polymorphisms in *FUT2* and *FUT3* genes have been reported [22], [35], [38] and will be discussed in “materials and methods” and “results” sections.

1.6 LEWIS ANTIGENS IN PANCREATIC CANCER

Extensive literature has linked aberrant expression of Lewis antigens with carcinogenesis [39], since they act as ligands for selectins, responsible for cell adhesion, angiogenesis and cancer invasion [21], [40].

The minimal sugar structure required for selectin binding is constituted by an α2,3-sialylated and α1,3/4 fucosylated tetrasaccharide based on a type 1 or a type 2 chain and the two isomers that fulfill this basic requirement are sLea (Siaα2,3Galβ1,3[Fucα1,4]GlcNAc) and sialyl Lewis x (sLex) (Siaα2,3Galβ1,4[Fucα1,3]GlcNAc [39], [41]. In particular, they are known to be the ligands for endothelial cell E-selectin, potentially responsible for cell adhesion and hematogenous metastasis [22], [24], [40].

The classic theory, the so-called incomplete synthesis and neo-synthesis processes, has been developed by Kannagi and colleagues [42], but it is no more convincing at present [34]. The incomplete synthesis process occurrs more often in early stages of cancer and leads to the biosynthesis of truncated structures, since the normal synthesis of complex glycans expressed in normal epithelial cells is impaired. Conversely, neo-synthesis is commonly observed in advanced stages of cancer, when several glycogenes are upregulated or downregulated and, thus, a *de novo* expression of certain antigens (such as sLea and sLex) takes place [30]. For example, the increased amount of CA19.9 in cancer could be due to the epigenetic silencing of the gene encoding for an α2-6 sialyltransferase, that is *ST6GALNAC6*, responsible for the attachment of an extra sialic acid residue on sLea and the development of disialyl

Lewis a (dsLea), normally expressed on the epithelial surface of digestive organs. This results in the predominance and accumulation of sLea, instead of dsLea [36].

1.7 CA19.9

In 1979, Koprowski and colleagues utilized hybridoma technology and discovered carbohydrate antigen 19.9 (CA19.9), originally isolated from a human colorectal cancer cell line SW1116, by using the mouse monoclonal antibody 1116-NS-19-9 [43]. CA19.9 was then discovered in the serum of patients with colon and pancreatic cancer in 1981 [44]. sLea is the tetrasaccharide NeuAcα2,3Galβ1,3[Fucα1,4]GlcNAc that constitutes the actual epitope of the CA19.9 antigen, which is constituted by a heterogeneous group of glycoconjugates, including N- or O- linked glycoproteins, mainly of mucin type, and glycosphingolipids [45], [46]. CA19.9 is normally produced by ductal cells in the pancreas, biliary system, and epithelial cells in the stomach, colon, uterus, and salivary glands [24].

Nowadays CA19.9 is the most common tumor-associated marker and it is the only one approved by the US Food and Drug Administration (FDA) for the management of patients diagnosed with PC [47]–[49]. In fact, it has been used for over 30 years for monitoring response to therapy, post-operative recurrence, disease progression, and prognosis in PC patients [12], [14], [36], [47].

Assessment of CA19.9 occurs in sera of patients through radio immuno assay (RIA) or enzyme-linked immunosorbent assay (ELISA) procedures, and elevation of its value is associated with various pathological conditions [50]. When it comes to cancer, it is generally believed that tumoral cells are able to overexpress CA19.9, but this is in contrast with what it is known about B3GALT5 for the production of CA19.9, at least on colon cancer, where the enzyme is down-regulated [51]–[53]. In literature, only

one research linked the expression of B3GALT5 with the expression of CA19.9 in PC, but the findings on 13 patients are the opposite, that is B3GALT5 is overexpressed in PC [54].

1.7.1 LIMITATIONS OF CA19.9 AS A PANCREATIC CANCER BIOMARKER

Despite multiple clinical applications for CA19.9 serum levels in PC patients, CA19.9 is not useful as a diagnostic tool, due to its low specificity (82-90%) and sensitivity (72-80%) [36], [55], [56]. In fact, it is also elevated in non-pancreatic cancers, such as stomach, colorectal, lung, thyroid, and biliary cancers, and in several benign conditions, such as cholangitis, obstructive jaundice, liver cirrhosis, pancreatitis, pulmonary, thyroidal and gynecologic diseases, and diabetes mellitus, and this results in a high false positive rate (10-30%). Moreover, it is elevated only in 65% of patients with resectable PDAC, mainly in late cancer stages, and up to 5-10% of the population is genetically negative [5], [18], [36], [57], [58]. Also, it cannot be used as a routine screening tool, due to its poor positive predictive value (0.9%) [29], [37], [59]. This is true also for individuals at higher risk of PC (hereditary pancreatitis, family history ofPC, Peutz-Jeghers syndrome), because CA19.9 serum levels fail to identify early/small tumors or precancer lesions in 10–15% of patients [36].

CA19.9 could have a diagnostic value only in case of symptomatic patients, that show weight loss, abdominal pain, and jaundice or when imaging studies indicate a tumor [60]. It is one of the most (ab)used serum tests performed in these recent years, largely exceeding the fair determination numbers recommended by the scientific literature [61], [62]

Moreover, recent interest in CA19.9 has increased, due to its role in monitoring the efficacy and the duration of the neoadjuvant therapy in PDAC [63] and in developing new treatment strategies by using monoclonal antibody with anticancer activity against it [5], [64]–[67]. That said, it is far from being a perfect biomarker for PC, so

there is an increased interest in searching for other molecules that can be alternatives or can complement CA19.9 [47], [68]–[70].

2. AIMS

Until now, only CA19.9 is used as tumor marker for PC, assuming that it is specifically (over)produced by cancer cells, as supposed by immunohistochemical data [71], [72] and according to the incomplete synthesis theory [42]. However, some crucial points have to be taken into account:

1. assumptions based on immunohistochemical data could be unreliable. In fact, immunohistochemistry (IHC) performed on mice tissues revealed a strong reactivity [25], which is not possible since mice lack FUT3 [25], [73], [74];
2. according to the incomplete synthesis theory developed by Kannagi and colleagues [42], sLea is the product of the silencing of *ST6GALNAC6*, and thus, dsLea. Expression of dsLea has never been analyzed on normal and tumoral pancreatic tissue, but our hypothesis is that dsLea on normal tissue is not abundant enough to justify the overexpression of sLea in PC tissue;
3. two glycosyltransferases are mandatory for the expression of type 1 chain Lewis antigens: a β1,3 galactosyltransferase and an α1,4 fucosyltransferase; in vitro and in vivo studies suggested that B3GALT5 and FUT3 are by far the two most relevant [51], [73], respectively. The expression levels of the other three glycosyltransferases, namely α2,3sialyltransferase, α1,2fucosyltransferase, and α2,6sialyltransferase, determine the amount of each antigen by competing with each other. In this regard, *ST3GAL3*, *FUT2*, and *ST6GALNAC6* were proposed as the candidate genes, respectively [75], [76];
4. the expression of B3GALT5 in PC tissue is controversial and needs to be clarified;
5. the use of cognate antigens of CA19.9, namely Lea and Leb, as management markers for PDAC, was never taken into consideration, even though there is no reason not to use them. In fact, Lewis antigens have a common precursor, and

then, depending on which glycogene is more expressed in each individual, each one of them can be more or less expressed.

Our hypothesis is that the tumoral duct-forming cells do not overproduce CA19.9, but the elevation of circulating antigens depends on the obstruction of neoplastic ducts and reverse of polarity occurring in malignant ductal cells (**figure 2**), as speculated in the work of Kalthoff et al. [77] and as seen in a nonmalignant disease such as chronic pancreatitis, where a stronger elevation in serum CA19.9 correlated with the obstruction of the main pancreatic ducts [78].

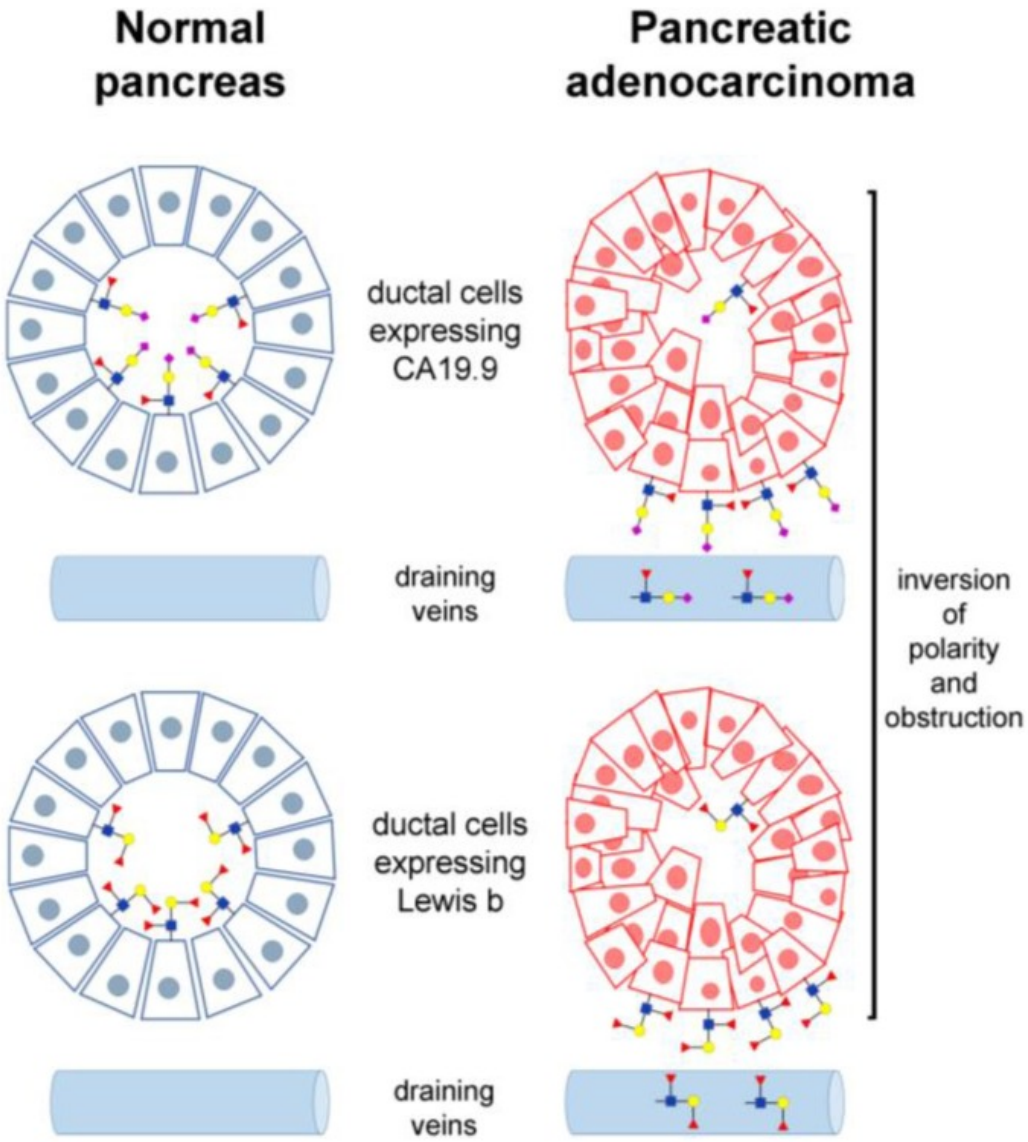


Figure 2. **Schematic representation of ductal cells in normal pancreas and in PDAC** [39]. The model suggests that inversion of polarity of malignant ductal cells and obstruction of the ducts occur in PDAC. This determines the reabsorption of the molecules normally secreted by the organ. Our hypothesis is that, since the individual glycosyltransferase expression pattern is not deregulated in cancer, serum CA19.9 levels will increase only in patients that express the antigen in the normal ducts (upper panel). This rationale can be applied also for Leb (lower panel) and Lea (not depicted). Sugars are depicted as in figure 1.

