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Carbohydrate antigens Lewis a and Lewis b act as tumor markers cooperating with CA19.9 in the management of PDAC patients

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ABSTRACT

Background: CA19.9 is the unique marker recommended for the preoperative staging and the follow-up of patients suffering from pancreatic ductal adenocarcinoma (PDAC) but up to 30% of PDAC patients maintain normal CA19.9 values and cannot be monitored in this way. Lewis a (Lea Galβ1,3[Fucα1,4]GlcNAc) and b (Leb, Fucα1,2Galβ1,3[Fucα1,4]GlcNAc) are antigens which are structurally similar to sialyl-Lewis a (Siaα2,3Galβ1,3[Fucα1,4]GlcNAc), the epitope of CA19.9.

Methods: We set an ELISA procedure determining the levels of Lea, Leb, and CA19.9 in the blood of healthy individuals or PDAC patients. Moreover, such antigens were also detected in cancer resections by immunofluorescence microscopy, and the levels of glycosyltransferase transcripts involved in Lewis antigen biosynthesis were determined by RT-qPCR.

Results: In our cohort of 116 healthy individuals, the distribution of circulating Lea and Leb was similar to that of CA19.9, allowing us to set putative cutoff values for both antigens. In a cohort of 115 PDAC patients, the differential distribution with respect to the controls was statistically significant for both antigens ($p < 0.001$). Out of 37 patients presenting normal CA19.9 values, 15 patients presented Lea or Leb above the cutoffs. By immunofluorescence, Lea, Leb and CA19.9 were all detected in cancer resections and expression levels were heterogeneous among patients in terms of intensity, localization and diffusion. The levels of relevant glycosyltransferase transcripts were found to be heterogeneous between cancers of different patients and no association was detectable with the levels of any circulating antigen.

Abbreviations: B3GALT5, β1,3 galactosyltransferase enzyme 5; CA19.9, carbohydrate antigen 19.9; dsLea, disialyl-Lewis a (Siaα2,3Galβ1,3[Fucα1,4][Siaα2,6]GlcNAc); ELISA, enzyme linked immunosorbent assay; Fuc, fucose; FUT1, GDP-Fuc to Gal α1,2 fucosyltransferase enzyme 1; FUT2, GDP-Fuc to Gal α1,2 fucosyltransferase enzyme 2; FUT3, GDP-Fuc to GlcNAc α1,3/4 fucosyltransferase enzyme 3; FUT5, GDP-Fuc to GlcNAc α1,3/4 fucosyltransferase enzyme 5; Gal, galactose; GalNAc, N-acetylgalactosamine; GAPDH, glyceraldeyde-3-phosphate dehydrogenase; GlcNAc, N-acetylglucosamine; HE, Hematoxylin-Eosin; IF, immunofluorescence; Lea, Lewis a: Galβ1,3[Fucα1,4]GlcNAc; Leb, Lewis b: Fucα1,2Galβ1,3[Fucα1,4]GlcNAc; PDAC, pancreatic ductal adenocarcinoma; RT-qPCR, reverse transcription quantitative polymerase chain reaction; sLea, sialyl-Lewis a: Siaα2,3Galβ1,3[Fucα1,4]GlcNAc; ST3GAL3, CMP-Sia to Gal α2,3 sialyltransferase enzyme 3; ST3GAL4, CMP-Sia to Gal α2,3 sialyltransferase enzyme 4; ST3GAL6, CMP-Sia to Gal α2,3 sialyltransferase enzyme 6; ST6GALNAC6, CMP-Sia to HexNAc α2,6 sialyltransferase enzyme 6.

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Conclusions: The concurrent quantification of Lea and Leb together with CA19.9 improves the management of PDAC patients.

1. Background

CA19.9 is one of the putative cancer antigens that is more abundantly quantified worldwide when suspecting gastrointestinal malignancies [1]. The applications which are supported by published data and recommended by medical associations are limited to the preoperative staging and follow-up of patients suffering from pancreatic ductal adenocarcinoma (PDAC) [2–4]. It is not sensitive or specific enough for diagnostic purposes, although it is frequently elevated in patients suffering from gastrointestinal malignancies and is widely used as a reference marker in research studies addressing novel putative markers [5,6]. The biological basis underlying CA19.9 elevation in cancer has not been clearly established. Experimental data in cancer cell lines and immunochemistry detection in tissue specimens from gastrointestinal tissues led to the hypothesis that the carbohydrate epitope of CA19.9, sialyl-Lewis a (sLea, Siaα2,3Galβ1,3[Fucα1,4]GlcNAc), is poorly synthesized in normal tissues but is synthesized in cancer due to the deranged expression of the glycosyltransferases involved in the biosynthesis (Fig. 1) [7]. In contrast, the analogue carbohydrate antigenic structures Lea, Leb, and dsLea could be typical of normal tissue and not relevant in cancer [8,9]. Consequently, the presence of circulating Lea and Leb has never been evaluated in cancer patients, nor in healthy individuals.

