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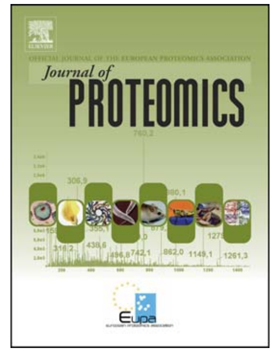

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Increased α1-3 fucosylation of α-1-acid glycoprotein (AGP) in pancreatic cancer

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Abbreviations: AAL, *Aleuria aurantia* lectin; AGP, α-1-acid glycoprotein; ChrP, chronic pancreatitis; ELLA, Enzyme-linked lectin assay; HC, healthy control; PDAC, pancreatic ductal adenocarcinoma; PhoSL, *Pholiota squarrosa* lectin; μZIC-HILIC-ESI-MS, zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry.

Keywords: α-1-acid glycoprotein; capillary electrophoresis; fucosylation; *Aleuria aurantia* lectin (AAL); mass spectrometry; pancreatic cancer.

Abstract:

Pancreatic cancer (PDAC) lacks reliable diagnostic biomarkers and the search for new biomarkers represents an important challenge. Previous results looking at a small cohort of patients showed an increase in α-1-acid glycoprotein (AGP) fucosylation in advanced PDAC using *N*-glycan sequencing. Here, we have analysed AGP glycoforms in a larger cohort using several analytical techniques including mass spectrometry (MS), capillary zone electrophoresis (CZE) and enzyme-linked lectin assays (ELLAs) for determining AGP glycoforms which could be PDAC associated. AGP from 31 serum samples, including healthy controls (HC), chronic pancreatitis (ChrP) and PDAC patients, was purified by immunoaffinity chromatography. Stable isotope labelling of AGP released *N*-glycans and their analysis by zwitterionic hydrophilic interaction capillary liquid chromatography electrospray MS (µZIC-HILIC-ESI-MS) showed an increase in AGP fucosylated glycoforms in PDAC compared to ChrP and HC. By CZE-UV analysis, relative concentrations of some of the AGP isoforms were found significantly different comparing PDAC and HC. Finally, ELLAs using *Aleuria aurantia* lectin displayed a significant increase in AGP fucosylation, before and after AGP neuraminidase treatment, in advanced PDAC compared to ChrP and HC, respectively. Altogether, these results indicate that α1-3 fucosylated glycoforms of AGP are increased in PDAC and could be potentially regarded as a PDAC biomarker.

Biological significance:

This work consists of a multidisciplinary approach that provides information on α-1-acid glycoprotein (AGP) glycosylation changes in disease state. The study has been performed using mass spectrometry, capillary zone electrophoresis and enzyme-linked lectin (ELLA) assay using *Aleuria aurantia* lectin. An increase of fucosylated AGP in pancreatic cancer patients' sera is described. ELLA would allow investigating in larger cohorts of patients the usefulness of α1-3 fucosylated AGP as a pancreatic cancer biomarker.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in Europe and United States [1]. Its low survival rate (7% in 5 years) is due to the intrinsic aggressiveness of this tumour and also to a late diagnosis, usually when metastasis has occurred [1, 2].

The only available biomarker for PDAC is CA19-9, but this marker is currently only used for monitoring disease progression because of its false positive rate [3]. Thus, it is important to search for new biomarkers able to differentiate PDAC from other benign diseases such as chronic pancreatitis (ChrP) which can also present high CA19-9 levels [4, 5].

Glycosylation is a post-translational modification present in very many proteins, and alterations in protein glycosylation are a key feature of malignant transformation and tumour progression [6, 7]. Those glycan alterations that are cancer associated usually affect the terminal structures of membrane-associated or secreted glycoproteins [8]. In this regard, glycomics and glycoproteomics have emerged as important tools in new biomarkers discovery [9, 10]. Previously, our group performed *N*-glycan sequencing of major serum acute phase proteins, including AGP, in a small cohort of serum samples from healthy controls and PDAC patients [11]. α-1-Acid glycoprotein (AGP), or orosomucoid, is a 41-43 kDa glycoprotein with 5 complex-type *N*-linked glycans resulting in a carbohydrate content of 45% of its molecular mass [12]. *N*-glycan analysis showed different bi-, tri- and tetraantennary sialylated structures, including several sialofucosylated ones [11, 13, 14]. Interestingly, a minor biantennary, disialylated, core fucosylated glycan of AGP was found specifically increased in advanced PDAC [11].

Recently, we have also analysed the relative levels of AGP-released glycan species of eight pathological samples (two ChrP and six PDAC) vs. a control pool. This was done by stable isotope labelling of the released glycans, separation of the various glycan species by zwitterionic hydrophilic interaction capillary liquid chromatography (µZIC-HILIC) and quantitation by electrospray mass spectrometry (ESI-MS). These previously obtained preliminary results showed an increase of several AGP fucosylated glycans in PDAC at advanced stages [15].

In the present work we have analysed the AGP glycosylation from serum samples in a larger cohort of individuals (N=31) using different complementary analytical approaches to explore the potential value of AGP glycoforms as PDAC biomarker. This study is further aimed at establishing an enzyme linked lectin assay (ELLA) as a quantitative method that could be easily transferred into clinics.

Within this paper, we have performed a relative quantification of the released AGP *N*-glycans using stable isotope labelling and subsequent µZIC-HILIC-MS. Further, AGP isoforms of the intact glycoprotein were analysed by capillary zone electrophoresis with UV detection (CZE-UV) and the different peaks, related with different carbohydrate moieties attached, were quantified and compared among groups. Finally, ELLAs using two different lectins specifically recognizing fucose were developed for determining differences in AGP glycoforms among groups of disease. These methods showed specific differences in AGP glycosylation comparing PDAC patients' serum with that of healthy controls and ChrP patients. In particular, significant increases in the **α1-3** fucosylation levels were found in the AGP from PDAC patients.

2. Materials and Methods

2.1 Serum samples

Serum samples were obtained from the Hospital Dr. Josep Trueta (Girona, Spain) following the standard operating procedures of its Ethics Committee. Samples corresponded to six healthy controls (two females and four males; age range 57-66), six chronic pancreatitis patients (three females and three males; age range 49-65), and nineteen pancreatic cancer patients (eight females and eleven males; age range 45-73; out of the cancer patients six were non-advanced stages, stage IB (T2N0M0), stage IIA (T3N0M0) and stage IIB (T1-2-3N1M0), and thirteen advanced stages, stage III (T4NxM0) and stage IV (T3NxM1) (Table 1). Patients were diagnosed by biopsy or image examination by the Digestive and Pathology Units.

2.2 Immunoaffinity column preparation

The immunoaffinity chromatography (IAC) column was made as previously described [16]. Briefly, Protein-Pak epoxy-silica of 40 µm particle size and 500 Å pore diameter (Millipore, Waters Chromatography Division, Milford, MA) was converted into diol-silica and oxidized to aldehyde-silica. The antibody goat anti-human AGP (Immune Systems Ltd, Paignton, Devon, UK) was transferred to coupling buffer (0.1 M sodium phosphate pH 5.7) and concentrated using centrifugal filter devices (Centricon 50, Amicon, Beverly, MA). The coupling was made by mixing the concentrated antibody and sodium cyanoborohydride with the supporting material for 65 h at 4 ºC. The unbound aldehyde groups were reduced with sodium borohydride. The antibody-derivatized Protein-Pak material in phosphate buffered saline (PBS) was packed into the column by the slurry method. A 3 cm PEEK-lined stainless steel tubing of 4.6 mm I.D. provided with 1/4 to 1/16 reduction end fittings and with 2 µm pore diameter frits were used as the immunoaffinity column hardware (Grace Davison Discover Sciences, Deerfield, IL).

2.3 AGP immunopurification from serum samples

To purify AGP from sera, a previously developed method [16] with slight modifications was applied as follows. 100 µL of each serum sample were incubated for 30 minutes with 1% (v/v) of protease Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO). Then 99 µL of water were added and the sera were pre-cleaned using a 0.22 µm Spin-X Centrifuge Tube Filter (Costar, Corning, NY) according to manufacturer's protocols.

The immunopurification with the column described above was performed in an Äkta-FPLC system (GE Healthcare, Buckinghamshire, United Kingdom). PBS was used as mobile phase at 0.5 mL/min flow rate, and 0.1 M glycine/HCl pH 2.2 was used as desorption buffer. The sample (200 µL) was injected and elution with desorption buffer was carried out for 3 min at 0.5 mL/min (after this time, the absorbance signal returned to the baseline level). The eluted sample (about 1.4 mL) was collected and neutralised by the addition of 0.1 M Na_2HPO_4. This neutralised fraction was re-injected in the IAC column in order to properly purify AGP [17]. Three further desorption steps were performed but only the two firsts elution fractions were collected and neutralised. Afterwards, each sample was concentrated and desalted using a Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA) in order to avoid any undesired interference of salt ions in the following procedures. A blank cycle was performed prior to each serum sample immunopurification run.

2.4 Protein quantification and purity determination

Protein concentration in each sample was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and using the extinction coefficient $E^{1\%} = 8.93$ at 278 nm. AGP purity after isolation from serum was confirmed for each sample by loading an aliquot of 100 ng of protein under reducing conditions on a 10% SDS-PAGE with detection by silver-staining.

