Longitudinal changes of serum protein N-Glycan levels for earlier detection of pancreatic cancer in high-risk individuals

Levink, I.J.M.; Klatte, D.C.F.; Hanna Sawires, R.G.; Vreeker, G.C.M.; Ibrahim, I.S.; Burgt, Y.E.M. van der; ... ; Mesker, W.E.

Pancreatology 22 (2022) 497–506

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Longitudinal changes of serum protein N-Glycan levels for earlier detection of pancreatic cancer in high-risk individuals

I.J.M. Levink [a, *, 1], D.C.F. Klatte [b, 1], R.G. Hanna-Sawires [c], G.C.M. Vreeker [d], I.S. Ibrahim [b], Y.E.M. van der Burgt [d], K.A. Overbeek [a], B.D.M. Koopmann [a], D.L. Cahen [a], G.M. Fuhler [a], M. Wuhrer [d], B.A. Bonsing [c], R.A.E.M. Tollenaar [c], F.P. Vleggaar [e], H.F.A. Vasen [b], M.E. van Leerdam [b, f], M.J. Bruno [a], W.E. Mesker [c]

[a] Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands
[b] Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[c] Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands
[d] Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, the Netherlands
[e] Department of Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht, the Netherlands
[f] Department of Gastrointestinal Oncology, Netherlands Cancer Institute, Amsterdam, the Netherlands

ARTICLE INFO

Article history:
Received 13 November 2021
Received in revised form
25 March 2022
Accepted 31 March 2022
Available online 4 April 2022

Keywords:
Pancreatic cancer
Glycosylation
Glycan biomarker
Early detection
Protein

ABSTRACT

Background: Surveillance of individuals at risk of developing pancreatic ductal adenocarcinoma (PDAC) has the potential to improve survival, yet early detection based on solely imaging modalities is challenging. We aimed to identify changes in serum glycosylation levels over time to earlier detect PDAC in high-risk individuals.

Methods: Individuals with a hereditary predisposition to develop PDAC were followed in two surveillance programs. Those, of which at least two consecutive serum samples were available, were included. Mass spectrometry analysis was performed to determine the total *N*-glycome for each consecutive sample. Potentially discriminating *N*-glycans were selected based on our previous cross-sectional analysis and relative abundances were calculated for each glycosylation feature.

Results: 165 individuals ("FPC-cohort" N = 119; Leiden cohort N = 46) were included. In total, 97 (59%) individuals had a genetic predisposition (77 *CDKN2A*, 15 *BRCA1/2*, 5 *STK11*) and 68 (41%) a family history of PDAC without a known genetic predisposition (>10-fold increased risk of developing PDAC). From each individual, a median number of 3 serum samples (IQR 3) was collected.

Ten individuals (6%) developed PDAC during 35 months of follow-up; nine (90%) of these patients carried a *CDKN2A* germline mutation. In PDAC cases, compared to all controls, glycosylation characteristics were increased (fucosylation, tri- and tetra-antennary structures, specific sialic linkage types), others decreased (complex-type diantennary and bisected glycans). The largest change over time was observed for tri-antennary fucosylated glycans, which were able to differentiate cases from controls with a specificity of 92%, sensitivity of 49% and accuracy of 90%.

Conclusion: Serum *N*-glycan monitoring may support early detection in a pancreas surveillance program.

1. Introduction

The majority of patients with pancreatic ductal adenocarcinoma (PDAC) are diagnosed at an incurable stage. Due to the lack of early recognizable symptoms, only 20% of patients are eligible for surgery at presentation [1]. Surveillance of patients at risk of developing PDAC may offer opportunities for early detection and improved survival [2].