Our previous studies did not fit with such model, suggesting to evaluate the presence and potential clinical significance of circulating Lea and Leb in PDAC patients [10,11]. To this purpose, we set an ELISA procedure and determined the amounts of both antigens in the blood of healthy individuals or PDAC patients, in comparison with that of CA19.9. We also quantitated the transcript levels of relevant glycosyltransferases involved in Lewis antigen biosynthesis by RT-qPCR performed on surgical resections from some of the patients. Sections of the same surgical specimens were also prepared to evaluate the expression of Lea, Leb and CA19.9 by IF using the corresponding antibodies.

2. Methods

2.1. Case selection and sample collection

Patients with a histological diagnosis of PDAC that underwent surgery were included (Supplementary Table S1). Briefly, 115 cases diagnosed in between 2012 and 2021 were studied, including 59 females and 56 males; the average age was 69 and the youngest and oldest cases were 40 and 82 years old, respectively. Sixteen patients were diagnosed and treated at San Paolo University Hospital (Milan, Italy), 92 were diagnosed and treated at Humanitas Research Hospital (Rozzano, Italy) and the other 7 were diagnosed at local centers nationwide and treated at the Humanitas Hospital. Total bilirubin values, potentially affecting CA19.9 levels [12], were > 3.0 mg/ml in seventeen patients, >1.5 and < 3.01 in 13 patients and < 1.5 mg/ml in 69 patients; bilirubin levels were not available for 16 patients. Thirty-one surgery specimens were available for RNA extraction, 15 collected by the Biological Resource Center at Humanitas Research Hospital and 16 at San Paolo University Hospital. Twenty-three specimens were available for glass slide preparation, 9 from Humanitas hospital and 14 from San Paolo University hospital, as prepared for routinary pathology in both cases. Aliquots of serum samples collected for routinary diagnostic purposes were frozen and maintained at −20 °C. Healthy control sera were collected from volunteers available at the institutions participating in the study.

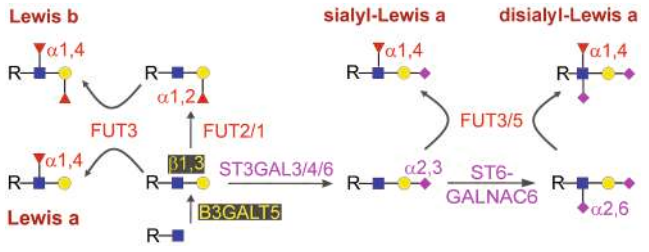

Fig. 1. Structure and biosynthesis of type 1 chain Lewis antigens. Monosaccharides are depicted according to the current representation: Blue square, GlcNAc, N-acetylglucosamine; yellow circle, Gal, galactose; Red triangle, Fuc, fucose; Pink diamond, sialic acid, Sia. Anomers, linkage positions, and enzymes involved in the reactions are indicated. Glycosyltransferases are named according to the HUGO recommendations.

2.2. Sandwich enzyme linked immunosorbent assay (ELISA) on patient sera

The detection of Lewis antigens in the serum was performed by a sandwich ELISA in 96 well plates (F8 Maxisorp Immuno-module, Nunc 469949). 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 Reagent for ELISA (BB) (1111258900,1 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 for 2.5 h at RT with 0.1 ml of a solution containing various volumes (2–10 μl) of serum diluted with BB. Serial dilutions of the spent media of COLO-205 cells, (1:2 to 1:1024 with the same original media) were used as calibrators; BB and regular media were used as negative controls. Standard calibrators available for CA19.9 (CanAg CA19-9 EIA, Fujirebio) were used according to the manufacturer's recommendations. Plates were washed 3 times with PBS-T (phosphate buffered saline containing 0.1 % Tween-20) and incubated with peroxidase-labeled secondary antibodies diluted with BB, either anti-CA19.9 (1:10,000), or anti-Lea antibody (1:20,000), 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) for 5–20 min at RT and stopped with 0.1 ml 1 N HCl. The resultant colors were evaluated in a microtiter plate reader at 450 nm. Peroxidase-labeled antibodies for detection were prepared from antibodies which were double-purified by protein-A Sepharose chromatography, 2.5 mg/ml, and using Lightning-Link® HRP Conjugation Kit (Innova Biosciences), according to the manufacturer's instructions. Anti-CA19.9 (hybridoma HB-8059, ATCC), anti-Lea (hybridoma HB-8324, ATCC) and anti-Leb (hybridoma HB-8326, ATCC) 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, as previously reported [11,13].