2.5 *N*-glycan analysis by zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (µZIC-HILIC- ESI- MS)

Relative quantitation of AGP glycosylation variants by stable isotope coded labelling of AGP released *N*-glycans was carried out using $^{12}C_6/^{13}C_6$ aniline and µZIC-HILIC-ESI-MS as previously described [18]. Briefly, purified AGP was reduced and subjected to enzymatic digestion by PNGase F. The released *N*-glycans were purified by solid phase extraction using Hypercarb cartridges (Thermo Fisher Scientific, Waltham, MA). The dried glycans were differently labelled with 0.35 M aniline and precipitated. For their chromatographic separation, a 1200 series capillary liquid chromatography system (Agilent Technologies, Waldbronn, Germany) was employed, using a capillary column (150 mm x 0.33 mm) filled with 5 µm particles with surface-immobilized zwitterionic sulfobetaine moieties (SeQuant, Umeå, Sweden). This HPLC system was coupled to a 6220 oa-TOF LC/MS mass spectrometer (Agilent Technologies) [15].

2.6 Capillary zone electrophoresis

Capillary zone electrophoresis with UV-Vis detection (CZE-UV) was performed by an Agilent 7100 CE instrument (Waldbronn, Germany) using a detection wavelength of 214nm. Agilent ChemStation software was used to control the instrument and to acquire and process data. The uncoated fused silica capillaries used (78.5 cm total length, 70 cm effective length, 50 µm i.d., 375 µm o.d.) were supplied by CM Scientific Limited (Silsden, United Kingdom). Separation was performed employing a method previously developed [16, 19]. Shortly, purified AGP of each sample diluted all at the same concentration were injected at the anodic end of the capillary for 30 s at 35 mbar. The background electrolyte consisted on 10 mM tricine, 10 mM NaCl, 10 mM sodium acetate, 7 M urea and 3.9 mM putrescine, pH 4.5. Separation voltage and temperature were 25 kV and 35º C, respectively. Peaks were aligned and assigned by using the effective electrophoretic mobility (μ_{eff}) as migration parameter for each peak and by comparison with the aligned AGP profile of the standard (G9885, Sigma-Aldrich) solution analysed on the same day. Each peak was quantified as the percentage of its corrected area (A_{corr}), which is calculated by multiplying the area counts by the migration velocity. The data is given as the percentage of corrected area (A_{corr} of a given peak in relation to the sum of A_{corr} of all peaks of the sample).

2.7 Enzymatic removal of sialic acid from purified AGP

One microgram of purified AGP at 0.2 mg/mL of each sample was digested overnight at 37ºC with 0.025 mg/mL of *Arthrobacter ureafaciens* neuraminidase (Roche Diagnostics, Mannheim, Germany) in 50 mM sodium phosphate pH 5.

2.8 Enzyme-linked lectin assay (ELLA) of purified AGP with *Aleuria aurantia* lectin (AAL) and *Pholiota squarrosa* lectin (PhoSL)

General fucosylation levels of purified AGP before and after treatment with neuraminidase were quantified by direct ELLA using biotinylated AAL (Vector Laboratories, Burlingame, CA). Core fucosylation levels were specifically determined using biotinylated PhoSL (kindly provided by Dr. Yuka Kobayashi from J-Oil mills, Inc., Yokohama, Japan). The procedure was optimized and performed as follows. All incubation steps, except the initial plate coating step with purified AGP, were performed at room temperature. Plate coating was performed with pure AGP samples diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) and incubated for 1 h at 37ºC in 96-well plates (Thermo Fisher Scientific). The amounts of purified AGP used were established for each assay considering the LOQ, and the dynamic range (between 1.1 ng/µL and 9.4 ng/µL for AAL assay and between 0.6 ng/µL and 15 ng/µL for PhoSL assay). Thus, the fucosylation analyses prior and after neuraminidase treatment were carried out with 2 ng/µL and 1 ng/µL AGP, respectively; and 8 ng/µL and 4 ng/µL AGP were used for core fucosylation determination. After washing the plate three times with washing buffer (0.9% NaCl solution, 0.05% Tween 20), wells were blocked for 1 h with 2%

polyvinylpirrolidone (PVP) (Sigma-Aldrich) in phosphate buffered saline (PBS). Wells were then washed and incubated for 2 h with either biotin labelled AAL at 4 µg/mL or biotin labelled PhoSL at 1µg/mL in lectin buffer (150 mM NaCl, 0.1 M Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Plates were washed and incubated for 1 h with streptavidin-horseradish peroxidase-conjugated (GE Healthcare Bio-Sciences, Uppsala, Sweden) diluted 1/5000 in PBS, 0.1% Tween20, 1% BSA for the AAL assay and with the streptavidin-horseradish peroxidase conjugated from the Vectastain ABC kit (Vector Laboratories) diluted according to manufacturer's instructions for the PhoSL assay. Plates were washed and colorimetric detection was performed using 100 µL/well of BM blue Peroxidase substrate soluble (Roche Diagnostics, Mannheim, Germany). Absorbance was read at 450 nm with a reference of 690 nm in an automated microplate reader (BIO-TEK, Winooski, VT) after stopping the reaction with 100 µL/well of 1 M H_2SO_4. In order to quantify the different glycan levels, a calibration curve of standard AGP was used. For each plate, duplicates of each sample were performed. The assays were conducted by triplicate, giving a total of 6 data points per sample. Only data with a $CV_{intraassay}$ <10% and a $CV_{interassay}$ <20% were considered for calculation of the mean values for each sample.

2.9 Statistical analysis

Mean or median comparisons of the variables were performed using the SPSS statistical software for Windows (version 19; SPSS Inc., Chicago, IL). The variables of each group (HC, ChrP and PDAC) were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests. When these criteria were accomplished (capillary zone electrophoresis data) intergroup differences were analyzed by means of ANOVA with Tukey post-test. Contrary, for non-normally distributed groups (data from lectin assays) Kruskal-Wallis test was performed. For all the analyses, $p<0.05$ was considered statistically significant.

3. Results

3.1 AGP immunopurification

AGP from serum samples was purified using an immunoaffinity column [16, 20] in order to assure that the glycans analysed by the following approaches belonged only to AGP and could not come from other serum proteins. Silver staining of a SDS-PAGE of the purified AGP fractions collected from the immunoaffinity column confirmed AGP purity (Fig. 1).

3.2 N-glycan analysis by µZIC-HILIC-ESI-MS

Glycan analysis by mass spectrometry is a technique widely used to determine glycan structures. In this study, we applied our described methodology involving isotope-coded labelling for improved quantification. Technically, this approach ionizes two differently labelled samples at the same time and compares intensity differences in the obtained profile. In this way we could monitor changes in the glycosylation pattern of AGP in control vs. patient samples by direct comparison. The levels of *N*-glycan structures released from AGP isolated from 6 ChrP and 12 PDAC samples, comprising 6 non-advanced and 6 advanced PDAC, were determined in comparison to a pool of control (Fig. 2A). The pool of control was prepared by mixing the same amount of AGP purified from four healthy control (HC; n=4, HC1, HC3; HC5 and HC6, Table 1) sera, followed by the subsequent release of the *N*-glycans with PNGase F and labelling. HILIC-HPLC combined with ESI-MS provided information regarding the monosaccharide composition of each glycan species considered. Several bi-, tri- and tetraantennary sialylated and sialofucosylated *N*-glycans were identified; their occurrence in AGP had been previously described by other authors [12,15, 21-23]. Bi-, tri- and tetraantennary glycans with one sialic acid, however, were not detected using this approach. Among the structures identified (Table 2), the following sialofucosylated structures were found: a biantennary, disialylated structure with one fucose (2Ant2Neu5Ac1Fuc), a triantennary, disialylated structure with one fucose (3Ant2Neu5Ac1Fuc), a triantennary, trisialylated structure with one fucose (3Ant3Neu5Ac1Fuc) and two fucoses (3Ant3Neu5Ac2Fuc), a tetraantennary trisialylated structure with one fucose (4Ant3Neu5Ac1Fuc) and two fucoses (4Ant3Neu5Ac2Fuc) and a tetraantennary tetrasialylated structure with one fucose (4Ant4Neu5Ac1Fuc), two fucoses (4Ant4Neu5Ac2Fuc) and three fucoses

(4Ant4Neu5Ac3Fuc). The absolute levels of the biantennary structure (2Ant2Neu5Ac1Fuc) were found being very low, accounting for less than the 0.7% of all *N*-glycan structures. This is of interest, as this structure is considered – as will be discussed later – being the only one with core α1-6-fucosylation according to the literature [11]. The rest of sialofucosylated structures, which is considered containing exclusively external (i.e. antenna-linked) α1-3-fucoses, because only type II structures (Galβ1-4GlcNAc) has been described in AGP [11], accounted for 19-68% of *N*-glycan structures, with different percentages depending on the samples. Di- and tri-fucosylated structures on AGP had not been described before by this technology. However, they had been previously reported by other authors using different methodologies [11, 21]. They accounted for 2-31% of *N*-glycan structures also depending on the samples.