So far, diagnosis of PDAC has been challenging in surveillance programs for individuals with an hereditary risk of developing PDAC that combine magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP) and endoscopic

* Corresponding author. Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Dr. Molewaterplein 40, 3015, GD, the Netherlands.
E-mail address: i.levink@erasmusmc.nl (I.J.M. Levink).
[1] Authors contributed equally

https://doi.org/10.1016/j.pan.2022.03.021

Abbreviations

AUC =	Area under the curve
CA19-9 =	Carbohydrate-antigen 19-9
CI =	Confidence interval
EUS =	Endoscopic ultrasound
EMC =	Erasmus Medical Center
FDR =	First-degree relative
FNA =	Fine-needle aspiration
FNB =	Fine-needle biopsy
FPC =	Familial pancreatic cancer
FTICR =	Fourier transform ion cyclotron resonance
IQR =	interquartile range
LUMC =	Leiden University Medical Center
MALDI =	Matrix-assisted laser desorption/ionization
MS =	Mass spectrometry
MRI =	Magnetic resonance imaging
MRCP =	Magnetic resonance cholangiopancreatography
PanIN =	Pancreatic intraepithelial neoplasia
IPMN =	Intraductal papillary mucinous neoplasm
PDAC =	Pancreatic ductal adenocarcinoma
SDR =	Second-degree relative

ultrasound (EUS) [3,4]. As a result, resection of abnormalities, which are – upon histological assessment – proven benign, has caused unnecessary harm due to overtreatment [5]. Novel molecular biomarkers are urgently needed to increase our diagnostic capabilities, resulting in more appropriate risk stratification, earlier recognition of malignant progression and personalized clinical management (i.e. intensified/reduced follow-up or surgery). Currently, for diagnosis of PDAC, fine-needle aspiration (FNA) or biopsy (FNB) is used. Although their diagnostic accuracy is relatively high [6], they rely on the ability to visualize the lesion and accurate sampling of smaller lesions (<10 mm) is challenging [7]. A biomarker that shows relative changes over time is specifically beneficial in high-risk individuals undergoing surveillance.

For PDAC, carbohydrate-antigen (CA) 19–9 is a commonly used serum biomarker. Structurally, CA19-9 is sialylated Lewis A antigen that is present on various proteins. Protein glycosylation is a post-translational modification that alters its functional properties. However, its use in clinical practice has been limited thus far to prediction of treatment response and detection of disease recurrence [8]. As a result of its imperfect diagnostic performance [9], implementation of CA19-9 in a surveillance program should be done with caution, as it may increase harm (due to unnecessary imaging and surgery), patient anxiety and health care costs.

As mass spectrometry (MS)-based protein glycosylation studies have demonstrated that specific glycosylation levels in total serum *N*-glycome profiles have potential in PDAC detection [8,10–13], our group set up a pipeline for the development of a glycan panel for surveillance purposes (Fig. 1). As a first step, we performed a cross-sectional analysis, comparing sporadic PDAC cases with healthy controls and identified 51 glycosylation traits (combinations of glycan structures according to biosynthetic pathways) that were differentially expressed between these groups. Of these, a preliminary panel of three glycosylation traits (CA2, A3F0L, CFa) was able to differentiate sporadic PDAC cases with an AUC of 0.81–0.88 (cross-sectionally) [14].

Our next step is validation of glycosylation traits in a longitudinal study to identify markers that are able to earlier detect asymptomatic PDAC in high-risk individuals undergoing surveillance. For this purpose, we performed *N*-glycome analysis on consecutive serum samples from high-risk individuals undergoing surveillance, and compared PDAC cases with controls.

2. Material and methods

2.1. Study design and pancreatic surveillance programs

From our previously identified 51 promising *N*-glycan markers [14], we evaluated the 13 (25%) best-performing glycosylation markers in serum that was consecutively collected in the course of two Dutch pancreatic surveillance programs (Fig. 1 in blue). Selected participants had at least two blood samples collected at different time points between 2007 and 2018.