2.3. RNA extraction and quantitative polymerase chain reaction

Specimens obtained upon surgery from 31 PDAC patients were snap-frozen and maintained under liquid nitrogen vapors. Frozen aliquots, about 80 mm^3, were homogenized in 0.25 ml Lysis Buffer (ReliaPrep RNA miniprep system, Promega) using a tissue lyser (TissueLyser LT, ID 85600 Qiagen) at 50o/s (Hz) for 3 min and centrifuged at 12,000 g for 1 min at RT. Supernatant was recovered and processed according to the manufacturer's protocol, including DNAse treatment. Elution was performed using 30 μl of nuclease free water heated at 70 °C.

First strand cDNA was synthesized from 1 to 4 μg of total RNA in a 20

µl reaction as reported [10]. Control reactions were prepared by omitting the reverse transcriptase. cDNAs (0.2–1.0 µl of first strand reactions) were amplified in a volume of 20 µl using Sybr TB Green Premix Ex Taq II (Tli RNase H Plus, ROX plus, RR82WR Takara) as reported [10]. Primer sequences are listed in Supplementary Table S2. The amounts of amplified target cDNAs were calculated as ΔCt with respect to GAPDH and expressed as $2^{-\Delta ct}$.

2.4. IF microscopy

IF was performed on 23 formalin-fixed paraffin-embedded 4 µm thick serial PDAC tissue sections obtained during surgery. 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; and anti-Leb 1 µg/ml; in addition, anti-dsLea (clone FH7, MAB10210 Merck) was used at a dilution of 1:200. Slides were incubated overnight at 4 °C. Polyclonal Rabbit Anti-Mouse Immunoglobulins/FITC (Dako, dilution 1:100) was used as a fluorophore-conjugated secondary antibody. Nuclei were subsequently counterstained with Hoechst 33,342 (Invitrogen). Serial dilutions of primary antibodies were tested on positive and negative controls to assess the effect of antibody concentrations. Fluorescence images were digitalized using NanoZoomer 2.0 (Hamamatsu Photonics, Japan).

2.5. Genomic DNA (gDNA) extraction

Frozen specimens of PDAC resections (4–6 mg) 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 and the obtained gDNA quantified by NanoDrop Spectrophotometer (Thermo Scientific).

2.6. Polymerase chain reaction (PCR) for genotyping FUT2 and FUT3

The two most common mutations on *FUT2* (A385T and G428A) and *FUT3* (T59G and T1067A) were investigated by PCR on gDNA [14,15]. For each specimen, 8 reactions are carried out with different primers pairs assembled as follows.

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. Sequences of the primers used are reported in supplementary Table S4. For each reaction, gDNAs (50 ng) were amplified in a volume of 25 µl using GoTaq G2 Flexi DNA polymerase (Promega) according to the manufacturer' protocol. Actin primers were present in each reaction as internal quality control. Amplification programs are summarized in supplementary Table S5

2.7. Statistical analysis

Univariate and descriptive statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc, La Jolla, California, USA) and the nonparametric Mann-Whitney test. In all tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Detection and quantification of circulating Lewis antigens

The normal range of circulating CA19.9 is commonly considered below 37 U/ml, while no information nor reference standard is available for Lea and Leb. We used the culture media of COLO-205 cells as a reference because we found that it contains adequate amounts of all three antigens. Various serial dilutions of the media provided a linear response by ELISA for each antigen (Supplementary Figure S1) and we defined 1 arbitrary unit (aU) of concentration the absorbance value obtained at a dilution of 1:512 of the culture medium. In the case of CA19.9, comparing COLO-205 media with a commercial reference standard, we found that 1 aU corresponds to 7.5 standard units (U), indicating that the concentration of CA19.9 in that media was about 4000 U/ml. In our cohort of 116 healthy individuals, 103 (89 %) presented CA19.9 values $\leq$ 37 U/ml, as expected, 10 presented values just above 37 U/ml and only three individuals presented values 2–4 times above the cutoff. In the case of Lea and Leb, we found a very similar distribution of values through the healthy population (Fig. 2, panel A). As a result, we assumed the putative cutoff values for Lea and Leb to be 11 and 123 aU/ml, respectively, corresponding to the same percentile as 37 U/ml for CA19.9. Of note, 9–10 individuals exceeded such values minimally and 3–4 individuals only presented values 3–4 times over their putative cutoffs, very similar to CA19.9.