Using the µZIC-HILIC-ESI-MS approach, differences between PDAC and ChrP samples were found in the relative levels of the nine sialofucosylated glycan structures (Fig. 2B). ChrP samples showed lower levels of all sialofucosylated structures when compared to healthy control (with the exception of one sample, ChrP1). In contrast, PDAC samples contained higher levels of the sialofucosylated glycans than the control pool, with the exception of one non-advanced PDAC (PDAC2) (Fig. 2B). The profile of the nine sialofucosylated glycans was different among the PDAC samples, and these structures were higher abundant in most of the advanced cancers than in the control pool. Four and five of the sialofucosylated structures of PDAC5 and PDAC13, respectively, were equally or less abundant than in the control pool while the rest of the structures showed higher levels than in the control pool. For each sample the ratio sum of fucosylated/sum of non-fucosylated structures was calculated and an increasing trend in this ratio was observed in PDAC samples (Fig. 3).

In contrast, no differences among groups could be detected when comparing the content of sialic acids and the extent of branching of *N*-glycan structures (data not shown).

3.3 Capillary electrophoresis analysis of AGP

Capillary zone electrophoresis with UV detection was able to separate purified AGP from each of the 31 serum samples (6 HC, 6 ChrP, 19 PDAC) into 12 peaks corresponding to isoforms. This is shown in Fig. 4A for a typical sample of PDAC. Migration in CZE is related to the charge to size ratio of the molecules and, therefore, differences in AGP glycosylation between AGP isoforms which contribute to changes in size and/or charge can modify the AGP electropherogram [17]. From the twelve peaks obtained, peak 1 was omitted in the analysis because of its weak signal which made it undetectable in some of the samples. The corrected area percentage values of the eleven analysed peaks were calculated for each of the serum samples and statistically different profiles between HC and PDAC sera were found (Table 3 and Fig. 4B). In particular, a decrease in the peak areas of isoforms with lower charge/size ratio (the earlier migrating peaks 4, 5 and 6) concomitant to an increase of the areas for AGP isoforms with higher charge/size ratio (peaks 8, 9 and 10) were observed when comparing the PDAC samples with HC.

ChrP samples showed an intermediate percentage of peak areas, non-significantly different from PDAC or HC peak areas. Interestingly, the peak area distribution of ChrP1 sample was more similar to some PDAC samples than to any ChrP sample (data not shown), a similar behaviour already detected when the *N*-glycans from ChrP1 were analysed by μZIC-HILIC-ESI-MS.

3.4 Fucosylation levels of AGP using AAL and PhoSL lectins

Fucosylation levels of AGP were analysed with an enzyme-linked lectin assay (ELLA) using *Aleuria aurantia* lectin (AAL) which recognizes fucoses α1-2, α1-3 or α1-6-linked [7] and with *Pholiota squarrosa* lectin (PhoSL) which recognizes only α1-6-linked fucose [24]. Both assays were optimized with standard AGP previously purified by immunoaffinity chromatography, and the amount of AGP/well for each assay was established according to the sensitivity (based on LOQ and LOD) and dynamic range of the corresponding AAL and PhoSL ELLAs.

General fucosylation levels of the different AGP samples were determined using AAL. In this case, 2 ng/μL of purified AGP/well from the cohort of 6 HC, 6 ChrP and 19 PDAC patients' sera were used. The ratio of fucosylated AGP was calculated dividing the value of fucosylated AGP -obtained from the ELLA- by the amount of AGP loaded in the ELLA (Fig. 5A and Table 4). As shown in Fig. 5A, the group of advanced PDAC showed significantly higher AGP fucosylated ratio than the ChrP patients. The lower reactivity of the ChrP group to this lectin may indicate either a lower AGP fucosylation level or a higher masking effect on the fucose by the presence of other monosaccharides (such as sialic acid attached nearby an external fucose). To better elucidate this issue, we investigated whether or not the removal of sialic acid affects AAL recognition. For that, all AGP samples were treated with neuraminidase, and ELLA with AAL was performed using 1 ng/µL of digested AGP/well. Doing so, higher fucosylation levels were obtained with neuraminidase treated AGP samples (calculated according to a calibration curve with non-treated standard AGP). Statistical analysis of fucosylation levels measured with AAL after neuraminidase digestion showed again a fucosylation increase in PDAC samples compared with HC and ChrP samples. This increase was more pronounced with AGP from advanced PDAC patients leading to fucosylation levels significantly different from those observed in HC (Fig. 5B and Table 4). Thus, sialic acid hinders the recognition by AAL, being the ratio of fucosylated AGP detected with AAL after sialic acid digestion higher than before the removal of the sialic acid.

Interestingly, the samples that showed higher fucosylation ratios in the ELLA with intact AGP were the same that showed higher fucosylation ratios in the ELLA with AGP after neuraminidase digestion, and *vice versa*. This could indicate homogeneous sialylation levels among samples, which agrees with our previous results obtained by µZIC-HILIC-ESI-MS that did not show significant differences in the sialic acid content of the AGP *N*-glycan structures among groups. Interestingly, the extreme value in the ChrP group of the AGP neuraminidase digested samples corresponded to ChrP1, the same sample that showed higher fucosylation levels by µZIC-HILIC-ESI-MS and a more different profile among the ChrP samples when analysed by CZE-UV. Also, the samples PDAC2 and PDAC13, which showed low levels of fucosylated structure by µZIC-HILIC-ESI-MS, were the PDAC samples with a lower AAL signal after sialic acid digestion.

The measure of core fucosylation (α1-6-linked fucose) levels of AGP *N*-glycans that are contained in the minor biantenary disialylated structure was addressed by an ELLA with PhoSL. For that, 4 ng/µL and 8 ng/µL of purified AGP /well were used. No differences in AGP core fucosylation levels among the three different groups were detected (data not shown). Thus, the increased fucosylation levels of AGP associated to PDAC is attributed to α1-3-linked fucoses.

4. Discussion

Fucosylation is one of the most important oligosaccharide modifications involved in cancer and inflammation [25]. Different studies link the presence of fucosylated epitopes on specific glycoproteins with cancer. In particular, Breborowicz and collaborators [26] demonstrated that fucosylated alpha-fetoprotein is more specific as a hepatocellular carcinoma (HCC) biomarker than alpha-fetoprotein (AFP) and nowadays fucosylated AFP (AFP-L3) is used for HCC risk assessment [27]. Likewise, other authors reported that high levels of fucosylated haptoglobin were produced in the advanced stages of several types of cancer such as ovarian, lung, breast and also pancreatic cancer [28, 29]. In PDAC, Shimomura and co-workers described recently a lectin assay using PhoSL to analyse α1-6-fucosylated haptoglobin in serum. They concluded that the increase in the fucosylation levels of haptoglobin in PDAC was more related to fucose linked α1-3 than α1-6 [30]. Remarkably, these results are in agreement with the ones we describe here for AGP, suggesting an increase in α1-3 fucosylated acute phase proteins in PDAC.

AGP is a highly glycosylated protein with 5 *N*-glycosylation sites. It is known to contain bi-, tri-, and tetraantennary structures. Biantennary structures can contain one or two sialic acids and the disialylated biantennary structure can contain a fucose. Regarding the fucosylated structure 2Ant2Neu5Ac1Fuc, our group previously showed that the fucose of this minor structure was α1-6-linked (core fucose), while the rest of tri- and tetraantennary fucosylated structures contained only external fucoses α1-3-linked [11]. Both *N*-glycan sequencing [11] and mass spectrometry [15] showed that this fucosylated biantennary structure represents a low proportion among all AGP glycan structures. AGP triantennary structures can be di- or trisialylated and one or two external fucose linked α1-3 can be present. For tetraantennary glycans, AGP show many different structures with 2, 3 or 4 sialic acids. The trisialylated tetraantenary structures can present one or two external fucoses and the tetrasialylated forms can present 1, 2 or 3 external fucoses linked α1-3[11]. Our previous studies using a smaller cohort of patients indicated that an increase in monofucosylated glycans could be associated to PDAC [11, 15]. Here, using a larger cohort of patients and extending the analysis to di- and tri-fucosylated glycans, we have shown an increase in most of the fucosylated structures of AGP released *N*-glycans in PDAC compared to ChrP and HC using µZIC-HILIC-ESI-MS. These results have been confirmed using an AAL lectin assay that recognizes fucosylated structures including α1-3 and α1-6 linked ones. Advanced PDAC showed a significant increase in AGP fucosylation compared with HC detected by AAL after sialic acid removal. The same outcome was found for PDAC vs. ChrP patients when no removal of sialic acids was performed. In addition, it must be pointed out that, although not being statistically significant, AGP from non-advanced PDAC sera also showed an increase tendency in fucosylation compared to non-malignant situations. No differences between PDAC, ChrP and HC could be detected using the specific core fucose recognizing lectin PhoSL. Both methods, MS and ELLA, have provided complementary information on AGP glycans. While MS identified and quantified the fucosylated glycans of AGP by the type of branching and the content of sialic acid, ELLAs with different binding specificity quantified the AGP fucosylated glycans distinguishing between fucose linkage positions. Altogether both methods have converged in that structures containing α1-3 linked fucose were found increased in PDAC. Thus, a quantitative ELLA based on AAL is a potential tool to detect fucosylated AGP. This methodology could be used to validate the usefulness of fucosylated AGP as a PDAC biomarker in a larger cohort.

CZE-UV analysis of AGP isoforms showed a general increase of peaks with larger charge/size ratio for cancer samples compared to chronic pancreatitis and controls. The same trend had been found in the CZE-UV analysis of AGP samples from serum of bladder cancer patients compared to controls [31]. The CZE-MS analysis of glycans and of the intact AGP for the same samples showed an increased expression of AGP isoforms with fucosylated tri- and tetraantennary glycans, and a decrease on tri- and tetraantennary non-fucosylated AGP isoforms in bladder cancer patients when compared to healthy controls [32]. Though it is not clear whether different fucosylation of the AGP isoforms or rather different degrees of sialylation are the cause of the shift observed in the quantitative isoform pattern by CZE-UV (Fig. 4 B), this shift can be seen as an additional diagnostic tool distinguishing the PDAC group from ChrP and HC groups.