The first cohort (FPC-cohort) concerns a collaboration between the University Medical Center Utrecht and the Erasmus MC University Medical Center Rotterdam (EMC), a Dutch branch of the international CAncer of the Pancreas Surveillance (CAPS) consortium [4]. This study was set up in 2007 and includes individuals with an estimated 10% or greater lifetime risk of PDAC. It encompasses germline mutation carriers of a known PDAC susceptibility gene (e.g., *CDKN2A, LKB1/STK11, BRCA1/2*), as well as familial pancreatic cancer (FPC) kindreds without a known gene mutation. The latter group was included after genetic testing and detailed evaluation of family history by a clinical geneticist. Individuals in this group have ≥2 blood relatives (who are first-degree relatives [FDR] to each other or FDR and/or second-degree relatives [SDR] with ≥1 under 50 years of age) or ≥ 3 blood relatives (who are FDR or SDR to each other) with PDAC. At inclusion, all individuals were 50–75 years of age or 10 years younger than the youngest age at which a blood relative developed PDAC. Individuals included in the FPC-cohort undergo surveillance with both EUS and MRI/MRCP and glucose testing [4]. Additionally, serum samples were stored in the biobank at each follow-up.

The second surveillance cohort is followed by the Leiden University Medical Center (LUMC) from the year 2000 onwards. The vast majority of this population comprises carriers with a proven germline *CDKN2A* (*p16-Leiden*) mutation, who are at an estimated 15–20% lifetime risk of PDAC [15]. In addition to *CDKN2A*, 1 high-risk individual was included with 3 family members with PDAC (1 FDR and 2 SDR). Starting at the age of 45 years, participants were offered annual MRI/MRCP [2]. Since 2012, blood samples were collected at regular screening intervals.

The institutional ethical review boards of participating centers (2007_024, Amsterdam University Medical Center; MEC-2021-448 EMC; MEC P00.107 LUMC) have approved the study, and the included individuals gave written informed consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki and Taipei.

2.2. Serum sample collection and plate design

Serum samples were collected consecutively and processed according to a standardized protocol [16]. Within 4 h after venipuncture, each blood sample was centrifuged for 10 min at 1000×*g* and collected serum was stored in 1.5 mL aliquots at −80 °C until further analysis. Before measurements were performed (i.e., serum N-glycome analysis), each sample was further aliquoted into 60 μL tubes, with one aliquot of each sample relocated into a 96-well plate format. For technical quality control of the spectra, each plate contained a minimum of six in-house standards and two blanks.