In the cohort of 115 PDAC patients (Fig. 2, panel B), CA19.9 was over 37 U/ml in 78 cases (68 %), Lea was over 11 aU/ml in 41 cases (35.6 %) and Leb was over 123 aU/ml in 39 case (34 %); the differential distribution with respect to the controls was statistically significant for all three antigens (CA19.9 $p < 0.0001$, Lea and Leb both $p < 0.001$). Interestingly, out of 37 patients presenting with CA19.9 $\leq$ 37 U/ml, 15 patients (40 %) presented Lea > 11 aU/ml or Leb > 125 aU/ml and two presented both Lea and Leb over the putative cutoff values (Fig. 2, panel C). Out of 78 patients with CA19.9 > 37 U/ml, 47 (60 %) presented Lea or Leb over their cutoff values. In particular, 33 presented the overexpression of circulating Lea, 30 overexpressed circulating Leb and 16 showed the overexpression of both. The remaining cases (31 patients) presented elevated CA19.9 alone.

To assess whether circulating molecules carry multiple Lewis antigens, sandwich ELISA was also performed using different antibodies for coating the plates and detecting the antigens. In the sera where amounts of multiple Lewis antigens are easily detectable, an antigen could be detected after coating the plate with an antibody against the other antigens. However, the fractions recovered with respect to coatings with the same antibody as for detection were very variable, spanning from less than 10 % to almost 100 % (Fig. 2, panel D). This suggests that carrier molecules frequently bear more than one Lewis antigen, but relevant amounts of one antigen are carried alone in some patients.

The levels of CA19.9 are affected by the genomic status of patients, due to the presence of null FUT3 and FUT2 alleles in the population [16,17]. Bi-allelic inactivating variants of FUT3 in particular make Lewis antigens undetectable. In our population, we found single Lewis antigens below the detection limit of our assay in various patients, but only five patients presented with all three antigens at undetectable levels. We were able to assess the genomic status of such patients and found no biallelic variants of frequent FUT3 mutations; one patient was heterozygous. We assessed the genomic status of another 11 patients and found two of them to be heterozygous for inactivating FUT3 alleles, while heterozygous inactivating FUT2 alleles were more frequent (9 out of 16).

3.2. IF detection of Lewis antigens in PDAC resections

Expression of the three Lewis antigens was also determined by IF on 23 cancer resections, representing patients with heterogeneous serum levels of the antigens. Lea and Leb were detected in cancer resections, as