As a consequence, the antigens are not secreted, but on the contrary, they are reabsorbed in the bloodstream. This is also the rationale for assessing the expression

levels of Lea and Leb, in addition to CA19.9, to improve the management of PDAC. In fact, as steted before, no specific association exists between cancer and CA19.9 with respect to Lea or Leb, because each one can be reabsorbed in the blood, according to their specific and individual expression [79]. That is, if the normal pancreatic ducts express and secrete high levels of CA19.9, upon malignant transformation the bloodstream does reabsorb large amounts of CA19.9. Rather, if the normal ducts mainly express and secrete Leb, or Lea, but low levels of CA19.9, upon malignant transformation the bloodstream cannot reabsorb relevant amounts of CA19.9, but probably does reabsorb Leb or Lea instead [34]. Of course, patients that do not have ductal obstruction (that are a minority) and/or loss of polarity, are expected to remain negative for all such antigens.

Another crucial aspect is that it is well known that null alleles of *FUT2* and *FUT3* are present in the human population [33], [36], [37], and this can affect the amounts of Lewis antigens. Patients with PDAC can not take advantage of their use for the follow up.

Therefore, the aims of this work are:

1. to verify if Lea and Leb can support CA19.9 in the management of PDAC, mainly in cases in which PDAC patients are negative for circulating CA19.9;
2. to assess the relationship between the amounts and pattern of Lewis antigens detected in the pancreatic tissue (both normal and neoplastic) and circulating in the blood of patients;
3. to confirm or to prove wrong the theory of Kalthoff, and, eventually, to define the mechanism underlying the overexpression of CA19.9 (and possibly of Lea and Leb) in PDAC.

3. MATERIALS AND METHODS

3.1 CASES SELECTION AND SAMPLE COLLECTION

This study includes patients with histologic diagnosis of PDAC. 19 patients' sera and 18 fragments of surgical resection (both normal and cancer parts) and 52 sera from healthy controls are being collected from San Paolo Hospital, together with 99 sera of PDAC patients from Humanitas Research Hospital.

Patients' sera are collected before surgery, while specimens of pancreatic tissue are being collected at surgery. For each patient, part of the sample is immediately fixed in neutral buffered formalin for 24 hours and processed, in order to perform immunofluorescence staining. Another part of the tissue specimen is immediately frozen in dry ice and kept at -80°C for RNA extraction and genomic DNA extraction.

3.2 RNA EXTRACTION

15 matched pairs of mRNAs from cancer and adjacent normal pancreas were processed for RNA extraction starting from frozen material. From each sample, one aliquote of about 80 mm^3 was cut, thawed and used as follows: samples were homogenized in 0.25 ml Lysis Buffer (ReliaPrep RNA miniprep system, Promega) using a tissue lyser (Qiagen) at 50 o/s (Hz) for 3 minutes and centrifuged at 12.000 rcf for 1 minute at RT. Supernatant was recovered and processed according to the manufacturer's protocol, including DNAse treatment. Elution was done with 30 µl of nuclease free water heated at 70 °C. RNA was quantitated by NanoDrop Spectrophotometer (Thermo Scientific).

3.3 GENOMIC DNA EXTRACTION

For genomic DNA (gDNA) extraction, samples were treated with 180 µl Buffer T1 and 25 µl of Proteinase K (Nucleo Spin Tissue, Genomic DNA from tissue, Machery-Nagel) at 56°C overnight, and then processed according to the manufacturer's protocol. Elution was done with 100 µl of nuclease free water. gDNA was quantitated by NanoDrop Spectrophotometer (Thermo Scientific).

3.4 SANDWICH ELISA ON PATIENTS' SERA

Detection of Lewis antigens in the sera of 118 patients and 52 healthy controls was performed by a sandwich ELISA in 96 well plates (Nunc F8 Maxisorp Immuno-module). Each well was covered at 4°C overnight with 0.1 ml of each capture antibody diluted at 4 µg/ml in Tris buffer 0.2 M pH 9.4. The following day, the unbound capture antibody was removed, 0.2 ml of Blocking Buffer (BB) (Roche) was added to each well and the plate was incubated for 75 min at RT. After that, BB was removed and each well was allowed to react 2.5 hours at RT either with 0.1 ml of human serum, or with 0.1 ml of serial dilution of the spent media of COLO-205 cells, which express large amount of all type 1 chain Lewis antigens and it is utilized as reference control for Lewis antigens, together with 25 µl of calibrators available for CA19.9 only (CanAg CA19-9 EIA, Fujirebio). Plates were washed 3 times with PBS-T (phosphate buffered saline containing 0.1% Tween-20) and incubated with peroxidase-labeled secondary antibodies either anti-CA19.9 (1:10.000), or anti-Lea antibody (1:20000), or anti-Leb antibody (1:5.000) for 75 min at RT. After washing 6 times with PBS-T, the reactions were developed using 0.1 ml TMB (Sigma), 5-20 min at RT, and stopped with 0.1 ml 1N HCl. The resultant colors were evaluated in a microtiter plate reader. Peroxidase-labeled antibodies for detection were prepared starting from antibodies double purified by protein-A Sepharose chromatography, 2.5 mg/ml, and using Lightning-

Link® HRP Conjugation Kit (Innova Biosciences), according to the manufacture's protocol. Anti-CA19.9 (ATCC HB-8059), anti-Lea (ATCC HB-8324) and anti-Leb (ATCC HB-8326) monoclonal antibodies were purified from the culture medium of the corresponding hybridomas by ammonium sulfate precipitation and affinity chromatography on a Protein A Sepharose column.

Linearity of detection for ELISA was assessed by the linear regression methods setting a correlation value $R^2 > 0.9$ (**figure 3**).

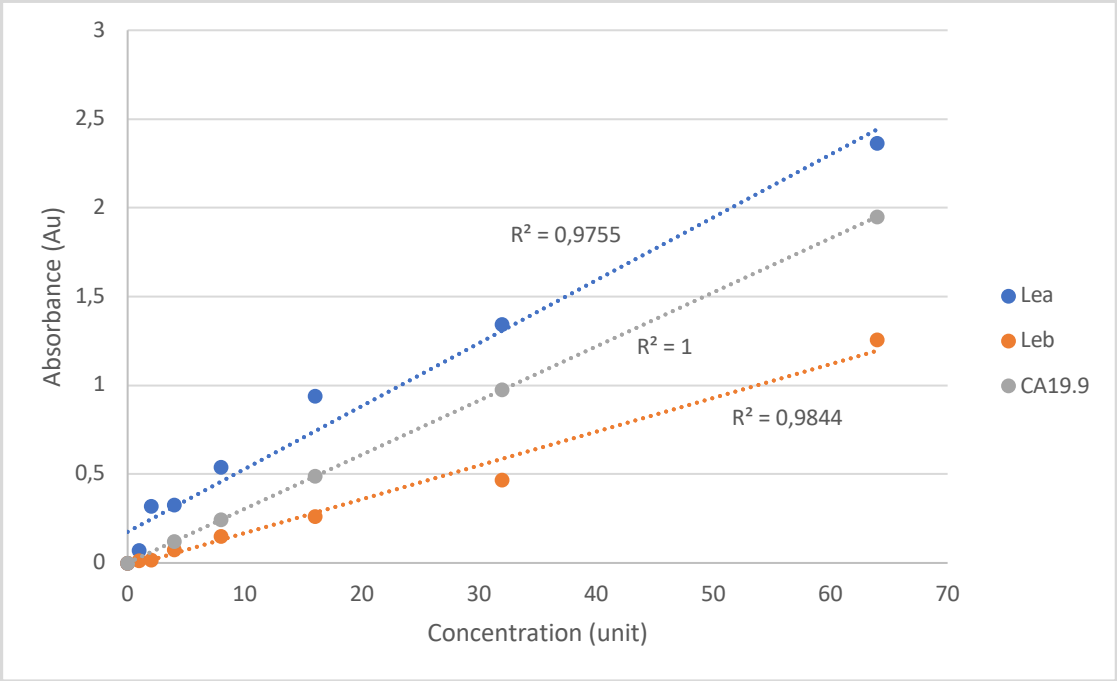


Figure 3. Linear regression method. Serial dilution of COLO-205 cells media is utilized as reference control for Lea and Leb, while CA19.9 calibrators (CanAg CA19-9 EIA, FujireBio) were utilized as reference control for CA19.9.