AGP, as well as alpha-fetoprotein and haptoglobin mentioned above, are acute phase proteins. Aberrantly glycosylated acute phase proteins may modulate macrophage activity in tumour microenvironment by interacting with its receptors and contribute to tumour cell survival, growth and metastasis [33]. Several studies propose AGP glycoforms for diagnosis and management of cancer.

Hashimoto and collaborators [34] showed by cross-affinity immunoelectrophoresis (CAIE) using AAL and concanavalin A that cancer patients who had fucosylated tri- and tetraantennary AGP for long periods after surgery had poor prognosis. Also, Mackiewicz and Mackiewicz [35] described a decrease in concanavalin-A reactivity of serum AGP from cancers originated in the liver, pancreas or bile duct. By contrast, these changes were not detected in metastatic liver tumours. AGP has also been analysed as a biomarker for breast cancer but the studies reveal that, although increased levels of AGP can be detected in plasma of patients with breast cancer, the concentration is unrelated to disease progression. However, monosaccharide composition and oligosaccharide profiles of the AGP glycosylation were found to differ between normal, non-invasive and invasive groups of breast cancer patients [36]. Likewise, Croce and collaborators reported a correlation between AGP and sLe^x expression in colorectal cancer tissues [37]. Also, specific changes in both expression and glycosylation of genetic variants of AGP in human malignant mesothelioma have been found [38]. Most of the methods reported in the aforementioned studies allow only a semi-quantification of the different AGP glycoforms. Here, we have used quantitative methods to analyse the N-glycans of purified AGP and have described fucosylation changes therein by mass spectrometry and ELLA. In order to avoid potential unspecific binding of other major serum fucosylated proteins that could produce unspecific signal in sandwich assays, here we have used immunoaffinity purified AGP for the accurate quantification of fucosylated AGP in the ELLA with AAL.

The mechanisms of post-translational modifications of AGP during diseases are still unknown. During last phase of acute inflammation, high levels of AGP are found in plasma but its glycan branching degree corresponded to that observed in non-inflamed status. Hence, different pathways regulating the expression of the protein on one hand and the modification of the oligosaccharide structures attached on the other hand may probably exist [21]. Fucosylation involves the action of fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporter [39]. The reason for the increased fucosylation of AGP in serum of patients with pancreatic cancer could be explained by different mechanisms, which are not exclusive one from the others. Although AGP is produced mainly by the liver, there are studies reporting AGP expression in other tissues as well [21, 37]. Also, there is the possibility of PDAC cells secreting factors that could induce the production of fucosylated AGP by the liver. This hypothesis has already been described by Okuyama and collaborators for another acute-phase protein, haptoglobin. They detected haptoglobin mRNA in a pancreatic adenocarcinoma cell line (PSN-1) and also described an increased production of fucosylated haptoglobin by hepatic cells (Hep3B) after stimulation with PSN-1 conditioned medium [40]. Moreover, inflammatory cytokines, such as interleukin-1β, have been reported to increase the secretion levels of AGP by HuH-7 hepatocyte cell line; and also to alter AGP sugar chains expression resulting in higher binding affinities to AAL, *Sambucus sieboldiana* agglutinin and concanavalin-A lectins [41]. In addition, the stimulation of HuH-7 cells with interleukin-1β induces an enhanced expression of the fucosyltransferase gene 6 (*FUT6*), which could explain the increase in α1-3 fucosylated structures [42]. In this regard, we have also described higher expression levels of *FUT3* and *FUT6* in PDAC tissues compared to normal tissues, which may account for the increase of α1-3 fucosylated structures in PDAC [43]. Discovering the mechanisms that produce these changes in AGP fucosylation in PDAC could help to develop novel targeted cancer therapies.

5. Conclusions

We have identified increased fucosylation levels of serum AGP in pancreatic cancer compared to healthy controls and chronic pancreatitis patients. *N*-glycan analysis showed an increase of the AGP sialofucosylated glycan structures in PDAC samples. We could be able to distinguish between HC and PDAC patient groups by capillary zone electrophoresis of AGP isoforms or by ELLA with AAL of neuraminidase digested AGP. We could also differentiate ChrP and PDAC patients by ELLA with AAL of purified AGP. These results suggest that AGP fucosylation levels could be useful as a PDAC marker, what should be confirmed in a larger cohort study in combination with the measure of other potential biomarkers. The different approaches used in this work show the suitability and complementarity of these techniques for the discovery of new potential biomarkers based on altered glycosylation of serum proteins.

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References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65:5-29.
[2] Freelove R, Walling AD. Pancreatic cancer: diagnosis and management. Am Fam Physician. 2006;73:485-92.
[3] Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011;378:607-20.
[4] Duffy MJ, Sturgeon C, Lamerz R, Haglund C, Holubec VL, Klapdor R, et al. Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report. Ann Oncol. 2010;21:441-7.
[5] Korkmaz M, Unal H, Selcuk H, Yilmaz U. Extraordinarily elevated serum levels of CA 19-9 and rapid decrease after successful therapy: a case report and review of literature. Turk J Gastroenterol. 2010;21:461-3.
[6] Stowell SR, Ju T, Cummings RD. Protein glycosylation in cancer. Annu Rev Pathol. 2015;10:473-510.
[7] Varki A KR, Toole BP. Glycosylation Changes in Cancer. Essentials of Glycobiology. In: Varki A CR, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editor. 2nd ed. New Yord: Cold Spring Harbour Laboratory Press; 2009.
[8] Christiansen MN, Chik J, Lee L, Anugraham M, Abrahams JL, Packer NH. Cell surface protein glycosylation in cancer. Proteomics. 2014;14:525-46.
[9] Peracaula R, Barrabes S, Sarrats A, Rudd PM, de Llorens R. Altered glycosylation in tumours focused to cancer diagnosis. Dis Markers. 2008;25:207-18.
[10] Pan S, Chen R, Aebersold R, Brentnall TA. Mass spectrometry based glycoproteomics--from a proteomics perspective. Mol Cell Proteomics. 2011;10:R110 003251.
[11] Sarrats A, Saldova R, Pla E, Fort E, Harvey DJ, Struwe WB, et al. Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis. Proteomics Clin Appl. 2010;4:432-48.
[12] Fournier T, Medjoubi NN, Porquet D. Alpha-1-acid glycoprotein. Biochim Biophys Acta. 2000;1482:157-71.
[13] Telford JE, Bones J, McManus C, Saldova R, Manning G, Doherty M, et al. Antipsychotic treatment of acute paranoid schizophrenia patients with olanzapine results in altered glycosylation of serum glycoproteins. J Proteome Res. 2012;11:3743-52.
[14] Saldova R, Royle L, Radcliffe CM, Abd Hamid UM, Evans R, Arnold JN, et al. Ovarian cancer is associated with changes in glycosylation in both acute-phase proteins and IgG. Glycobiology. 2007;17:1344-56.
[15] Gimenez E, Balmana M, Figueras J, Fort E, Bolos C, Sanz-Nebot V, et al. Quantitative analysis of N-glycans from human alfa-acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis. Anal Chim Acta. 2015;866:59-68.
[16] Puerta A, Martin-Alvarez PJ, Ongay S, Diez-Masa JC, de Frutos M. Immunoaffinity, capillary electrophoresis, and statistics for studying intact alpha 1-acid glycoprotein isoforms as an atherothrombosis biomarker. Methods Mol Biol. 2013;919:215-30.