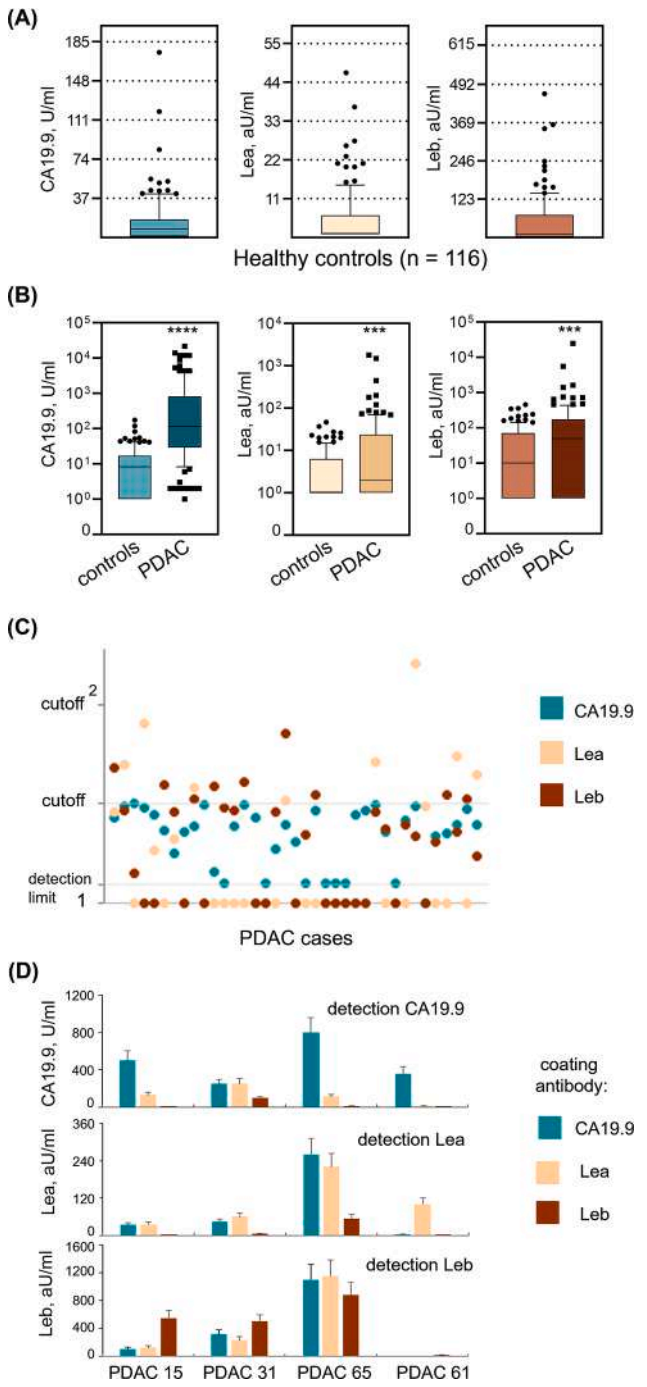

Fig. 2. Detection of circulating Lewis antigens. (A) CA19.9, Lea and Leb were quantified by sandwich ELISA in the blood of healthy controls. Putative cutoffs for Lea and Leb were tentatively set at 11 (Lea) and 123 (Leb) arbitrary units (aU) per ml following the distribution of CA19.9 levels in the same healthy population (cutoff 37 U/ml, 89th centile). (B) The three antigens were also quantified in PDAC patients and the obtained values compared with those of panel A. Statistical differences between healthy controls and PDAC patients were evaluated using the Mann-Whitney test. (C) The levels of Lea and Leb antigens in the individual blood of 37 PDAC patients presenting normal CA19.9 values are reported as the fold increase in the relative cutoff values, expressed in a logarithmic scale. (D) In patients showing elevation of multiple Lewis antigens, the levels of each antigen were determined using the proper detection antibody and the three different coating antibodies. Four representative PDAC cases were shown. Values are the mean ± standard deviations of three independent assays.

well as CA19.9, but expression levels and patterns were extremely heterogeneous between patients in terms of intensity, localization and diffusion. In the areas maintaining ductal-shaped structure, ductal epithelium is frequently stained. CA19.9 and Lea antibodies frequently stain the same structures, while Leb stains others. Apycal localization in polarized cells is typical, but reversed polarity or diffuse localization are also frequent. Serum levels of each antigen seem not to be correlated with the levels or predominance of staining in tissue. Relevant examples are shown in Fig. 3, the corresponding serum values of the antigens are reported in Table 1.

We have assessed the expression of dsLea in PDAC patients and found that it strictly overlaps with that of CA19.9. In some PDAC resections, including an adjacent normal pancreas, we have found that dsLea is actually expressed and predominates over CA19.9 in the well differentiated tissue (Supplementary Figure S2).

3.3. Expression levels in PDAC resections of transcripts coding glycosyltransferases involved in Lewis antigen biosynthesis

Expression levels of 9 glycosyltransferase transcripts potentially involved in Lewis antigen biosynthesis were determined in 31 PDAC resections from patients presenting heterogeneous levels of circulating antigens. Circulating CA19.9 was below 43 U/ml in 16 cases and ranged from 51 to 4400 U/ml in the remaining 15 patients. Lea was below 10 aU/ml in 16 cases and ranged from 22 to 1800 aU/ml in the others. Leb was below 90 aU/ml in 19 cases and ranged from 147 to 720 aU/ml in the other 12. The levels of glycosyltransferase transcripts were heterogeneous between cancers of different patients (Fig. 4, panel A) and no association was detectable with the levels of any circulating antigen (Fig. 4, panel B). Significant associations were found between various glycosyltransferase transcripts: the strongest between FUT3 and ST3GAL3 and then between B3GALT5 and FUT2, ST3GAL3 and ST6GALNAC6, FUT3 and ST6GALNAC6.

4. Discussion

We have found that the carbohydrate epitopes Lea and Leb are tumor markers which are able to complement CA19.9 in the management of PDAC patients.