3.5 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was prepared and DNase treated with a commercially available kit (ReliaPrep cell RNA miniprep, Promega), was quantitated by NanoDrop Spectrophotometer (Thermo Scientific) and reverse transcribed. First strand cDNA

was synthesized from 1 to 4 µg of total RNA in a 20 µl volume by Moloney Murine Leukemia virus reverse transcriptase (2500 U/ml, USB-Affymetrix), 0.4 µM oligo-(d)T_{12-18} primer, the supplied buffer, and 1000U/ml human placental RNase inhibitor. Reactions were kept at 37°C for 45 min and then at 42°C for 45 min. Control reactions were prepared by omitting the reverse transcriptase. Quantitative Real-Time polymerase chain reaction (qPCR) was performed by amplifying cDNAs (0.2–1.0 µL of first strand reactions) in a volume of 20 µl using Sybr Premix Ex Taq (Tli RNase H Plus, Takara), ROX as reference dye and StepOnePlus instrument (Applied Biosystem Life Technologies). Primer sequences are listed below in **table 2**. Annealing temperature was 60°C. Amplification program included 20 sec at 95°C, 40 cycles consisting in 3 sec at 95°C, 30 sec at 60°C, and a last step 15 sec at 95°C and 1 min at 60°C. The amounts of amplified target cDNAs were calculated as ΔCt with respect to GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

FUT1	
Forward	AGC AGC TCG GCC ATG TGG
Reverse	TCT GAT TAC CAA ACC GGC CA
FUT2	
Forward	TGA GGT GCC TGC CCA ACC
Reverse	CTG CTG AAC GTG AAA TAT AGT GG
FUT3	
Forward	GGA GCT TTG GTA AGC AGG AG
Reverse	CAC AGC CAC CAG CAG CTG
B3GALT5	
Forward	CTC TTA CCC AGC AAA AAA TGG
Reverse	GGA AGG GAG GTG TCT GCC
ST3GAL3	
Forward	CTC TGG GGT CAC GAA TTG AC
Reverse	TGC TCA GGC CGC TGC ATG
ST3GAL4	
Forward	CTC TAA CGT CTT TGG CAA CTA C
Reverse	CGG CAC CTG AGG CTC TG
ST3GAL6	
Forward	GGA ACG AAT GTC TAT TGG GTG
Reverse	AAG TCG AAA ATA TTC CGC TGA TG
ST6GALNAC6	
Forward	TGA GTA GCA ACA AAG AGC AGC
Reverse	GAG AGG GCA GTG TCT TGT TG
GAPDH	
Forward	GGA GAA GGC TGG GGC TC
Reverse	GGC ATG GAC TGT GGT CAT G

Table 2. **PCR primers for quantification of specific transcripts by qPCR.**

3.6 POLYMERASE CHAIN REACTION FOR GENOTYPING *FUT2* AND *FUT3*

Detection of the most common mutations on *FUT2* (A385T and G428A) and *FUT3* (T59G and T1067A) [32], [38] were performed by polymerase chain reaction (PCR) on gDNA of 18 PDAC patients. For each patient and the corresponding gDNA, 8 reactions are carried out, in order to verify if mutations are present or not, and, when present, if they are heterozygous or homozygous. For each reaction, gDNA (50 ng) was

amplified in a volume of 25 µl using GoTaq G2 Flexi DNA Polymerase (Promega) and supplied Green Buffer. Actin primers were present in each reaction as internal quality control. Primer sequences are listed below in **table 3**. Amplification program is summarized in **table 4**.

Actin	
Forward	TGA ACC CCA AGG CCA ACC G
Reverse	CTG CTT GCT GAT CCA CAT CTG
***FUT2* WT for A385T mutation**	
Forward	AGG AGG AAT ACC GCC ACA
***FUT2* mutated for A385T**	
Forward	GAG GAG GAA TAC CGC CAC
***FUT2* WT for G428A mutation**	
Forward	GCT ACC CCT GCT CCT GG
***FUT2* mutated for G428A**	
Forward	CGG CTA CCC CTG CTC CTA
***FUT2* reverse common**	
Reverse	GGC TGC CTC TGG CTT AAA G
***FUT3* WT for T59G mutation**	
Forward	CGC TGT CTG GCC GCA CT
***FUT3* mutated for T59G**	
Forward	CGC TGT CTG GCC GCA CG
***FUT3* reverse common for WT and mutated T59G**	
Reverse	GGA GTC GCT GCG GTA GG
***FUT3* WT for T1067A mutation**	
Reverse	CAG GTG AAC CAA GCC GCT
***FUT3* mutated for T1067A**	
Reverse	CAG GTG AAC CAA GCC GCT T
***FUT3* forward common for WT and mutated T1067A**	
Forward	GGT GGA CGT GTA CGG ACG

Table 3. PCR primers for detection of specific mutations in *FUT2* and *FUT3* genes. Schematic list of primers organization in the 8 different reactions is reported as follow: 1. *FUT2* WT for A385T + *FUT2*

reverse common; 2. *FUT2* mutated for A385T + *FUT2* reverse common; 3. *FUT2* WT for G428A mutation + *FUT2* reverse common; 4. *FUT2* mutated for G428A + *FUT2* reverse common; 5. *FUT3* WT for T59G mutation + *FUT3* reverse common for WT and mutated T59G; 6. *FUT3* mutated for T59G + *FUT3* reverse common for WT and mutated T59G; 7. *FUT3* WT for T1067A mutation + *FUT3* forward common for WT and mutated T1067A; 8. *FUT3* mutated for T1067A + *FUT3* forward common for WT and mutated T1067A. *FUT2* forward primers, *FUT3* forward WT/mutated for T59G primers, and *FUT3* reverse WT/mutated for T1067A primers are designed to cover the sequence where it could be the specific mutation. *FUT2* reverse primer, *FUT3* reverse WT/mutated for T59G primers, and *FUT3* forward WT/mutated for T1067A primers are common for the related sequences.

Annealing 62°C	**Annealing 64°C**	**Annealing 62°C**
1. 94°C 3 min	1. 94°C 0,1 sec	1. 94°C 0,1 sec
2. 94°C 1 min	2. 94°C 50 sec	2. 94°C 1 min
3. 66°C 1 min	3. 64°C 1 min	3. 62°C 1 min
4. 72°C 1 min 30 sec	4. 72°C 1 min 30 sec	4. 72°C 1 min 30 sec
5. repeat twice from step 2 to step 4	5. repeat twice from step 2 to step 4	5. repeat 26 times from step 2 to step 4
6. 94°C 2 min	6. 94°C 2 min	6. 72°C 8 min
7. Go to annealing 64°C program	7. Go to annealing 62°C final program	7. 10°C infinite

Table 4. PCR amplification programs. They consist in 3 different steps, that have to be performed sequentially.

3.7 IMMUNOFLUORESCENCE ON PANCREATIC TISSUE SECTIONS

Immunofluorescence (IF) was performed on 18 matched formalin-fixed paraffin-embedded 4 µm thick PDAC tissue sections, from cancer and adjacent normal pancreas (see details in “results” section). Sections were deparaffinized in xylene and rehydrated through a graded series of alcohols. Primary antibodies were those above reported for ELISA, and were used at the following concentrations: anti-CA19.9, 0.4 µg/ml; anti-Lea 1 µg/ml; anti-Leb 1 µg/ml. Slides were incubated overnight at 4°C. Polyclonal Rabbit Anti-Mouse Immunoglobulins/FITC (fluorescein isothiocyanate)

(Dako, dilution 1:80) was used as fluorophore-conjugated secondary antibody. Nuclei were subsequently counterstained with DAPI (4',6-diamidino-2-phenylindole, Invitrogen). Serial dilutions of primary antibodies were tested on positive and negative controls and on a colon sample to assess the effect of antibody concentrations. Fluorescence images were viewed and captured using NanoZoomer 2.0 (Hamamatsu Photonics, Japan).

4. RESULTS

4.1 DETECTION OF LEWIS ANTIGENS IN PATIENTS' SERA

To assess the levels of Lewis antigens in patients' sera, ELISA procedure was performed on sera of 118 PDAC patients and 52 healthy controls. As far as concerns CA19.9 levels, recommended upper limit has been set at 37 U/ml [38], [80], even if surgeons and clinicians base medical decisions on a higher value, that is 120 U/ml, considered the cut-off of the real positivity. Contrary to CA19.9, there is not a normal range of values for Lea and Leb. This range was deduced by screening sera of healthy controls and an arbitrary maximum level was defined. The distributions of the sera values of the sLea and Lea are showed in figure 3, panel A and B. Regarding CA19.9, most of the controls (n=33) are under the cut-off value, a substantial number of controls (n=17) is under the value of 120 U/ml, and few controls are over the value of 120 U/ml (n=2). Something similar occurs for Lea, and the cut-off value was set at an arbitrary value of 37 U/ml (see **figure 4** for details), but it is not yet known the threshold of borderline values that could be much lower than 120 U/ml. Evaluation of Leb is still in progress and cannot be reported in the present thesis.

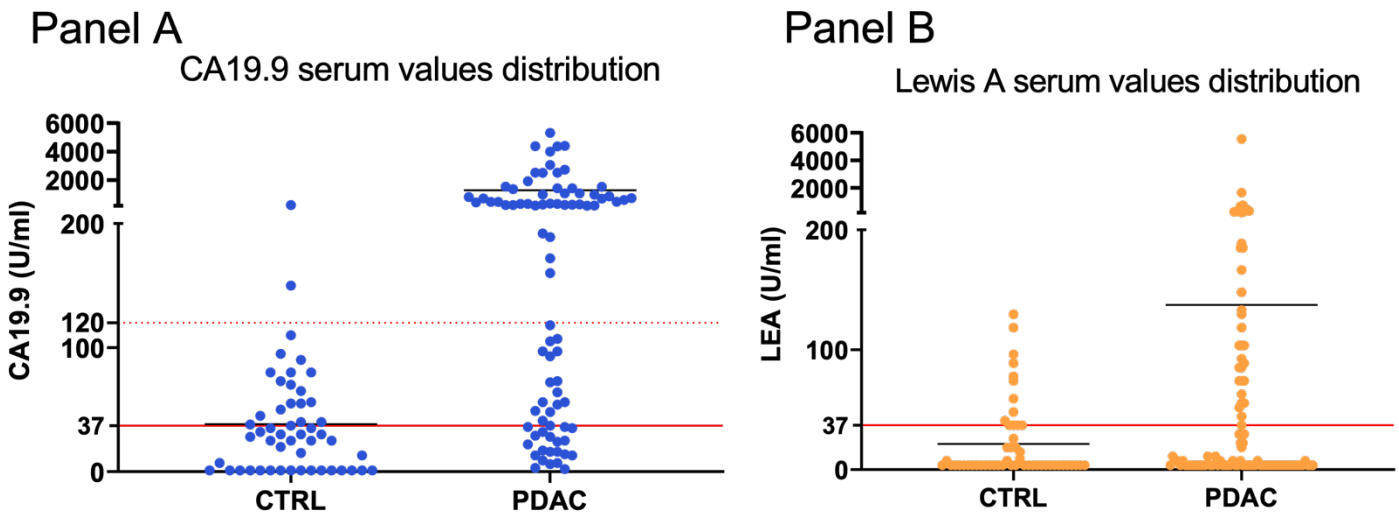


Figure 4. Distribution of CA19.9 and Lea levels in both control and PDAC sera. Panel A: CA19.9 values in the cohorts match literature data since only 2/52 control values (3.8%) are over 120 U/ml, while about a half PDAC values (63/118, 53,4%) are over 120 U/ml. Panel B: as far as concerns Lea, the cut-off was fixed at the arbitrary absorbance value that placed 84,6% of controls (44/52) below such value, and made 37 units according to the CA19.9 scale. Among PDAC patients, 42/118 (35,6%) are over the cut-off value.

In our cohort, 57 out of 118 patients (48.3%) are CA19.9 positive (>37 U/ml), 5 out of 118 (6.8%) are CA19.9 negative (≤37 U/ml) and Lea positive (>37 U/ml), and 9 out of 118 (8.5%) are CA19.9 borderline (>37 and ≤120 U/ml) and Lea positive (>37 U/ml). Consequently, we conclude that the follow-up of 14 out of 118 PDAC patients (12%) could be better monitored using Lea and CA19.9 together instead of CA19.9 alone (**figures 5 and 6**).