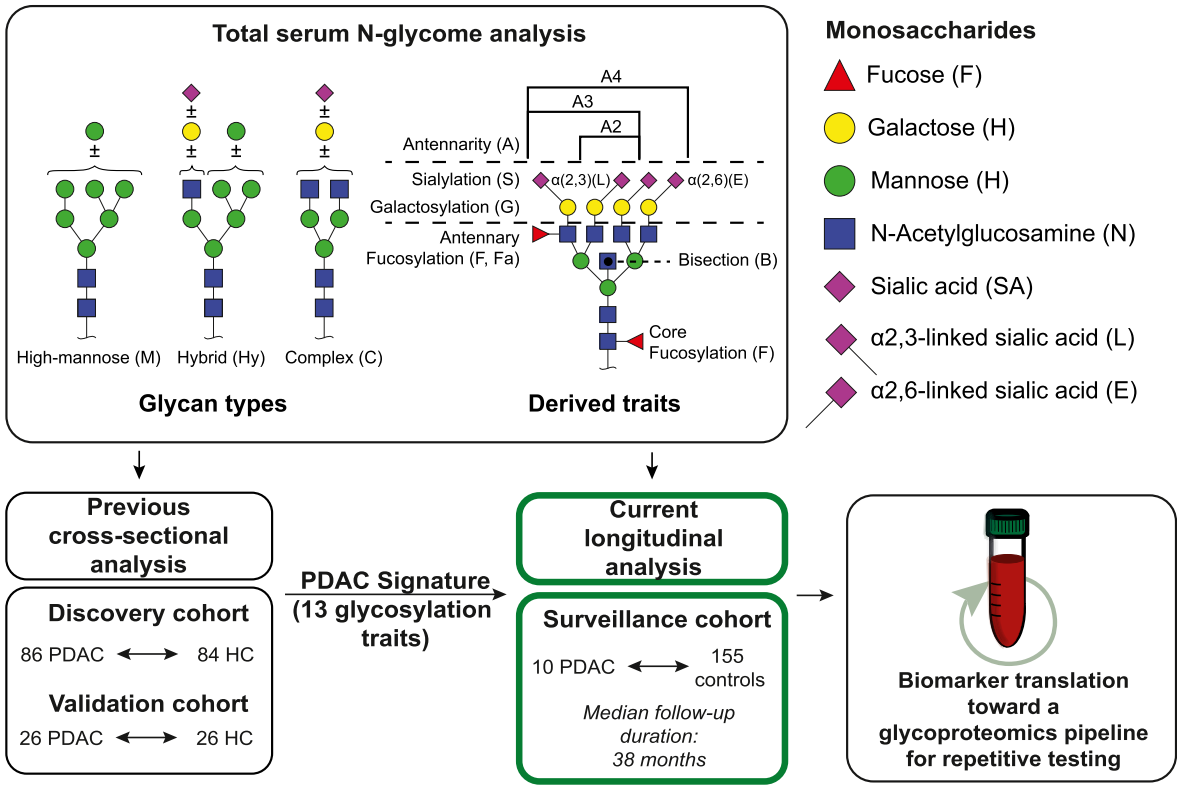


Fig. 1. Pipeline showing the development of a glycan-based biomarker panel for surveillance purposes. First, MS-based total *N*-glycome analysis provides relative abundances of single N-glycans from all serum proteins. Three commonly occurring N-glycan structures are distinguished, namely high-mannose, hybrid and complex-type. From this data, glycosylation traits are calculated that reflect biosynthetic pathways. The abundance of these glycosylation traits was previously described in a cross-sectional cohort.[1] Blue boxes indicate the current longitudinal analysis. In order to implement glycosylation-based biomarkers in clinical practice (for repetitive monitoring in a surveillance cohort), one or multiple protein-glycosylation trait combinations (glycoproteoform) need to be selected. PDAC = pancreatic ductal adenocarcinoma; HC = healthy control.

2.3. Serum sample preparation and mass spectrometry analysis of glycans

Six microliter of serum was subjected to analysis according to a previously reported protocol [16]. The procedure consisted of various steps that were carried out in a standardized manner on a Hamilton liquid handling platform. The first step (global release of N-glycans) was performed manually using the enzyme PNGase F (Roche Diagnostics, Mannheim, Germany). All sialic acid residues were chemically derivatized into stable end-products using an in-house developed ethylesterification protocol [16]. Thus introduced mass differences allow differentiation between α2,3- and α2,6-linked species. Next, the glycans were purified using cotton-based hydrophilic interaction liquid chromatography (HILIC) micro-tips and eluted and premixed with sDHB Matrix-assisted laser desorption/ionization (MALDI) matrix (5 mg/mL in 99% ACN with 1 mM NaOH). The glycans were spotted onto a MALDI target plate (800/384 MTP AnchorChip, Bruker Daltonics, Bremen, Germany) and measured on a Bruker 15T solariX XR Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The system was controlled by ftmsControl version 2.1.0 and spectra in an m/z-range from 1011.86 to 5000.00 were recorded with 1 M data points (i.e., transient length of 2.307 s). DataAnalysis Software 4.2 (Bruker Daltonics, Bremen, Germany) was used for the visualization and data analysis of all MALDI-FTICR spectra. The relative abundances of different *N*-glycans were determined from MS-data using in-house developed MassyTools (version 0.1.8.1) software [16], and subsequently combined in glycosylation traits on the basis of common structural features (Fig. 1; Supplemental Table S1).

2.4. Data processing and analysis

Clinical characteristics were described as medians with inter-quartile range (IQR) or percentages. Statistical significance was assessed with Mann-Whitney *U* test for continuous data and Fisher's exact or χ^2 test for categorical variables.