Our data indicate that the serum levels of Lea and Leb circulating in PDAC patients are significantly higher than in healthy individuals. Although CA19.9 is more sensitive, about 40 % of those presenting normal values of CA19.9 are instead positive for Lea or Leb, presenting values over the putative cutoff. Consequently, a relevant number of PDAC patients that cannot be staged and monitored by CA19.9, could be managed through Lea and Leb. In our first approach, we set the cutoff at the same percentile as CA19.9 and we have found that a sandwich ELISA can detect an antigen after coating the plate with an antibody directed against one of the other antigens in some patients, suggesting that Lea and Leb are frequently carried in the bloodstream by the same molecules carrying CA19.9.

Using IF microscopy, we also found that Lea and Leb are commonly expressed in surgical resections from PDAC patients, as is CA19.9, confirming that PDAC produces all three antigens, presumably decorating the same glycoproteins. At this regard, circulating Lewis antigens [18,19] as well as those secreted in the culture media of cancer cell lines [20,21], are reported to be mainly carried by high molecular weight glycoprotein (mucins), which are well detected by sandwich ELISA, requiring multivalent antigens. Those expressed in tissues and cell lines include lower molecular weight glycoproteins that are poorly detected by ELISA but instead by dot-blot or even western-blot [10]. dsLea is also expressed by PDAC, without relevant regulation with respect to the adjacent normal pancreas.

Tissue and serum expression does not seem to be strictly related to any antigen. Moreover, in cancer resections, the levels of glycosyltransferase transcripts involved in Lewis antigen biosynthesis are highly