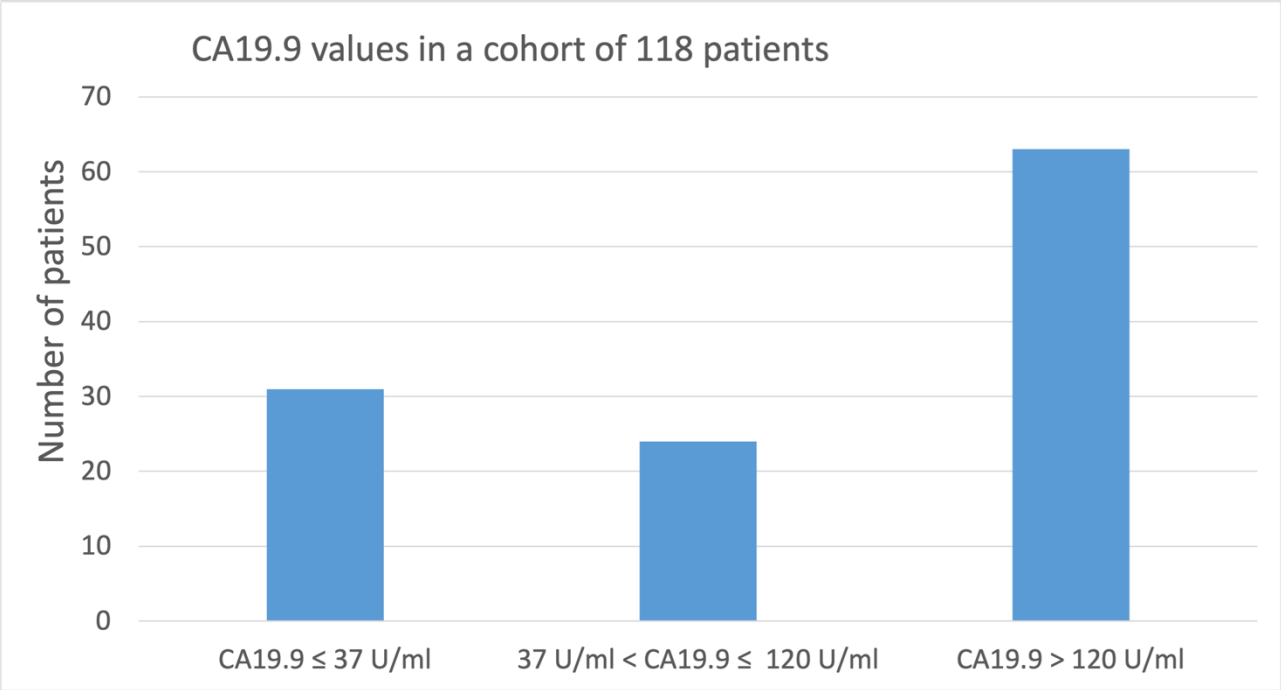


Figure 5. CA19.9 values in a cohort of 118 patients. 118 PDAC patients have been tested for CA19.9. 55 patients (46,6%) resulted negative or borderline (CA19.9 ≤ 120 U/ml), while 63 patients (53,4%) resulted positive (CA19.9 > 120 U/ml).

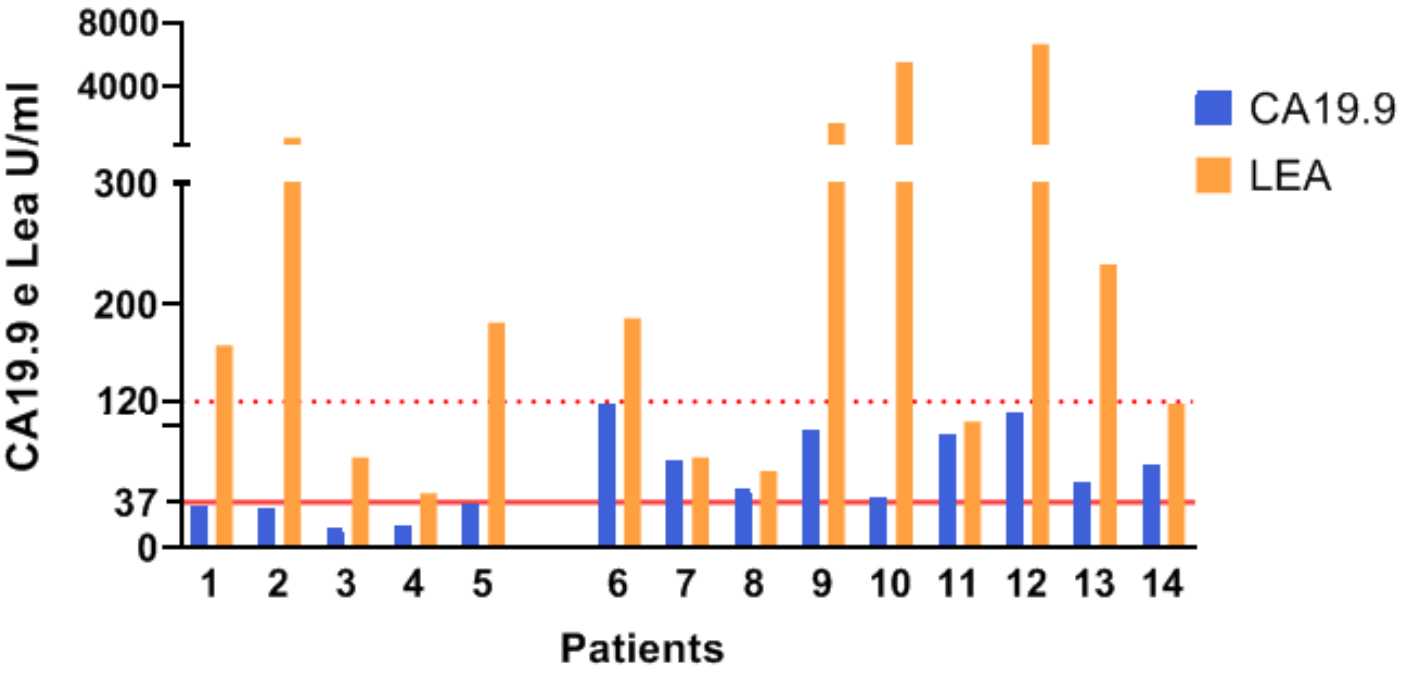


Figure 6. PDAC patients that can be monitored with Lea. This chart shows the PDAC patients who could be only or better monitored using Lea antigen in addition to CA19.9, in fact 14/118 patients (12%) are CA19.9 negative (patients 1-5) or borderline (patients 6-14) and Lea positive.

4.2 DETECTION OF GLYCOSYLTRANSFERASE TRANSCRIPTS IN PANCREATIC TISSUES

Expression levels of selected glycogenes (FUT1, FUT2, FUT3, B3GALT5, ST3GAL3, ST3GAL4, ST3GAL6, ST6GALNAC6) involved in the biosynthesis of type 1 chain Lewis antigens were assessed by qPCR in 14 matched normal and cancer specimens of PDAC patients that underwent surgery (**figure 7**).

Most of the glycogenes (FUT1, FUT2, B3GALT5, ST3GAL4, ST3GAL6) do not show a significant difference in expression between normal and tumoral tissues. Among them, it is worth considering FUT2 and B3GALT5, that play a pivotal role in the biosynthesis of the Lewis antigens. FUT2 presents a slight increase in the expression in the tumoral part, while on the opposite, B3GALT5 seems to behave like in colon carcinoma, when there is a down-regulation of the transcript [34].

On the other hand, glycogenes like ST6GALNAC6, FUT3, and ST3GAL3 show a significant change in their expression: ST6GALNAC6 is involved in the production of dsLea, normally expressed on the epithelial surface of digestive organs, and its mRNA expression is downregulated in PDAC tissue, as hypothesized [81] but never confirmed; the last two pivotal enzymes, FUT3 and ST3GAL3, showed a significant down-regulation in the tumoral tissue.

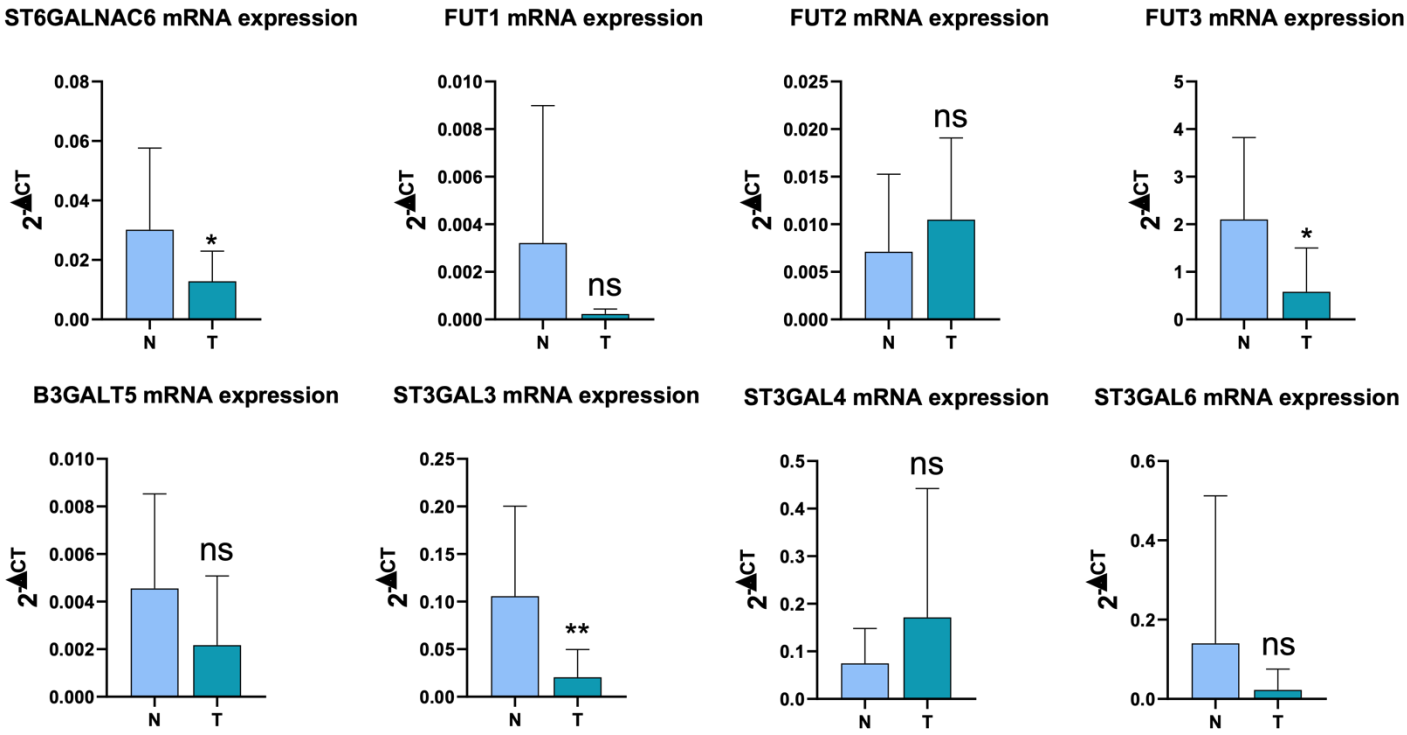


Figure 7. **mRNA expression level of all the glycogenes involved in the biosynthesis of Lewis antigens**, both in the normal pancreatic tissue and in their tumoral counterpart. Results are calculated as the relative fold gene expression $2^{(-\Delta\Delta CT)}$.