Candidate glycosylation traits were selected based on published data from our group comparing protein glycosylation in sporadic PDAC cases and healthy controls [14]. The mean abundance values (relative to total abundance per sample) of selected glycosylation traits were plotted over time per investigated group (cases *vs* controls) with 95% confidence interval (CI). The median glycosylation levels per investigated group at both baseline (t_0) and last follow-up (before PDAC development) were assessed and a ratio was calculated by dividing median levels of cases by that of controls at both timepoints. Subsequently, change of glycosylation over time was quantified by calculating the difference between the most recent (before PDAC development for cases) and first measurement normalized by time between samples (in months). Subsequently, this metric was compared between cases and controls (Mann-Whitney U) for the 13 glycosylation traits (p-values not shown). P-values < 0.004 were considered significant (Bonferroni correction for multiple testing). To calculate the diagnostic performance of difference over time, ROC curves were generated (and area under the curves (AUCs) calculated) for those glycosylation traits that showed a different change over time (Mann-Whitney U; $p < 0.05$). To estimate sensitivity and specificity, two locations on the curve were selected aiming: 1) high sensitivity (minimum specificity ≥40%); and 2) high specificity (minimum sensitivity ≥40%). CIs for

sensitivity, specificity and accuracy are based on the cumulative probabilities of the binomial distribution ("exact" Clopper-Pearson CIs).

Additionally, as changes in glycosylation levels may also reflect the presence of other malignancies, we visualized and compared glycosylation traits over time of cases developing PDAC, controls with another malignancy, and controls who underwent pancreatic surgery for benign disease.

Data was analyzed with Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL). Data were visualized using R (version 4.0.2; package "Tidyverse") and GraphPad (GraphPad Prism version 9, La Jolla, CA).

3. Results

3.1. Comparison of cohorts

In total, 165 patients met the inclusion criteria, 119 from the FPC-cohort and 46 from the Leiden cohort. Patient characteristics did not differ regarding age (FPC-cohort median 52 years [IQR 14]; Leiden cohort 53 [IQR 14]; p = 0.84), gender (FPC-cohort 39% male, Leiden cohort 44%; p = 0.60) and BMI (25 kg/m^2 [IQR 5.7]; 26 kg/m^2 [IQR 4.8]; p = 0.35; Table 1). Due to differences in inclusion criteria, the Leiden cohort predominantly consisted of *CDKN2A* mutation carriers (97.8%), while the FPC-cohort was more heterogeneous, containing both mutations carriers (*CDKN2A* 26.9%, *BRCA2* 11.8%, *BRCA1* 0.8%, *STK11* 4.2%) and mutation-negative FPC kindreds (56.3%; Table 1). 52.1% of the members of the FPC-cohort had 3 or more family members with PDAC, this number was lower in the Leiden cohort (26.1%; p = 0.001).

At baseline, the majority of patients in both cohorts had no abnormalities on imaging (FPC-cohort n = 70 [58.8%]; Leiden cohort n = 39 [84.8%]). 7 participants (5.9%) of the FPC-cohort had an indeterminate lesion, for which the follow-up interval was shortened to 3 months, but none of these lesions developed to PDAC. Participants were followed for a median of 35 months (IQR 61) in the FPC-cohort and 42 months (IQR 37; p = 0.23) in the Leiden cohort. During surveillance, more samples were collected in the FPC-cohort (median 4 [IQR 5]) than in the Leiden cohort (median 2 [IQR 0]; Table 1).

3.2. Surveillance outcomes

During follow-up, 10 participants (6.1%) developed PDAC. Nine (90%) cases were *CDKN2A* mutation carriers and one was a *STK11* gene mutation carrier (Table 2). At baseline, only 1 case (P4 from the Leiden cohort) had a feature suspected for malignancy. All 10 cases underwent surgery within 4 months after diagnosis. Four individuals had stage 1 disease (AJCC 8th edition), while the remainder had stage 2 or higher. One of the cases with stage 1 (P1) had a concomitant primary cancer in the cervix (T2bN1Mx). Two cases were interval cancers, detected because of jaundice. The others were detected at time of surveillance. For the majority of cases (8/10), the last serum collection had been performed less than six months before diagnosis (Table 2).