[17] Ongay S, Neususs C, Vaas S, Diez-Masa JC, de Frutos M. Evaluation of the effect of the immunopurification-based procedures on the CZE-UV and CZE-ESI-TOF-MS determination of isoforms of intact alpha-1-acid glycoprotein from human serum. Electrophoresis. 2010;31:1796-804.
[18] Gimenez E, Sanz-Nebot V, Rizzi A. Relative quantitation of glycosylation variants by stable isotope labeling of enzymatically released N-glycans using [12C]/[13C] aniline and ZIC-HILIC-ESI-TOF-MS. Anal Bioanal Chem. 2013;405:7307-19.
[19] Lacunza I, Sanz J, Diez-Masa JC, de Frutos M. CZE of human alpha-1-acid glycoprotein for qualitative and quantitative comparison of samples from different pathological conditions. Electrophoresis. 2006;27:4205-14.
[20] Puerta A, Diez-Masa JC, Martin-Alvarez PJ, Martin-Ventura JL, Barbas C, Tunon J, et al. Study of the capillary electrophoresis profile of intact alpha-1-acid glycoprotein isoforms as a biomarker of atherothrombosis. Analyst. 2011;136:816-22.
[21] Ceciliani F, Pocacqua V. The acute phase protein alpha1-acid glycoprotein: a model for altered glycosylation during diseases. Curr Protein Pept Sci. 2007;8:91-108.
[22] Ongay S, Neususs C. Isoform differentiation of intact AGP from human serum by capillary electrophoresis-mass spectrometry. Anal Bioanal Chem. 2010;398:845-55.
[23] Nakano M, Kakehi K, Tsai MH, Lee YC. Detailed structural features of glycan chains derived from alpha1-acid glycoproteins of several different animals: the presence of hypersialylated, O-acetylated sialic acids but not disialyl residues. Glycobiology. 2004;14:431-41.
[24] Kobayashi Y, Tateno H, Dohra H, Moriwaki K, Miyoshi E, Hirabayashi J, et al. A novel core fucose-specific lectin from the mushroom Pholiota squarrosa. The Journal of biological chemistry. 2012;287:33973-82.
[25] Miyoshi E, Moriwaki K, Terao N, Tan CC, Terao M, Nakagawa T, et al. Fucosylation is a promising target for cancer diagnosis and therapy. Biomolecules. 2012;2:34-45.
[26] Breborowicz J, Mackiewicz A, Breborowicz D. Microheterogeneity of alpha-fetoprotein in patient serum as demonstrated by lectin affino-electrophoresis. Scand J Immunol. 1981;14:15-20.
[27] Wong RJ, Ahmed A, Gish RG. Elevated alpha-fetoprotein: differential diagnosis - hepatocellular carcinoma and other disorders. Clin Liver Dis. 2015;19:309-23.
[28] Turner GA. Haptoglobin. A potential reporter molecule for glycosylation changes in disease. Adv Exp Med Biol. 1995;376:231-8.
[29] Matsumoto H, Shinzaki S, Narisada M, Kawamoto S, Kuwamoto K, Moriwaki K, et al. Clinical application of a lectin-antibody ELISA to measure fucosylated haptoglobin in sera of patients with pancreatic cancer. Clin Chem Lab Med. 2010;48:505-12.
[30] Shimomura M, Nakayama K, Azuma K, Terao N, Nishino K, Takamatsu S, et al. Establishment of a novel lectin-antibody ELISA system to determine core-fucosylated haptoglobin. Clin Chim Acta. 2015;446:30-6.
[31] Ongay S. Development of immunopurification and capillary electrophoresis methodologies for the study of glycoproteins as potential cancer biomarkers. Madrid: Universidad Autónoma de Madrid; 2010.
[32] Ongay S, Martin-Alvarez PJ, Neususs C, de Frutos M. Statistical evaluation of CZE-UV and CZE-ESI-MS data of intact alpha-1-acid glycoprotein isoforms for their use as potential biomarkers in bladder cancer. Electrophoresis. 2010;31:3314-25.
[33] Dempsey E, Rudd PM. Acute phase glycoproteins: bystanders or participants in carcinogenesis? Ann N Y Acad Sci. 2012;1253:122-32.
[34] Hashimoto S, Asao T, Takahashi J, Yagihashi Y, Nishimura T, Saniabadi AR, et al. alpha1-acid glycoprotein fucosylation as a marker of carcinoma progression and prognosis. Cancer. 2004;101:2825-36.
[35] Mackiewicz A, Mackiewicz K. Glycoforms of serum alpha 1-acid glycoprotein as markers of inflammation and cancer. Glycoconj J. 1995;12:241-7.
[36] Smith KD, Magliocco AM, Matthews-Smith G, Behan J. Alpha-1-Acid Glycoprotein (AGP) as a Potential Biomarker for Breast Cancer: INTECH Open Access Publisher; 2012.
[37] Croce MV, Salice VC, Lacunza E, Segal-Eiras A. Alpha 1-acid glycoprotein (AGP): a possible carrier of sialyl lewis X (slewis X) antigen in colorectal carcinoma. Histol Histopathol. 2005;20:91-7.

[38] Herve F, Duche JC, Jaurand MC. Changes in expression and microheterogeneity of the genetic variants of human alpha1-acid glycoprotein in malignant mesothelioma. J Chromatogr B Biomed Sci Appl. 1998;715:111-23.
[39] Miyoshi E, Moriwaki K, Nakagawa T. Biological function of fucosylation in cancer biology. J Biochem. 2008;143:725-9.
[40] Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, et al. Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer. 2006;118:2803-8.
[41] Azuma Y, Murata M, Matsumoto K. Alteration of sugar chains on alpha(1)-acid glycoprotein secreted following cytokine stimulation of HuH-7 cells in vitro. Clin Chim Acta. 2000;294:93-103.
[42] Higai K, Miyazaki N, Azuma Y, Matsumoto K. Interleukin-1beta induces sialyl Lewis X on hepatocellular carcinoma HuH-7 cells via enhanced expression of ST3Gal IV and FUT VI gene. FEBS Lett. 2006;580:6069-75.
[43] Perez-Garay M, Arteta B, Llop E, Cobler L, Pages L, Ortiz R, et al. alpha2,3-Sialyltransferase ST3Gal IV promotes migration and metastasis in pancreatic adenocarcinoma cells and tends to be highly expressed in pancreatic adenocarcinoma tissues. Int J Biochem Cell Biol. 2013;45:1748-57.

TABLE 1: Clinical and pathological characteristics of the patients

Category	Cases	Stage (TNM system)	Gender	Age	Age Average	±SD	Range
Healthy control (HC)	HC1**		male	59	60.67	3.61	57-66
	HC2		female	63			
	HC3**		male	62			
	HC4		male	57			
	HC5**		male	66			
	HC6**		female	57			
Chronic pancreatitis (ChrP)	ChrP1		male	59	59.50	5.54	49-65
	ChrP2*		female	62			
	ChrP3		male	62			
	ChrP4		female	49			
	ChrP5*		male	65			
	ChrP6		female	60			
Non-advanced PDAC	PDAC1	IB (T2N0M0)	male	63	59.67	3.88	53-63
	PDAC2*	IIA (T3N0M0)	female	62			
	PDAC3*	IIB (T3N1M0)	male	57			
	PDAC4	IIA (T3N0M0)	male	61			
	PDAC5	IIB (T3N1M0)	male	62			
	PDAC6	IIB (T3N1M0)	female	53			
Advanced PDAC	PDAC7	III (T4NxM0)	male	58	60.46	8.01	45-73
	PDAC8	III (T4NxM0)	female	59			
	PDAC9	III (T4NxM0)	male	52			
	PDAC10	III (T4NxM0)	female	69			
	PDAC11*	III (T4N1M0)	male	67			
	PDAC12*	III (T4N0M0)	male	55			
	PDAC13	III (T4N0M0)	male	66			
	PDAC14	IV (TxNxM1)	male	52			
	PDAC15	IV (T3N1M1)	female	67			
	PDAC16	IV (TxNxM1)	female	61			
	PDAC17	IV (T3N1M1)	female	45			
	PDAC18*	IV (T3N1M1)	male	73			
	PDAC19*	IV (T3N1M1)	female	62			

* samples of the previous study [15].
** samples that constitute the pool of HC for the *N*-glycan analysis by µZIC-HILIC-ESI-MS.
HC, healthy control; ChrP, chronic pancreatitis; PDAC pancreatic ductal adenocarcinoma.

TABLE 2: Relative molar ratios of AGP *N*-glycan structures determined by µZIC-HILIC-ESI-MS for each individual patient and the mean and SD for each group of patients.