4.3 DETECTION OF *FUT2* AND *FUT3* MUTATIONS ON gDNA

Genotyping of *FUT2* and *FUT3* genes was provided for 18 patients on their pancreatic tissue by PCR on gDNA. Investigated mutations were A385T (missense mutation) and G428A (nonsense mutation) for *FUT2*, and T59G and T1067A (both missense mutations) for *FUT3*. As far as concerns *FUT2*, more than a half of patients (11 out of 18, 61%) have heterozygous inactivating mutation G428A, while one patient is homozygous for the same mutation; on the other side, 3 patients out of 18 (17%) are heterozygous for both the mutations of *FUT3* (T59G and T1067A). **Table 5** summarizes and correlates the patients with the respective *FUT2* and *FUT3* genes status. **Figures 8A and 8B** show some examples of PCR product yield belonging to patients with different mutations on agarose gel.

	FUT2	***FUT3***
PZ 1	Heterozygous for G428A	WT
PZ 2	WT	WT
PZ 3	Heterozygous for G428A	Heterozygous for both T59G and T1067A
PZ 4	Heterozygous for G428A	WT
PZ 5	Heterozygous for G428A	Heterozygous for both T59G and T1067A
PZ 6	WT	WT
PZ 7	WT	WT
PZ 8	Heterozygous for G428A	WT
PZ 9	WT	WT
PZ 10	WT	WT
PZ 11	Heterozygous for G428A	Heterozygous for both T59G and T1067A
PZ 12	Heterozygous for G428A	WT
PZ 13	WT	WT
PZ 14	WT	WT
PZ 15	Heterozygous for G428A	WT
PZ 16	Heterozygous for G428A	WT
PZ 17	Heterozygous for G428A	WT
PZ 18	Homozygous for G428A	WT

Table 5. Schematic representation of *FUT2* and *FUT3* status in 18 PDAC patients. WT: wild type.

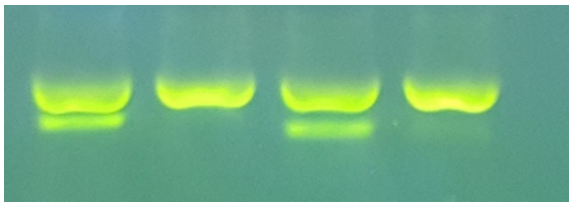
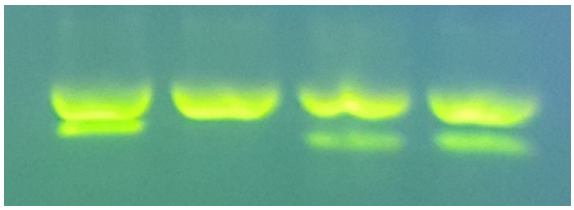
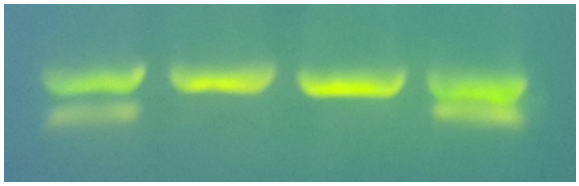
	Row 1 pWT	2 pMUT	3 pWT	4 pMUT
***FUT2* status** **PZ 2**	**Panel A** ***Actin*** ***FUT2***			
***FUT2* status** **PZ 3**	**Panel B** ***Actin*** ***FUT2***			
***FUT2* status** **PZ 18**	**Panel C** ***Actin*** ***FUT2***			

Figure 8A. *FUT2* mutations in gDNA samples. PCR was performed in order to detect *FUT2* mutations in gDNA samples. PCR reactions were loaded on agarose gel and *FUT2* status was evaluated. 3 different combinations are depicted in the figure. Upper bands represent *actin*, used as quality internal control, while lower bands (when present) represent the gene status. Rows correspond to the type of mutation. Row 1: *FUT2* is WT and it does not contain the A385T mutation; row 2: *FUT2* with the A385T mutation; row 3: *FUT2* is WT and it does not contain the G428A mutation; row 4: *FUT2* with the G428A mutation. Panel A: patient 2 is *FUT2* WT for both the mutations, in fact row 2 and row 4 do not show any band (except for the actine ones). Panel B: patient 3 is heterozygous for the *FUT2* G428A mutation, in fact both rows 3 and 4 show a band (except for the actine ones). Panel C: patient 18 is homozygous for the *FUT2* G428A mutation, in fact no band is present in row 3 (except

for the actine one). pWT (primer wild type) were used in row 1 and 3, while pMUT (primer mutation) were used in row 2 and 4.

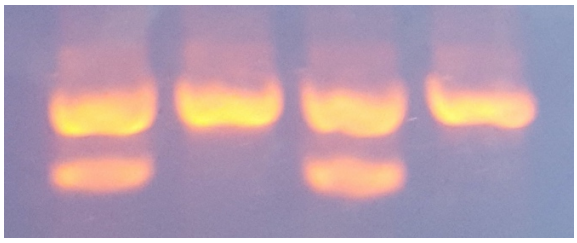
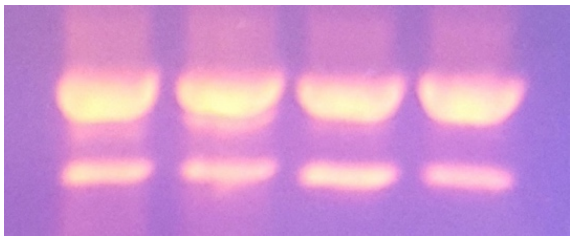
	Row	**1**	**2**	**3**	**4**
		pWT	pMUT	pWT	pMUT
***FUT3* status** **PZ 6**	**Panel A** ***Actin*** ***FUT3***				
***FUT3* status** **PZ 3**	**Panel B** ***Actin*** ***FUT3***				

Figure 8B. *FUT3* mutations in gDNA samples. PCR was performed in order to detect *FUT3* mutations in gDNA samples. PCR reactions were loaded on agarose gel and *FUT3* status was evaluated. 2 different combinations are depicted in the figure. Upper bands represent *actin*, used as quality internal control, while lower band (when present) represents the gene status. Rows correspond to the type of mutation. Row 1: *FUT3* is WT and it does not contain the T59G mutation; row 2: *FUT3* with the T59G mutation; row 3: *FUT3* is WT and it does not contain the T1067A mutation; row 4: *FUT3* with the T1067A mutation. Panel A: patient 6 is *FUT3* WT for both the mutations, in fact line 2 and line 4 do not show any band. Panel B: patient 3 is both heterozygous for *FUT3* T59G and T1067A mutations, in fact both line 2 and 4 show the bands. pWT (primer wild type) were used in row 1 and 3, while pMUT (primer mutation) were used in row 2 and 4.

4.4 DETECTION OF LEWIS ANTIGENS IN PANCREATIC SAMPLES BY IMMUNOFLUORESCENCE ON TISSUE SECTIONS

Expression of type 1 chain Lewis antigens on pancreatic tissue was assessed by IF. Normal and cancer tissue slices were evaluated by a pathologist according to the following criteria:

1. percentage of Lewis antigens diffusion in the tissue (score goes from 0% to 100%)
2. intensity of expression of Lewis antigens (score goes from 0 to 3)

It was not possible to investigate both the normal and matched tumoral part for all the patients due to lack of tissue, in fact the complete evaluation was possible only on 13 patients out of 18, while evaluation of normal part alone and tumoral part alone was conducted respectively on 3 patients and on 2 patients out of 18.

Taking into consideration only the 13 patients with matched normal and tumoral tissues, and comparing the intensity of expression and the diffusion in the parenchima and in the ducts (both in the membranes and in the secretum) of CA19.9, Lea, and Leb, it is possible to notice that generally there is a higher score of diffusion and intensity in the tumoral tissue for all the three Lewis antigens. In particular, patients with low grade diffusion of CA19.9 in normal pancreatic tissue are 10, while patients with high grade diffusion are 0; when it comes to the tumor, there are only 2 patients with low grade diffusion, while the patients with high grade diffusion increased from 0 to 9. Intensity of expression follows the same trend: several normal cases are score 1 and just one case is score 3, but when it comes to cancer the number of score 3 intensity increases to 7 (**figure 9**).

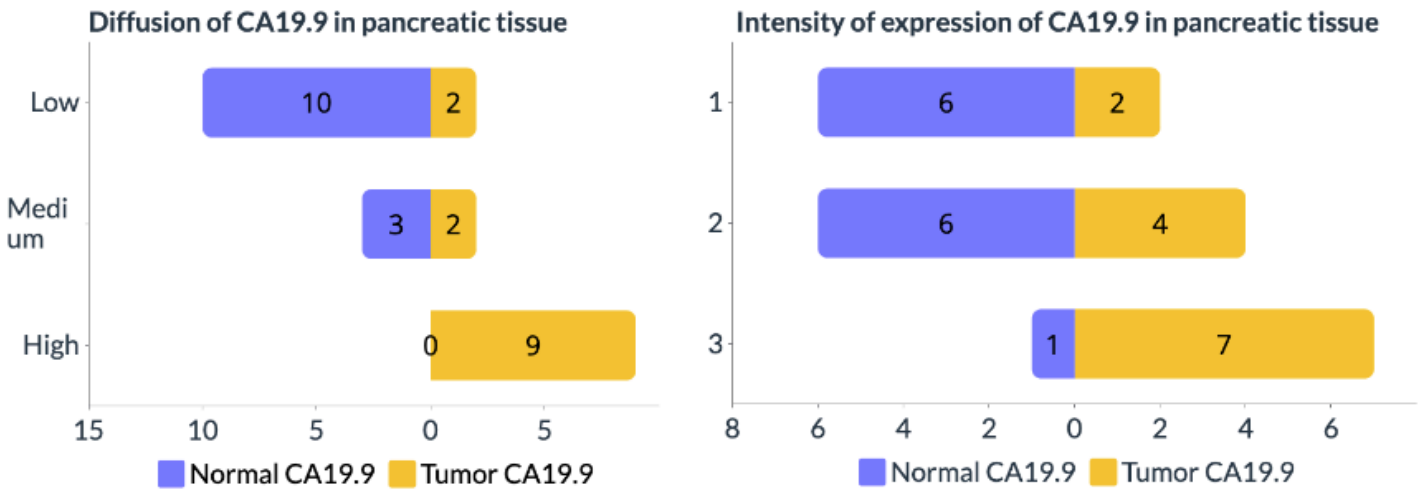


Figure 9. Grade of diffusion and the intensity of expression of CA19.9 in pancreatic tissue. Normal tissue of patients is depicted in purple, while PDAC tissue of patients is represented in yellow. The trend is similar for both the diffusion and the intensity of expression, in fact the number of patients with low grade/score 1 decrease in normal tissue and the number of patients with high grade/score 3 increases in PDAC tissue.