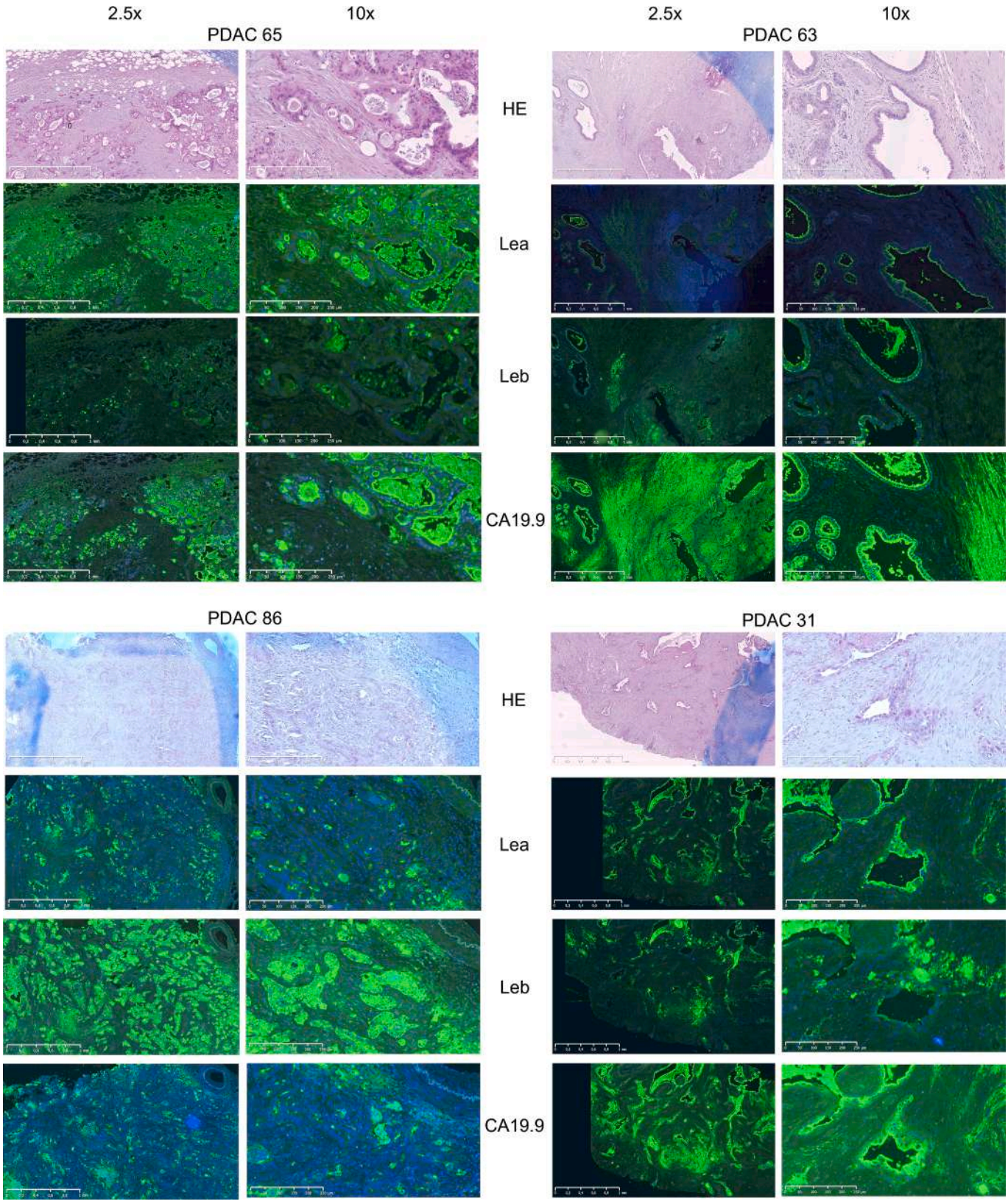

Fig. 3. Detection of CA19.9, Lea and Leb on PDAC resections by immunofluorescence microscopy. Paraffin-embedded serial slices of PDAC resections were deparaffinized and each one treated with Hematoxylin-Eosin (HE) or with antibodies directed to the three antigens, followed by FITC-labeled secondary antibody, as detailed under "Materials and Methods". Nuclei were counterstained with Hoechst 33,342 as a reference. Images were taken at different magnifications to show the staining, even at the cellular level. Four representative PDAC cases were shown. Supplementary Table 3 reports the levels of the same antigens measured in the blood of such patients. Scale bars are 1 mm (2.5x magnification) or 250 μm (10x magnification).

Table 1
Levels of Lewis antigens circulating in the blood of the PDAC patients whose resections were analyzed by IF microscopy in Fig. 2.

	Lea aU/ml (aU/ml x cutoff^{-1})	Leb aU/ml (aU/ml x cutoff^{-1})	CA19.9 U/ml (U/ml x cutoff^{-1})
PDAC 65	180 (16.3)	720 (5.8)	1068 (28.8)
PDAC 86	50, (4.5)	90 (0.72)	35 (0.94)
PDAC 31	80 (7.3)	405 (3.3)	189 (5.1)
PDAC 63	87 (7.9)	650 (5.2)	4384 (118)

heterogeneous and not correlated with the levels of antigens, neither circulating nor expressed in the tissue. Taken together, our data suggest that Lea, Leb and CA19.9 undergo a similar cancer-associated mechanism of resorption in the bloodstream which is not related to the amounts of antigens synthesized in the tissue, nor to the levels of glycosyltransferase transcripts, suggesting that Lewis antigen accumulation and resorption in the blood is not simply a matter of glycosylation, but instead of the more complex derangement of mucin traffic. In this regard, Lea and Leb share the same limits and merits as CA19.9 and should be used in a complementary manner. The automated procedure for CA19.9 determination currently available could be extended to Lea and Leb through potentially easy adaptation because of several similarities. Both anti-Lea and anti-Leb antibodies are mouse monoclonal IgG, as is anti-CA19.9, both bind antigens carried by the same molecules carrying CA19.9, both react efficiently in a sandwich ELISA requiring the same volume of serum or plasma as anti-CA19.9, as reported by one of the most commonly used automation system (Roche Cobas Elecsys, https://elabdoc-prod.roche.com/eLD/api/downloads/97be00ff-6a2d-ef11-2491-005056a772fd?countryIsoCode=XG). Once automated, the parallel determination of Lea, Leb and CA19.9 could be performed routinely. In our PDAC population, 22 cases present normal values for all three antigens in a way that is not due to the most frequent recessive inactivating FUT3 variants. Measurement of the three antigens together in large cohorts of PDAC patients has the potential to improve the management of PDAC patients and to shed light on the putative clinical significance of their concurrent, partial or absent elevation.