		Chronic Pancreatitis							Pancreatic Adenocarcinoma													
									Non-advanced							Advanced						
		ChrP1	**ChrP2**	**ChrP3**	**ChrP4**	**ChrP5**	**ChrP6**	**Mean (±SD)**	**PDAC 1**	**PDAC 2**	**PDAC 3**	**PDAC 4**	**PDAC 5**	**PDAC 6**	**Mean (±SD)**	**PDAC 11**	**PDAC 12**	**PDAC 13**	**PDAC 17**	**PDAC 18**	**PDAC 19**	**Mean (±SD)**
Non-fucosylated structures	**2Ant2Neu5Ac**	1.34	0.95	0.66	0.79	0.73	0.68	**0.86 (±0.26)**	1.69	0.92	0.45	0.93	1.58	1.00	**1.10 (±0.46)**	1.24	0.76	1.38	1.09	0.52	0.68	**0.95 (±0.34)**
	3Ant2Neu5Ac	0.89	0.67	0.63	0.77	0.62	0.78	**0.73 (±0.11)**	0.87	0.92	0.36	0.67	1.37	1.12	**0.88 (±0.35)**	0.82	0.54	1.29	0.94	0.39	0.59	**0.76 (±0.32)**
	3Ant3Neu5Ac	1.01	0.73	0.63	0.72	0.69	0.90	**0.78 (±0.15)**	0.92	0.87	0.37	0.79	1.59	1.23	**0.96 (±0.41)**	0.93	0.62	1.33	1.02	0.52	0.91	**0.89 (±0.29)**
	4Ant3Neu5Ac	1.10	0.65	0.79	0.79	0.62	0.82	**0.79 (±0.17)**	0.70	1.09	0.34	0.51	1.06	1.25	**0.82 (±0.36)**	0.83	0.41	1.33	1.03	0.40	0.73	**0.79 (±0.36)**
	4Ant4Neu5Ac	1.16	0.57	0.72	0.75	0.68	0.77	**0.77 (±0.20)**	0.71	1.13	0.26	0.46	1.01	1.01	**0.76 (±0.35)**	0.92	0.49	1.35	1.10	0.38	0.66	**0.81 (±0.37)**
Fucosylated structures	**2Ant2Neu5Ac1Fuc**	2.33	0.53	0.78	0.60	1.03	0.85	**1.02 (±0.67)**	3.09	0.81	0.61	1.31	1.12	1.05	**1.33 (±0.89)**	2.14	1.93	1.16	1.07	0.98	1.74	**1.50 (±0.49)**
	3Ant2Neu5Ac1Fuc	1.40	0.80	0.68	0.55	0.68	0.85	**0.83 (±0.30)**	1.70	0.75	1.12	1.36	1.26	1.47	**1.28 (±0.32)**	1.15	1.33	0.85	1.44	1.32	1.69	**1.30 (±0.28)**
	3Ant3Neu5Ac1Fuc	1.83	0.75	0.62	0.56	0.64	0.86	**0.87 (±0.48)**	1.89	0.63	1.19	2.11	1.33	1.46	**1.43 (±0.53)**	1.33	1.26	0.96	1.28	1.14	1.66	**1.27 (±0.23)**
	3Ant3Neu5Ac2Fuc	1.80	0.54	0.59	0.50	0.31	0.64	**0.73 (±0.54)**	2.95	0.52	2.02	2.82	1.08	1.35	**1.79 (±0.98)**	1.92	1.76	0.49	1.20	2.31	2.41	**1.68 (±0.72)**
	4Ant3Neu5Ac1Fuc	2.06	0.85	0.79	0.55	0.58	0.86	**0.95 (±0.56)**	1.94	0.74	1.19	2.11	0.91	1.77	**1.44 (±0.57)**	1.33	1.59	0.92	1.81	1.20	1.84	**1.45 (±0.36)**
	4Ant3Neu5Ac2Fuc	2.30	0.46	0.77	0.45	0.52	0.67	**0.86 (±0.71)**	3.40	0.67	2.33	5.20	1.09	2.09	**2.46 (±1.65)**	1.64	3.03	0.51	1.70	2.72	2.72	**2.05 (±0.95)**
	4Ant4Neu5Ac1Fuc	2.00	0.79	0.81	0.63	0.73	0.88	**0.97 (±0.51)**	1.62	0.85	0.97	1.52	0.97	1.23	**1.19 (±0.32)**	1.53	1.34	1.08	1.33	0.99	1.82	**1.35 (±0.30)**
	4Ant4Neu5Ac2Fuc	2.22	0.77	0.71	0.56	0.48	0.82	**0.93 (±0.65)**	2.05	0.66	3.97	2.59	0.82	1.29	**1.90 (±1.25)**	1.56	2.64	0.75	1.43	1.98	2.47	**1.81 (±0.70)**
	4Ant4Neu5Ac3Fuc	1.74	0.44	0.51	0.53	0.32	0.71	**0.71 (±0.52)**	1.99	0.05	1.83	3.42	0.52	1.02	**1.47 (±1.21)**	1.48	3.06	0.33	1.27	2.08	2.62	**1.81 (±0.99)**

TABLE 3: Capillary zone electrophoresis. Mean values of the percentage of the peak corrected area for each group of patients.

		Mean (±SD)			p-value (ANOVA)		
	peak	**HC**	**ChrP**	**PDAC**	**HC vs ChrP**	**HC vs PDAC**	**ChrP vs PDAC**
Capillary Electrophoresis (CE-UV)	**p2**	0.99 (±0.33)	0.72 (±0.35)	0.75 (±0.23)	0.229	0.194	0.966
	p3	2.66 (±0.92)	1.85 (±0.93)	1.75 (±0.72)	0.208	0.056	0.959
	p4	6.54 (±1.86)	4.79 (±1.81)	4.38 (±1.57)	0.186	**0.027***	0.857
	p5	13.42 (±2.77)	10.62 (±2.59)	9.75 (±2.81)	0.204	**0.022***	0.781
	p6	21.42 (±2.41)	19.00 (±2.18)	17.83 (±3.00)	0.301	**0.026***	0.644
	p7	23.98 (±1.74)	24.35 (±0.79)	23.66 (±1.38)	0.885	0.868	0.528
	p8	18.07 (±3.36)	21.02 (±2.70)	21.88 (±2.72)	0.189	**0.021***	0.798
	p9	9.1 (±2.57)	12.01 (±2.92)	13.51 (±3.59)	0.296	**0.022***	0.606
	p10	2.93 (±1.20)	4.33 (±1.37)	5.11 (±2.09)	0.401	**0.045***	0.643
	p11	0.84 (±0.43)	1.05 (±0.35)	1.25 (±0.51)	0.727	0.166	0.628
	p12	0.36 (-)	0.38 (±0.04)	0.45 (±0.13)	-	-	-

*statistically significant (p<0.05)

TABLE 4: *Aleuria aurantia* lectin assays. Mean values of the ratios of fucosylated AGP/total AGP before and after neuraminidase digestion.

		Mean (±SD)				p-value (Kruskal-Wallis)					
		HC	**ChrP**	**Non-advanced PDAC**	**Advanced PDAC**	**HC vs ChrP**	**HC vs non-advanced PDAC**	**HC vs advanced PDAC**	**ChrP vs non-advanced PDAC**	**ChrP vs advanced PDAC**	**Non-advanced PDAC vs advanced PDAC**
Lectin Assay (AAL)	**AGP**	0.87 (±0.25)	0.68 (±0.20)	1.09 (±0.65)	1.45 (±0.74)	0.273	0.715	0.154	0.522	**0.025***	0.303
	neuraminidase-digested AGP	4.31 (±1.92)	4.68 (±4.05)	5.99 (±3.74)	8.31 (±3.84)	0.423	0.670	**0.028***	0.286	0.096	0.213

*statistically significant (p<0.05)

Figure Captions

Figure 1. Purity profiles of serum AGP isolated by immunoaffinity chromatography. Silver staining of 10% SDS-PAGE showing twelve representative samples.

Figure 2. µZIC-HILIC-ESI-MS analysis of aniline-labelled *N*-glycans released from AGP. (A) Extracted ion chromatograms of *N*-glycan species 4Ant4Neu5Ac2Fuc released from control AGP (dotted line) and PDAC (i) or ChrP (ii) samples (solid line). (B) Experimental molar ratios determined for the sialofucosylated *N*-glycans of AGP. A molar ratio value of 1 is obtained when the glycan in the pathological sample have the same peak area as the glycan detected in the pool of healthy control samples.

Figure 3. Ratio of fucosylated/non-fucosylated AGP glycan structures. This was determined by dividing the molar ratios obtained by *N*-glycan µZIC-HILIC-ESI-MS analysis of fucosylated structures by the corresponding values of non-fucosylated structures. *N*-glycans with the same number of antennae are considered together and the averages of each group of samples are depicted as histograms with ± standard error (SE). HC does not present SE since it corresponds to a pool of samples and is analysed by µZIC-HILIC-ESI-MS as a single one.

Figure 4. AGP analysis by capillary electrophoresis. (A) Example of capillary electrophoresis profile of purified serum AGP. Each identified peak is labelled according to its migration time from p1 (the first to migrate) to p12 (the last one to elute). (B) Statistical comparison of percentage of corrected area of each peak of serum AGP from different samples grouped in HC, ChrP and PDAC analysed by CE-UV. ANOVA test with Tukey post-test was used ($p<0.05$ was considered statistically significant, labelled as *).

Figure 5. ELLA analysis of purified AGP using *Aleuria aurantia* lectin (AAL). Boxplots describe the median and the samples distribution within a group. (A) Ratio of fucosylated AGP/total AGP analysed

by ELLA. (B) Ratio of fucosylated AGP/total AGP by direct ELLA after neuraminidase treatment for removal of sialic acids. Non-parametric Kruskal-Wallis test was used for statistical analysis ($p<0.05$ was considered statistically significant, labelled as *). Outlier data are indicated as ο and extreme values as +.