Figures 10 and 11 describe diffusion and intensity of the antigen, respectively Lea and Leb. As far as concerns Lea, the pattern of expression is very similar to the one of CA19.9 (several cases in which the normal tissue is low grade diffusion and score 1 intensity, and several cases in which the PDAC tissue is high grade diffusion and score 3 intensity).

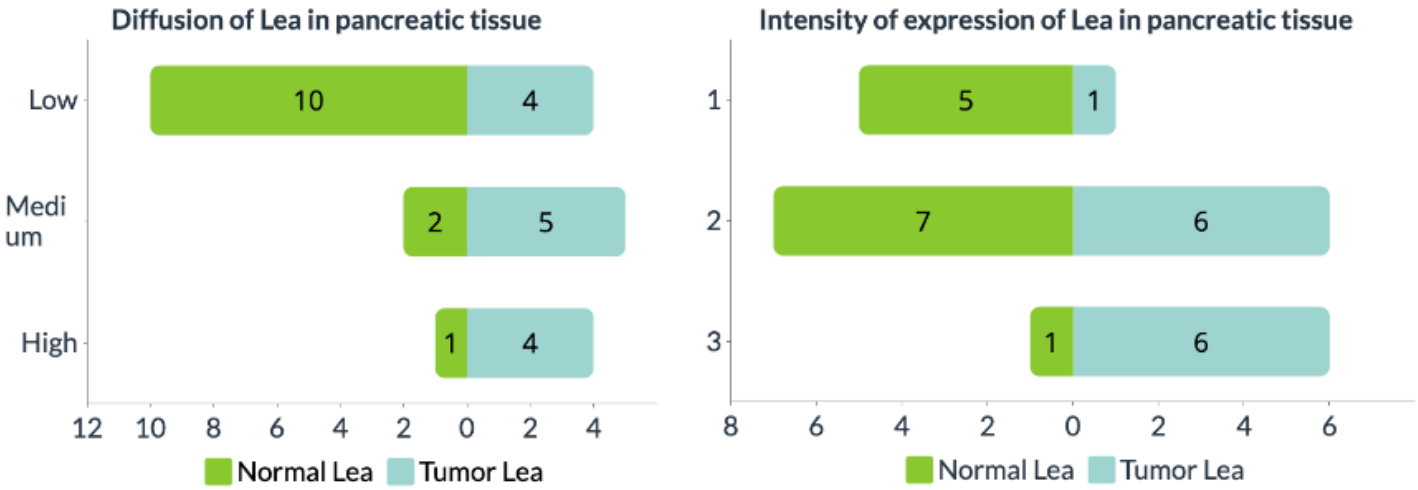


Figure 10. Grade of diffusion and the intensity of expression of Lea in pancreatic tissue. Normal tissue of patients is depicted in green, while PDAC tissue of patients is represented in light blue. Both the charts are very similar to the ones of CA19.9.

Leb presents some differences with respect to CA19.9 and Lea only for the intensity category, in fact it appears that there are a few numbers of normal tissues (n=4) with score 1, and consistent number of normal tissues (n=5) with score 3. In particular, the number of patients with score 1 decrease of just one patient, while the number of patients with score 3 remains stable when tumoral part is taken into consideration.

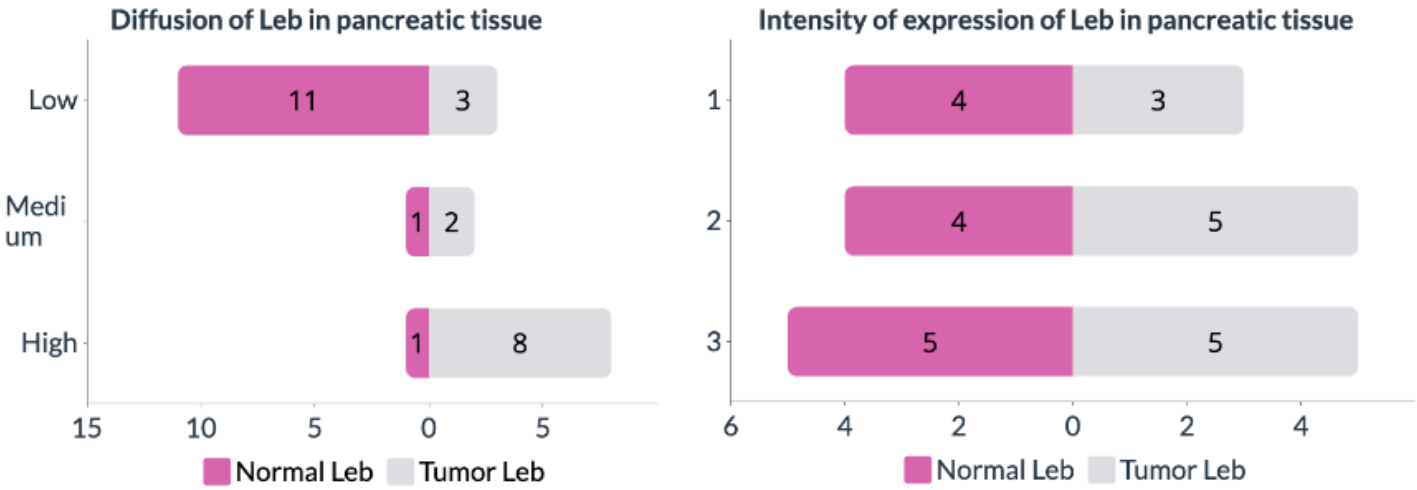


Figure 11. Grade of diffusion and the intensity of expression of Leb in pancreatic tissue. Normal tissue of patients is depicted in pink, while PDAC tissue of patients is represented in grey. Diffusion of Leb in the tissue is similar to the one of CA19.9 and Lea, while there is an evident difference when it comes to the intensity of expression. In fact, there is not a change in the number of patients that have score 3 in normal tissue and in tumoral tissue, and the same is basically valid also for score 1 and 2.

In the following figures (**figure 12 and 13**), normal and cancer tissues of a patient are taken as example. It is possible to notice that, as previously reported, CA19.9 and Lea behave similarly, in fact they are barely visible in the normal tissue. Instead, Leb is not visible in the parenchima, but it is recognizable in the ducts (both in the membranes and in the secretum).

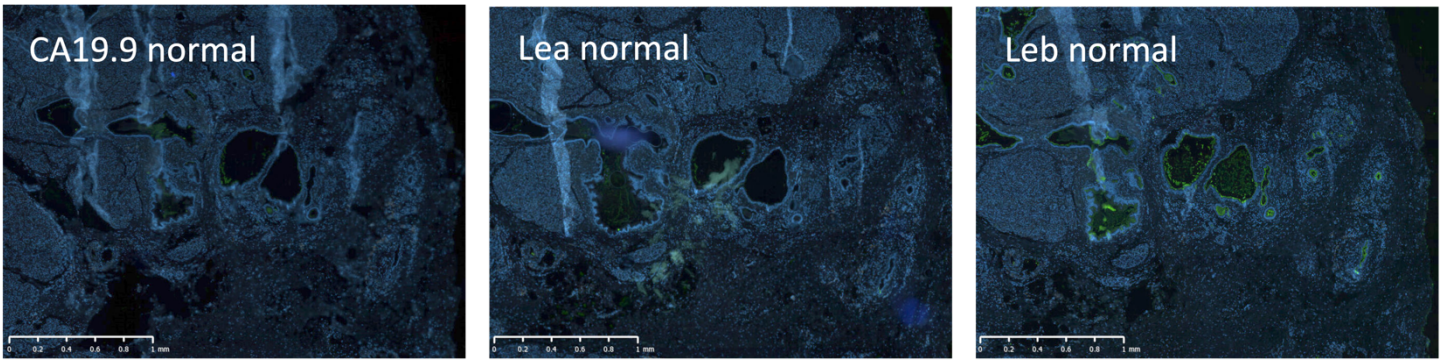


Figure 12. Detection of CA19.9, Lea and Leb in normal pancreas sections using anti-CA19.9 (ATCC HB-8059), anti-Lea (ATCC HB-8324) and anti-Leb (ATCC HB-8326) monoclonal antibodies. For IF detection, nuclei were blue-counterstained with DAPI as a reference. Scale bar is 1 mm. The pictures represent an example of the expression of the Lewis antigens in the normal tissue: CA19.9 and Lea are very poorly expressed, while Leb is expressed only in the pancreatic ducts (both in the membranes and in the secretum).

The tumoral counterpart of the same patient's tissue has been evaluated. The diffusion of all the three Lewis antigen is clearly visible in the parenchima and in the ducts, and also intensity of expression is intensified.

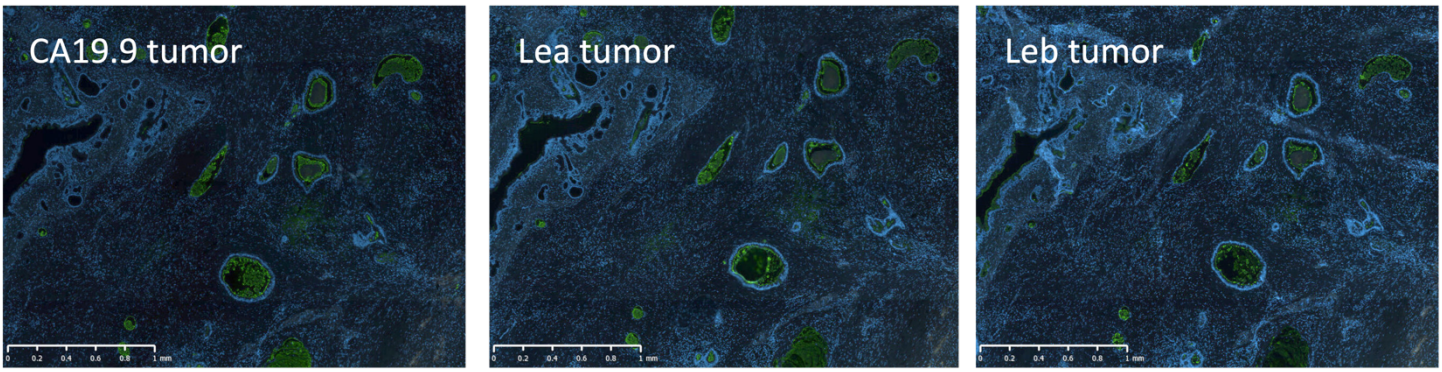


Figure 13. Detection of CA19.9, Lea and Leb in the tumoral counterpart of the same patient's tissue of figure 12. The Lewis antigens are now visible, in fact they are well distributed in the pancreatic parenchima and ducts. Scale bar is 1 mm.