5. Ethics statement

Ethical approval was obtained from the San Paolo Hospital, Milano, Italy, Ethics Committee (Protocol 1564/2018) and Humanitas Research

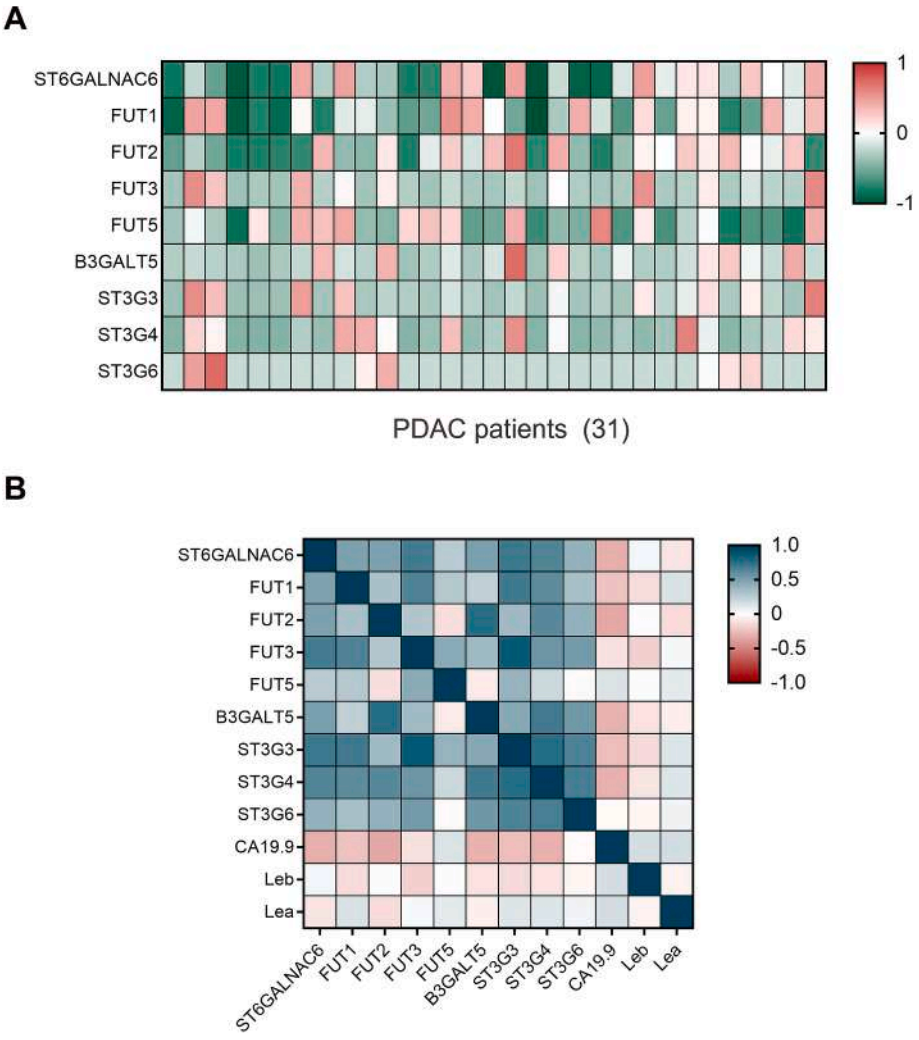

Fig. 4. Expression of transcripts coding glycosyltransferases involved in Lewis antigen biosynthesis in PDAC resections. Total RNA was extracted from 31 PDAC patients expressing heterogeneous levels of circulating Lewis antigens, reverse-transcribed and submitted to quantitative PCR using the specific primer pairs reported in Supplementary Table S2. (A) The levels of each transcript were measured as $2^{-\Delta Ct}$ with respect to GAPDH. The obtained values were presented as a z-score for each patient in a heatmap. (B) The same values were plotted one against the other and against the levels of the corresponding antigens circulating in the blood of the same patients. The results are presented as a heatmap showing the Pearson correlation coefficient.

Hospital, Ethics Committee (Protocol 953/20). All participants provided written informed consent.

CRediT authorship contribution statement

Rossella Indellicato: Writing – original draft, Investigation, Data curation, Conceptualization. **Michele Dei Cas:** Writing – review & editing, Formal analysis, Data curation. **Aida Zulueta:** Investigation. **Anna Caretti:** Writing – review & editing, Investigation. **Delfina Tosi:** Visualization, Investigation. **Claudia Cigala:** Resources. **Gaetano Bulfamante:** Project administration. **Enrico De Nicola:** Resources. **Giovanna Scifo:** Resources. **Enrico Opocher:** Resources. **Daniela Pistillo:** Project administration. **Gennaro Nappo:** Resources, Data curation. **Alessandro Zerbi:** Conceptualization. **Marco Trinchera:** Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Serum and resection specimens collected from patients undergoing surgery at Humanitas Hospital were collected through the Biological Resource Center at Humanitas Research Hospital.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.119990.

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