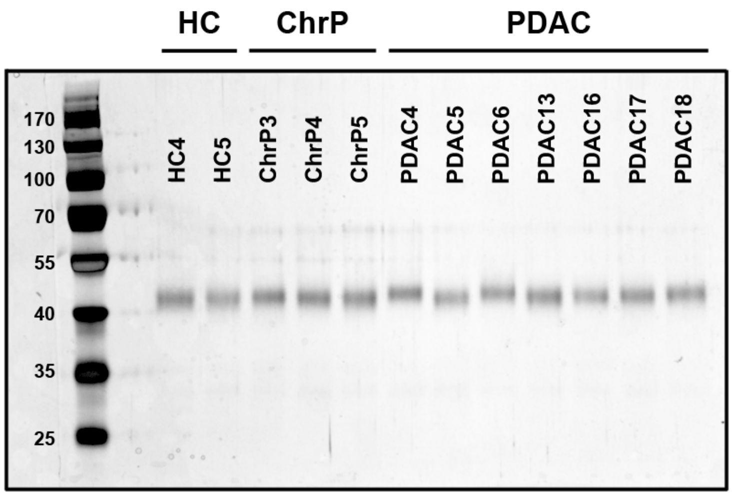

HC
ChrP
PDAC
170
130
100
70
55
40
35
25
HC4
HC5
ChrP3
ChrP4
ChrP5
PDAC4
PDAC5
PDAC6
PDAC13
PDAC16
PDAC17
PDAC18

Fig. 1

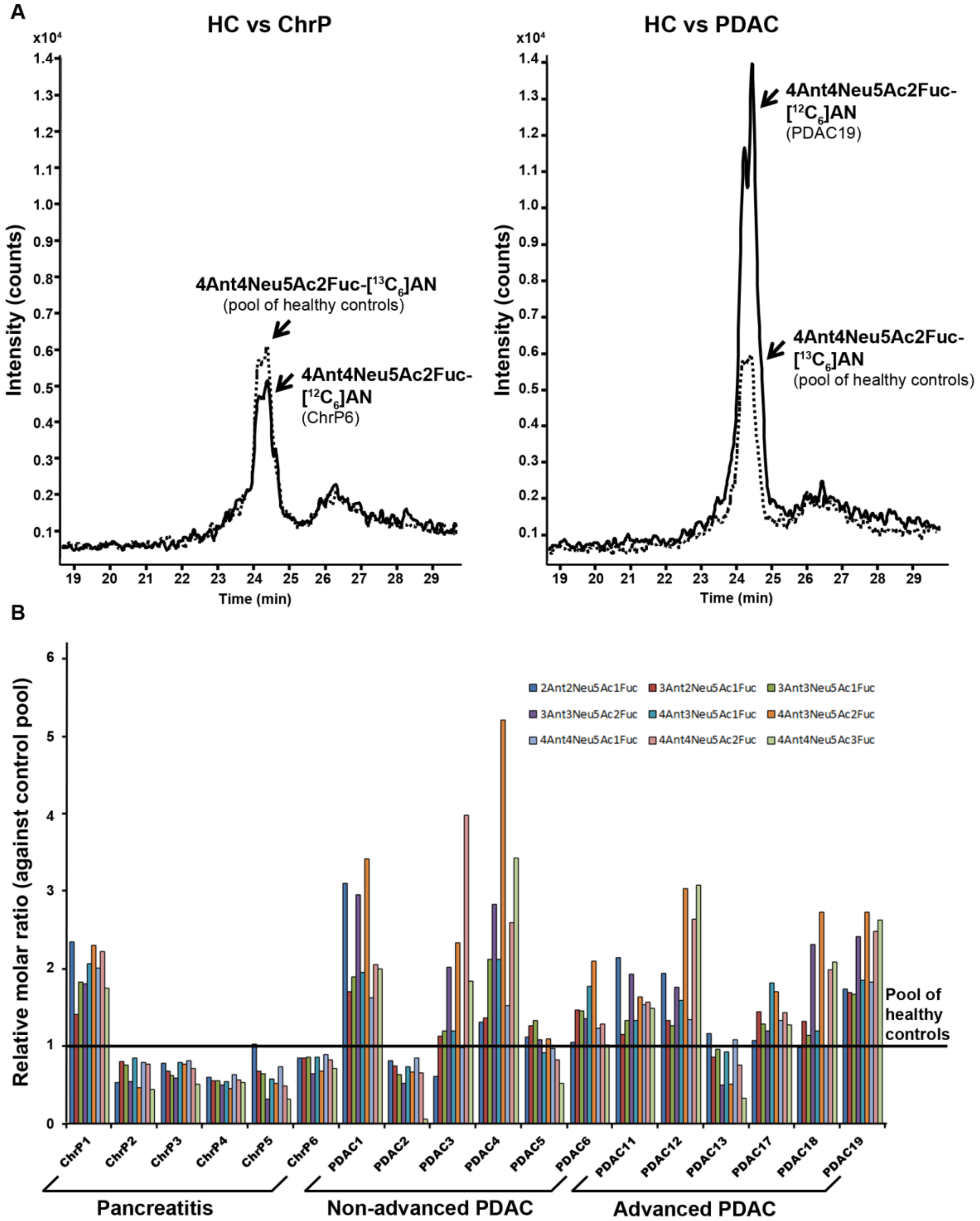

A
HC vs ChrP
HC vs PDAC
x10⁴
Intensity (counts)
4Ant4Neu5Ac2Fuc-[13C6]AN
(pool of healthy controls)
4Ant4Neu5Ac2Fuc-
[12C6]AN
(ChrP6)
(PDAC19)
Time (min)
B
Relative molar ratio (against control pool)
2Ant2Neu5Ac1Fuc
3Ant2Neu5Ac1Fuc
3Ant3Neu5Ac1Fuc
3Ant3Neu5Ac2Fuc
4Ant3Neu5Ac1Fuc
4Ant3Neu5Ac2Fuc
4Ant4Neu5Ac1Fuc
4Ant4Neu5Ac2Fuc
4Ant4Neu5Ac3Fuc
Pool of
healthy
controls
ChrP1
ChrP2
ChrP3
ChrP4
ChrP5
ChrP6
PDAC1
PDAC2
PDAC3
PDAC4
PDAC5
PDAC6
PDAC11
PDAC12
PDAC13
PDAC17
PDAC18
PDAC19
Pancreatitis
Non-advanced PDAC
Advanced PDAC

Fig. 2

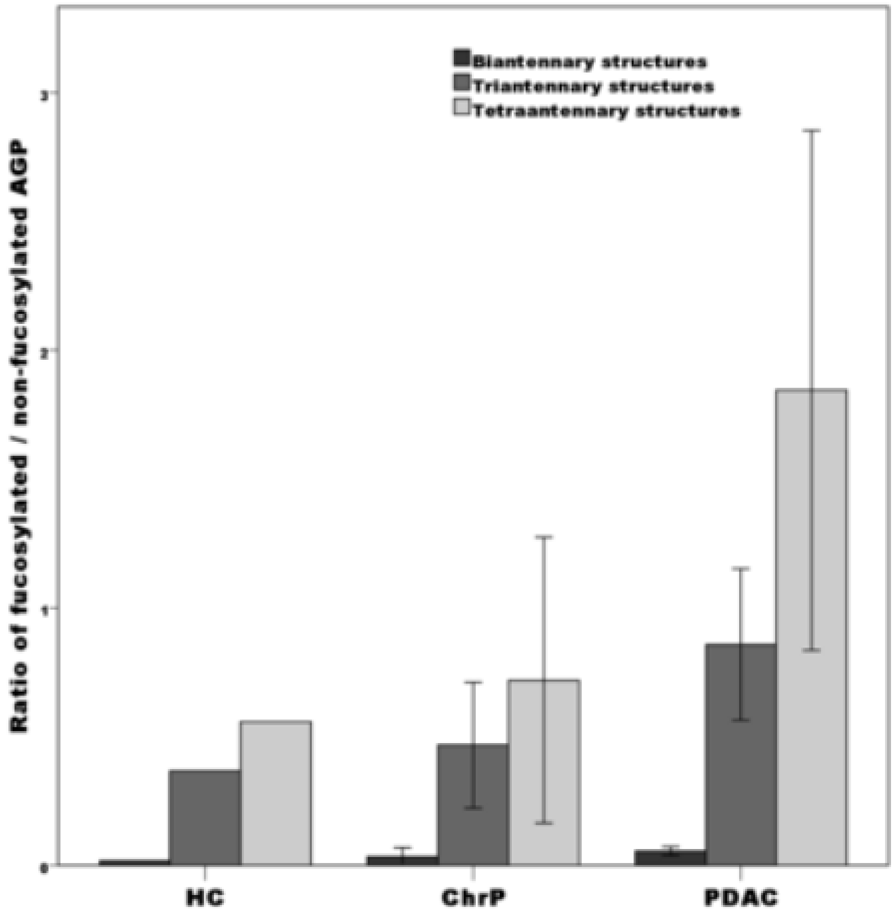

Biantennary structures
Triantennary structures
Tetraantennary structures
Ratio of fucosylated / non-fucosylated AGP
3
2
1
0
HC
ChrP
PDAC