Inversion of polarity of malignant ductal cells and obstruction of the ducts in PDAC were evaluated to determine if they could be part of the mechanism that allows the

increase of CA19.9 and, eventually, the other Lewis antigens in the serum. For each cancer tissue sample, about 20% of the ducts displayed at least inversion of polarity. The diffusion of the antigens in the external side of the ductal cells that spread in the parenchima is clearly visible, and eventually Lewis antigens are reabsorbed in the closest blood vessels. It is not possible to detect the obstruction of the ducts properly, but it is common knowledge that the majority of PDAC have this feature. In **figure 14**, some examples of normal and cancer ducts are depicted.

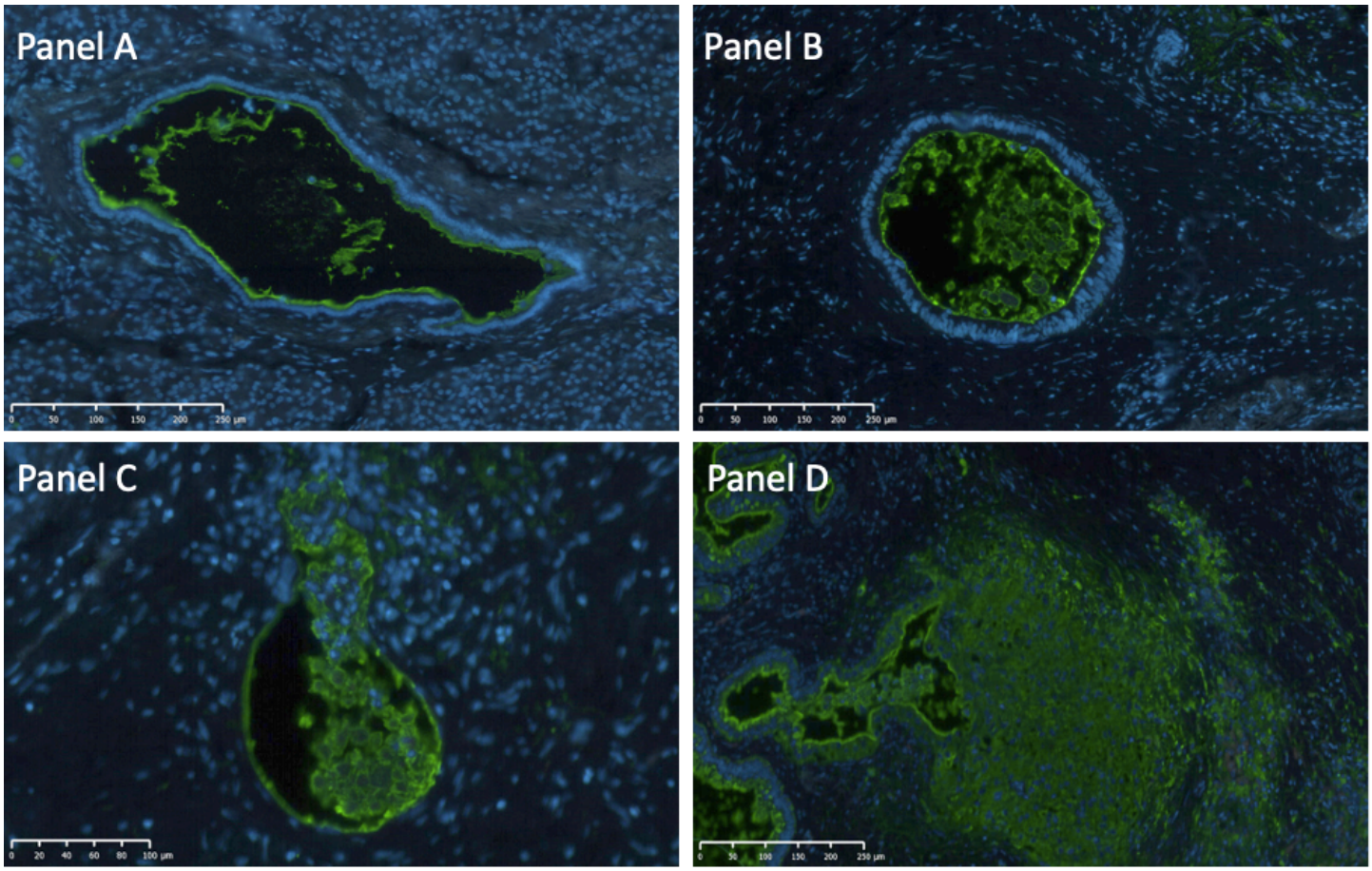


Figure 14. Examples of normal and tumoral ducts. Panel A: typical normal duct, with ductal cells in line and the antigen visible in the internal border of the duct and inside of it. Panel B: tumoral duct, with disorganized ductal cells and a higher volume of antigen inside the duct. Panel C and D: other examples of tumoral ducts, with inversion of polarity of the ductal cells and the consequent diffusion of the antigen in the parenchima.

4.5 CORRELATION BETWEEN TRANSCRIPTS EXPRESSION AND LEWIS ANTIGENS EXPRESSION IN CANCER TISSUES

Values of serum antigen were correlated with diffusion and intensity of expression of the antigens in cancer tissue (**figures 15A and 15B**), to see if high values in the serum correspond to high expression in the tissue, and viceversa. It appears that there is no specific correlation between CA19.9 sera values with intensity of expression of the antigen, but correlation between CA19.9 sera values and the diffusion of the antigen in cancer tissues appears proportional when it comes to high diffusion of the antigen in the tissue, in fact most of the positive patients (10 out of 15) have positive or bordeline sera values. Regarding Lea, both diffusion and intensity of expression of the antigen seem not to correlate with Lea sera values.

It will be necessary to expand the number of enrolled patients to confirm these data, since patients in our cohort are in a very limited number.

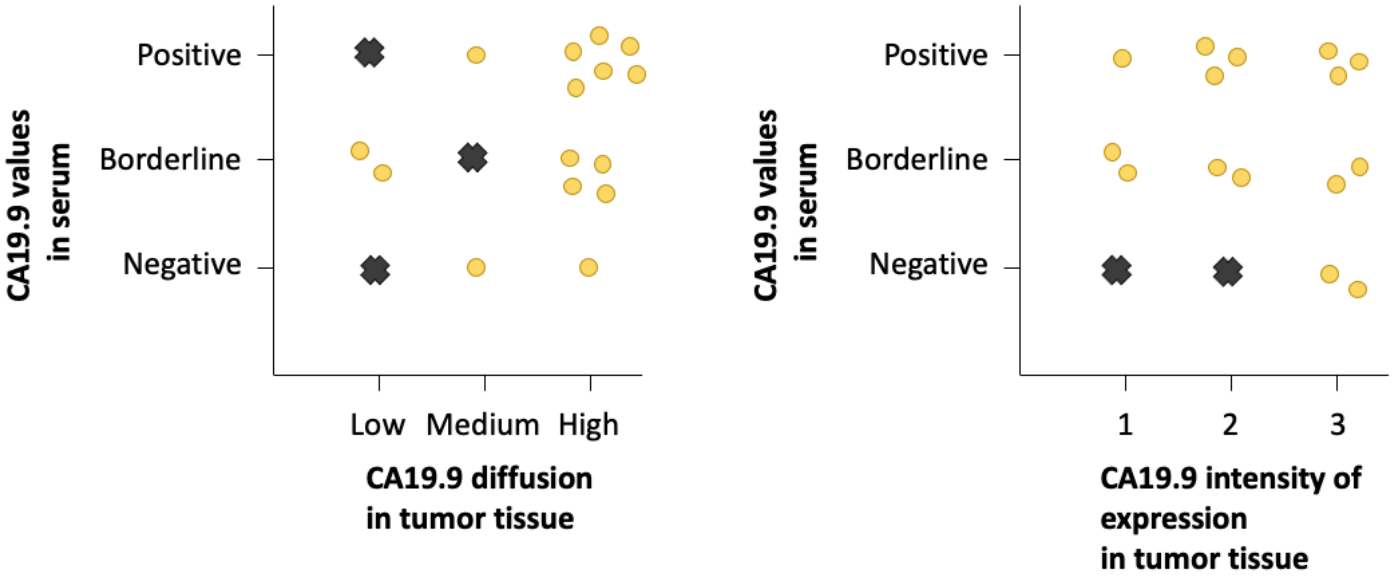


Figure 15A. Correlation between sera values of CA19.9 and diffusion/intensity of expression in the tissue. The graphic in the left represents the correlation between the sera values of CA19.9 and the diffusion of the antigen in cancer tissues, while the one in the right depicts the correlation between the sera values of CA19.9 and the intensity of expression of the antigen in cancer tissues. Each dot represents a patient, while black crosses represent missing values. It seems like the first association is proportional. The second association is quite randomic, and high sera values can be linked to grade 1 intensity of expression, and viceversa.

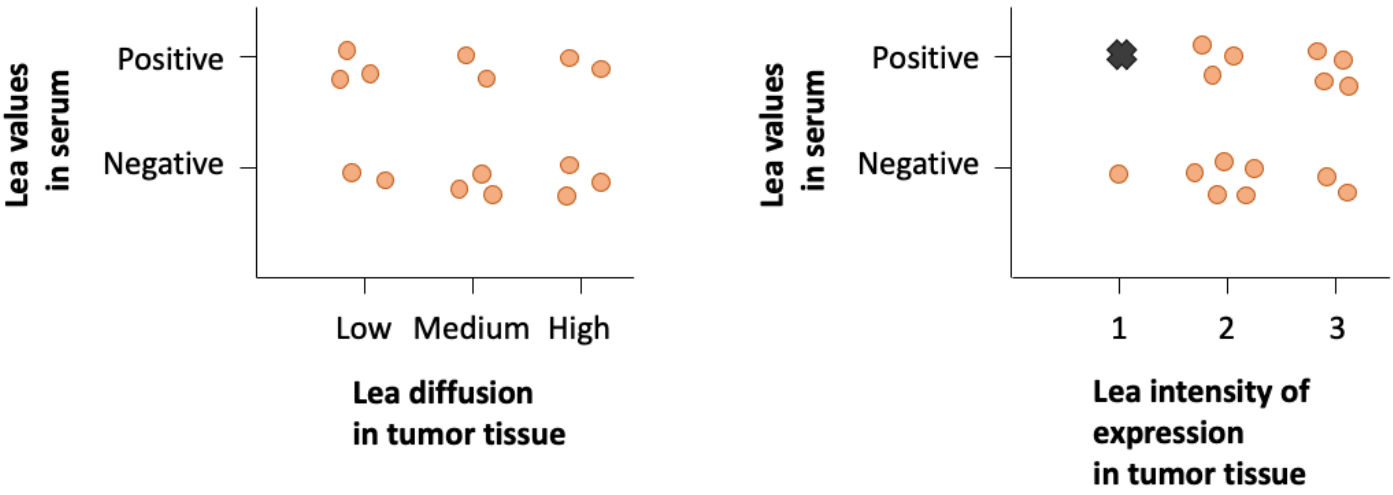


Figure 15B. Correlation between sera values of Lea and diffusion/intensity of expression in the tissue. As for the previous graphic, here it is depicted the correlation between the sera values of Lea and the diffusion of Lea in cancer tissues (left graphic), and correlation between the sera values of Lea and the intensity of expression of the antigen in cancer tissues (right graphic). Each dot represents a patient, while black crosses represent missing values. It appears that the association is quite randomic, and high sera values can be linked to low diffusion/grade 1 intensity, and viceversa.

5. DISCUSSION

Currently, management of PC patients and therapeutical decisions are based on the values of CA19.9, one of the most used biomarkers, belonging to the family of Lewis antigens. CA19.9 displays several limitations: the rate of false positive (10-30%) [36], the genetically negative patients (5-10%) [57], and the increase only when the tumor is at late stage (65% of cases) [18] contribute to the necessity to find new biomarkers, able to support CA19.9. Cognate Lewis antigens (Lea and Leb) have never been taken into consideration, even if no specific association exists between PC and CA19.9 with respect to Lea or Leb, because each one can be reabsorbed in the blood, according to their specific and individual expression [79].

Evaluation of serological levels of Lewis antigens was carried out with ELISA and it proved that Lea could be a valid ally of CA19.9 in the management of PDAC patients, whose serological levels of CA19.9 remain negative or slightly positive. In fact, the majority of negative controls (44/52, 84,6%) were placed under the arbitrary cut-off value of 37 U/ml, while a substantial difference can be seen in PDAC patients, where 55/118 (46,4%) resulted negative or borderline and 63/118 (53,4%) resulted positive.

Therefore, 14 patients over 118 (12%) could have been monitored with Lea instead of CA19.9 when CA19.9 displayed a negative value, or together with CA19.9 when CA19.9 displayed a borderline value. Further research on a wider number of patients and additional clinical studies (i.e. difference between Lea values before and after surgery, correlation with therapeutical approaches) need to be carried out to confirm this finding.

Genotyping of *FUT2* and *FUT3* assessed the gene status of the patients. *FUT2* status could affect the level of the Leb transcript, while *FUT3* status could affect the levels of all three Lewis antigens. In our cohort, 10 patients out of 18 resulted with a heterozygous mutation on *FUT2*, and one patient with a homozygous mutation. It is

not possible to compare the *FUT2* genetic status with serological value of Leb due to the ongoing processing of samples, but assessment of Leb on pancreatic tissues showed that no antigen is present in the homozygous patient's tissue, while a discrete variety of tissue values (low, medium, high diffusion/intensity of the antigen) can be observed in the heterozygous patients, exactly like WT patients, without any specific statistical correlation.

3 patients out of 18 have overlapping heterozygous mutation of *FUT2* and heterozygous mutations on *FUT3*. It is quite common that *FUT3* exhibits two mutations on the same allele, and this is confirmed by the presence of the Lewis antigens in the pancreatic tissue of all the patients who display these mutations. As for *FUT2*, also *FUT3* gene status includes low, medium, and high values of Lewis antigens, without any specific correlation between patients with WT and mutated *FUT3*. This could mean that one functioning allele of these two genes is more than sufficient to synthesizes the Lewis antigens in variable amount, according to specific and individual gene expression.

qPCR experiments were performed on glycogenes known to be involved in the type 1 chain Lewis antigens biosynthesis. The most relevant and necessary are B3GALT5, ST3GAL3, FUT2, and FUT3, while all the others contribute to a less extent. In particular, FUT1 can direct the α-2 fucosylation on type 1 chain Lewis antigens [82], and ST3GAL4/6 are the candidates that, more likely, can add the sialic acid to Galβ1-3GlcNAc. In fact, they act preferentially on the type 2 (Galβ1-4GlcNAc) disaccharide sequence [83], and our recent study demonstrated that other ST3GALs must flank ST3GAL3 in the biosynthesis of type 1 chain Lewis antigens, since CA19.9 was found in the sera of two patients with a nonsense variant of ST3GAL3 [84]. qPCR results suggested that the expression levels of glycosyltransferases do not affect the amounts of the Lewis antigens detected in the pancreatic tissue of PDAC patients, in fact most of the glycogenes (FUT1, FUT2, B3GALT5, ST3GAL4, ST3GAL6) do not show any

statistically significant variation in their expression between normal and cancer tissues. FUT3 and ST3GAL3 are statistically significant downregulated in the tumoral tissue, but this do not correspond to a minor distribution of the Lewis antigens, that present an increased diffusion and intensity instead. Also ST6GALNAC6, responsible for the attachment of an extra sialic acid residue on sLea and the development of dsLea, resulted downregulated. This could favor the accumulation of sLea instead of dsLea in the tumoral tissue, but research of dsLea on cancer tissues needs to be carried out in order to confirm the data.

IF of CA19.9, Lea and Leb on 18 PDAC tissue sections and their normal counterparts suggests that in the normal tissue they are all poorly expressed, but, among them, Leb is the most visible in the pancreatic ducts, in both the membranes and the secretum. For all the three Lewis antigens there is a higher score of diffusion in the tumoral tissue, and intensity of expression follows the same trend. Previous results [79] found out that the expression pattern of Lewis antigens was maintained in cancer in two PDAC patients, in accordance with the theory that speculate that only if the normal pancreatic ducts express and secrete high levels of CA19.9, upon malignant transformation does the bloodstream reabsorb large amounts of CA19.9. Rather, if the normal ducts mainly express and secrete Leb, or Lea, but low levels of CA19.9, upon malignant transformation the bloodstream cannot reabsorb relevant amounts of CA19.9, but probably does reabsorb Leb or Lea instead [34]. Instead, in our study there is no such association, i.e. if the normal tissue of a patient expresses more Lea with respect to Leb and CA19.9, not necessarily Lea would be the most expressed antigen in the tumoral counterpart. It does not seem to be a rationale behind this, neither a way to predict which antigen will be mostly expressed upon malignant transformation. The mechanism leading to an increase of the antigens expressed in the tumoral tissue is largely unknown at present, but we speculate that the inversion of polarity of the malignant ductal cells and the obstruction of the ducts could lead to

the more intense diffusion of the antigens in the tissue and the consequent reabsorption of the antigens in the bloodstream. To confirm this hypothesis, it would be necessary to evaluate a higher number of samples.

Some other variable must contribute to the increase of Lewis antigens in the bloodstream during carcinogenesis, since tissue antigens levels poorly correlate with serum antigens levels. One hypothesis is related to the type of Lewis antigens carrier molecules: sandwich ELISA is sensitive to high MW proteins carrying multiple epitopes (as mucins), but not to smaller antigenically monovalent proteins, which are instead better detected by dot-blotting [84], [85], and not to glycosphingolipids, detectable with mass spectrometry. The role of carrier molecules deserves further investigations.

6. CONCLUSIONS

Epidemiologic studies reveal that pancreatic tumor is projected to be the second-leading cause of cancer death by 2030 in the US [5]. A better management of this disease is impelling, since even if diagnostic and therapeutic approaches improved in these recent years, a substantial number of patients remain excluded from an appropriate management. This thesis aims to prove that alternative biomarkers can flank the well-known CA19.9 in this task.

Our data suggest that serum determination of cognate type 1 chain Lewis antigen, that is Lea, is able to better monitor 12% of PDAC patients, that resulted CA19.9-negative or borderline, in fact it could be an alternative marker in patients that are CA19.9 negative, and a complementary marker in patients that are CA19.9 borderline. Experimental work on an additional Lewis antigen, that is Leb, is still in progress.

Genotyping of *FUT2* and *FUT3* showed that the levels of the Lewis antigens in the tissue are comparable between patients with WT and variant (inactive) fucosyltransferases. In fact, it appears that one functioning allele is more than sufficient to synthesizes the Lewis antigens in variable amount, according to individual gene expression. In our cohort, just one patient carried a homozygous mutation on *FUT2* gene, and, as a matter of fact, Leb was not detectable in the tissue.

Similarly, qPCR experiments suggested that the expression levels of glycosyltransferases do not affect the amounts of the Lewis antigens detected in the pancreatic tissue of PDAC patients, in fact, even if ST3GAL3 and FUT3 transcripts resulted downregulated in PDAC tissue, all three Lewis antigens are generally more diffuse and more intense with respect to the normal counterpart.

IF of CA19.9, Lea and Leb on 18 PDAC tissue sections and their normal counterparts showed that Lewis antigens are all poorly expressed in the normal tissue and there is

an apparently randomic increase in the tumoral counterpart, that is, not related to the level of gene transcripts involved in their biosynthesis and to the *FUT2-3* gene status. Factors that can contribute to a higher diffusion and intensity in the tumoral tissue and to a higher levels of Lewis antigens in the bloodstream are the inversion of polarity of malignant ductal cells and obstruction of ducts, but this cannot be the only explanation, since most of the ducts do not display any of these features. It is probable that other factors contribute to this difference, for example the carrier molecules: generally, ELISA is just able to detect high MW proteins carrying multiple epitopes (as mucins), but not smaller antigenically monovalent proteins and glycosphingolipids.

Further investigations, in a wider cohort of patients, should be carried out, as well as clinical investigations to confirm the employment of Lea as serum marker.

The journey for a biomarker from bench to clinic is long and arduous and there remains many obstacles to overcome, but it seems like these studies are on the right path to the employment of new biomarker that can help CA19.9 in the management of PDAC patients.

7. SCIENTIFIC PRODUCTION

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Indellicato R., Trinchera M. Epigenetic regulation of glycosylation. *Adv Exp Med Biol*. 2021; 1325:173-186. Doi: 10.1007/978-3-030-70115-4_8

Indellicato R., Trinchera M. Epigenetic regulation of glycosylation in cancer and other diseases. *Int J Mol Sci.* 2021 mar 15;22(6):2980. Doi: 10.3390/ijms22062980

8. SCIENTIFIC ACTIVITIES

- Tutorato nell'ambito del Corso di Studio di Scienze e Sicurezza Chimica-Tossicologiche dell'Ambiente: 31 ore di attività nell'ambito dell'insegnamento di Microbiologia e Igiene (from 3/6/2020 to 12/6/2020)

- Tutorato nell'ambito del Corso di Studio di Safety Assessment of Xenobiotics and Biotechnological Products: 20 ore di attività nell'ambito dell'insegnamento di Environmental Microbiology and Biotechnological Remediation (from 8/5/2020 to 26/5/2020)

- Partecipazione al "Corso introduttivo alla sperimentazione animale" organizzato dall'Università degli Studi di Milano (from 16/9/2019 to 19/9/2019)

9. OTHER ACTIVITIES

- Maternity leave from 15/12/2020 to 29/9/2021
- Maternity leave from 29/8/2022 to 29/1/2023

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