Fig. 3

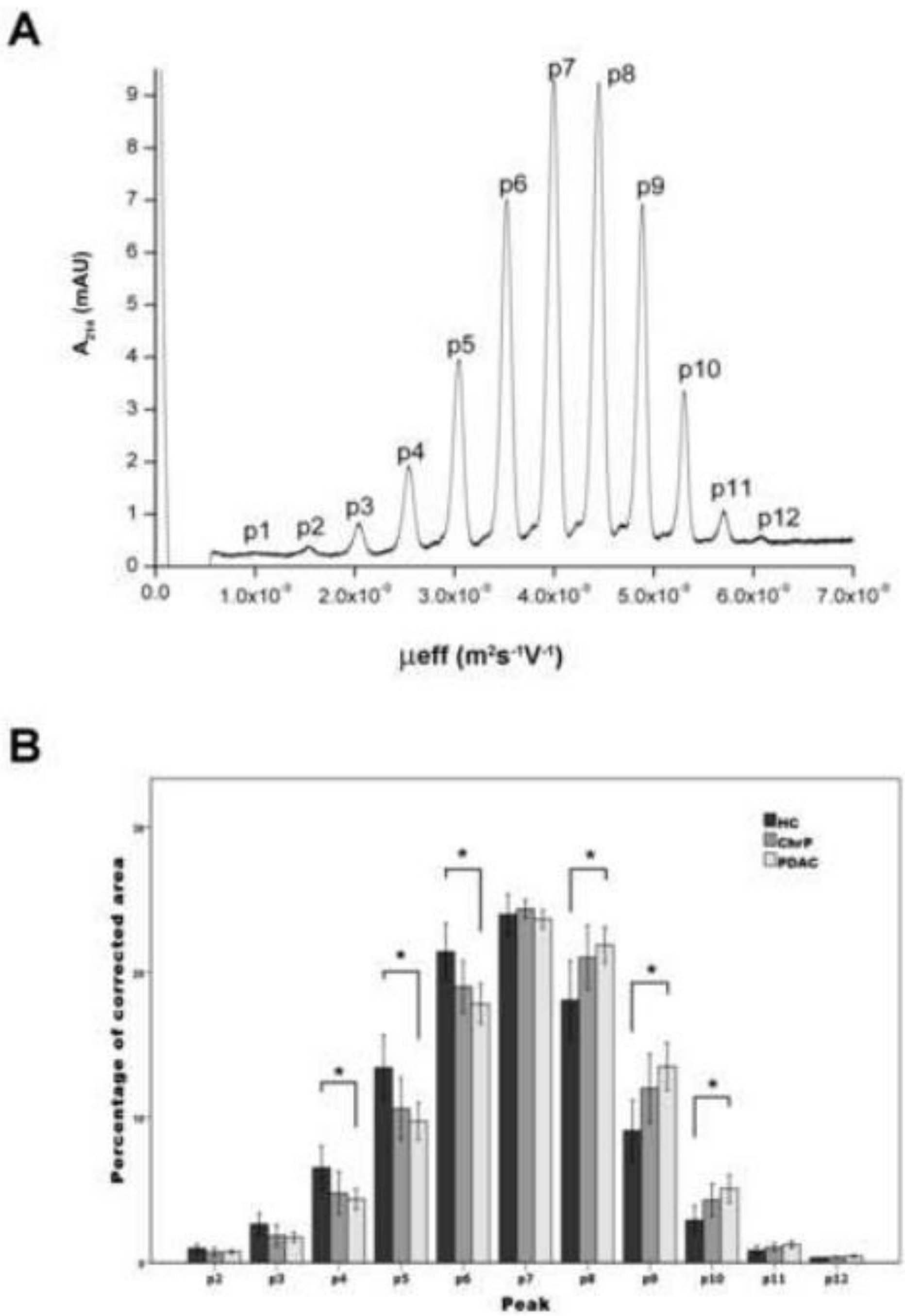

A
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
p11
p12
A214 (mAU)
μeff (m²s⁻¹V⁻¹)
B
HC
ChrP
PDAC
Percentage of corrected area
Peak
p2
p3
p4
p5
p6
p7
p8
p9
p10
p11
p12

Fig. 4

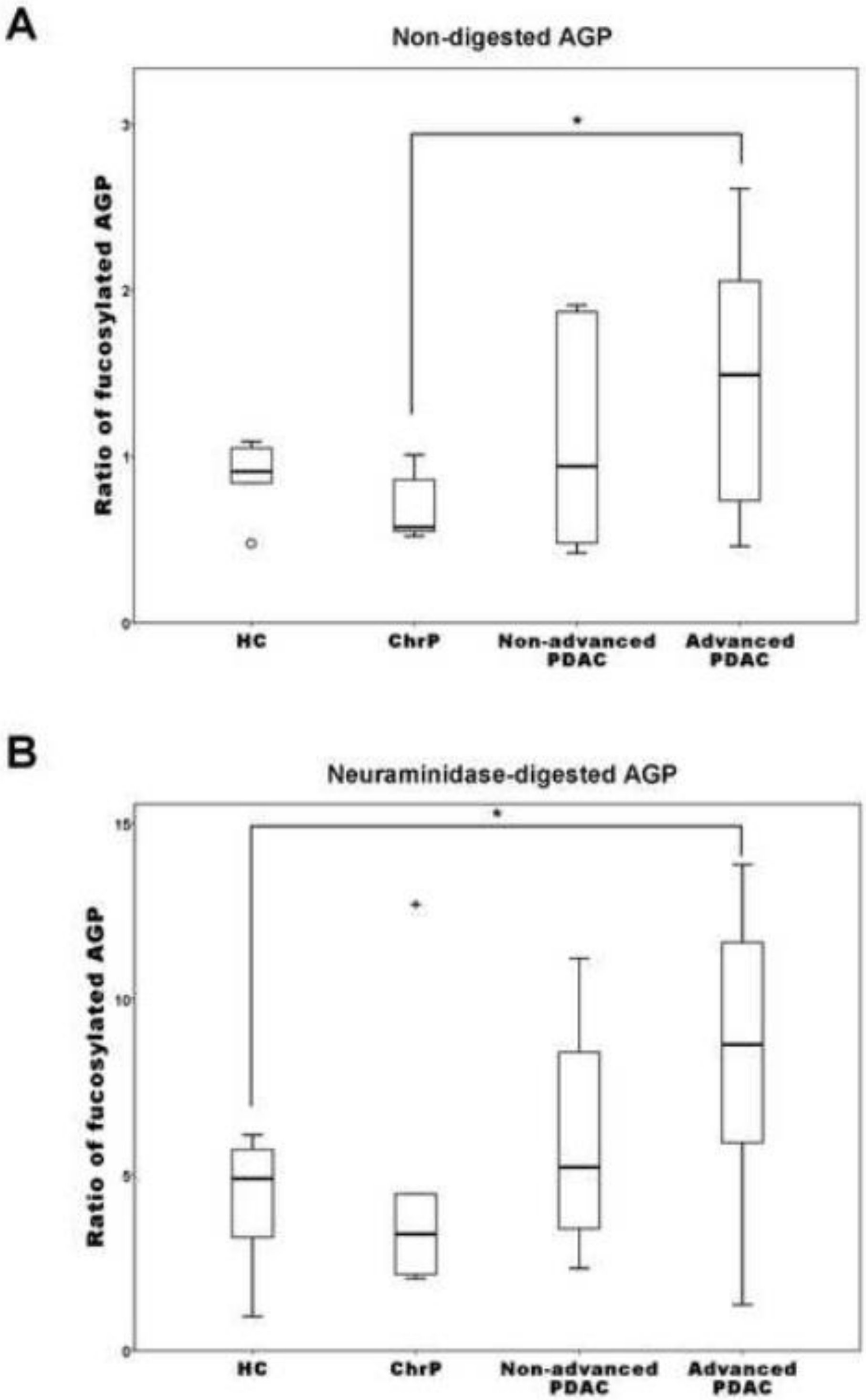
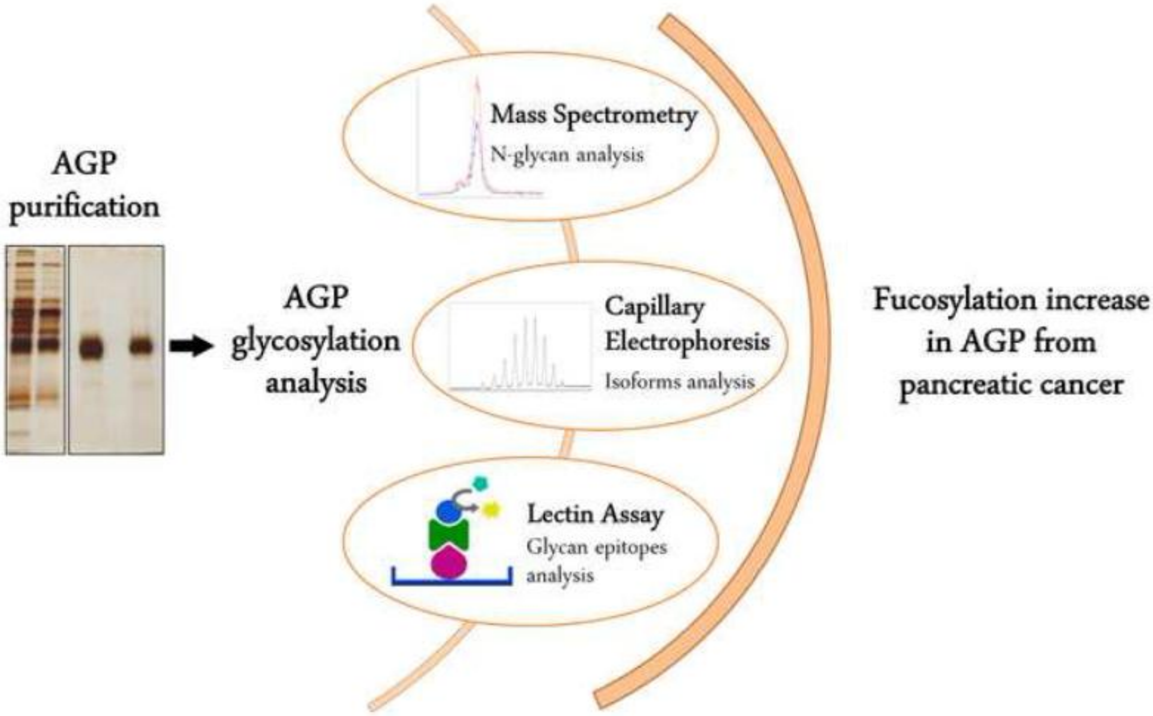

A
Non-digested AGP
Ratio of fucosylated AGP
0
1
2
3
*
HC
ChrP
Non-advanced PDAC
Advanced PDAC
B
Neuraminidase-digested AGP
Ratio of fucosylated AGP
0
5
10
15
*
HC
ChrP
Non-advanced PDAC
Advanced PDAC

Fig. 5

Graphical abstract



Highlights

- Analysis of serum alpha-1 acid glycoprotein (AGP) glycosylation in pancreatic disease.
- Complementary analytical techniques, MS, CE and lectin assays to determine AGP glycoforms.
- AGP fucosylation increase in pancreatic cancer *versus* pancreatitis and healthy controls.