Of 155 controls, eight underwent surgery for falsely suspected PDAC ('Benign surgery controls'; n = 8; 5.2%; Table 2). Of these, five individuals had precursor lesions (Pancreatic intraepithelial neoplasia [PanIN] or intraductal papillary mucinous neoplasm [IPMN]) or a pancreatic neuro-endocrine tumor (NET). Benign surgery controls were analyzed as a separate group, as changed glycosylation traits may already be visible in precursor lesions. For 6/8 benign surgery controls, the last collection was performed less than six months before diagnosis. During the study period, 9 controls developed a malignancy other than PDAC (3 melanoma; 3 breast cancer; 1 esophageal cancer; 1 lymphoma; 1 duodenal NET; Table 2).

Table 1
Clinical characteristics.

	CAPS cohort n = 119	Leiden cohort n = 46	p-value
Baseline information			
Age in years, median (IQR)	52 (14)	53 (14)	0.84
Male sex, n (%)	46 (38.7)	20 (43.5)	0.60
BMI in kg/m^2, median (IQR)	25.3 (5.7)	26.2 (4.8)	0.35
Gene mutation, n (%)	52 (34.7)	45 (97.8)	<0.0001
CDKN2A p16	32 (26.9)	45 (97.8)	
BRCA2 + ≥ 2 blood relatives with PDAC	14 (11.8)	0 (0.0)	
BRCA1 + ≥ 2 blood relatives with PDAC	1 (0.8)	0 (0.0)	
STK11	5 (4.2)	0 (0.0)	
Total number of any degree blood relatives with PDAC, n (%)			0.001
0	13 (10.9)	12 (26.1)	
1	9 (7.6)	12 (26.1)	
2	35 (29.4)	10 (21.7)	
3 or more	62 (52.1)	12 (26.1)	
Diabetes mellitus, n (%)	3 (2.5)	0 (0.0)	NA
Working diagnosis at baseline, n (%)			<0.0001
No abnormalities	70 (58.8)	39 (84.8)	
Unspecified cyst	18 (15.1)	2 (4.3)	
SB-IPMN	20 (16.8)	4 (8.7)	
MD-IPMN	1 (0.8)	0 (0.0)	
pNET	1 (0.8)	0 (0.0)	
Duodenum NET	1 (0.8)	0 (0.0)	
Chronic pancreatitis	1 (0.8)	0 (0.0)	
Indeterminate, not suspect for malignancy	7 (5.9)	0 (0.0)	
Suspicion of malignancy	0 (0.0)	1 (2.1)	
Follow-up information			
Number of follow-up visits per individual, median (IQR)	4 (5)	2 (0.3)	<0.0001
Follow-up duration in months, median (IQR)	35 (61)	42 (37)	0.23
Individuals who developed PDAC, n (%)	3 (2.5)	7 (15.2)	0.005

IQR = interquartile range, IPMN = Intraductal papillary mucinous neoplasm, SB-IPMN = side-branch IPMN, MD-IPMN = main-duct IPMN, (p)NET = (pancreatic) neuroendocrine tumor, PDAC = pancreatic ductal adenocarcinoma.

Table 2
Details of individuals who underwent surgery and developed other (non-pancreatic) malignancies.

Patient	Cohort	Pathological outcome	TNM stage AJCC 8th edition	Gender/Age at diagnosis, years	Risk category	Nr. of samples analyzed before esection/total	Time between last blood sample and diagnosis (months)	Time between baseline and diagnosis/surgery (months)
PDAC cases								
P1	FPC	PDAC + Cervixca	T1aN0M0	F/54	*STK11*	3/4[b]	6	25/29
P2	Leiden	PDAC	T1aN0M0	M/50	*CDKN2A*	2/2	16	27/31
P3	Leiden	PDAC	T1aN0M0	F/62	*CDKN2A*	2/2	1	36/39
P4	Leiden	PDAC	T1bN0M0	M/64	*CDKN2A*	2/2	0	3/6
P5	FPC	PDAC	T1cN1M0	M/50	*CDKN2A*	4/4	2	24/25
P6	FPC	PDAC	T2N1M0	M/55	*CDKN2A*	4/4	12	41/44
P7	Leiden	PDAC	T2N2M0	F/69	*CDKN2A*	2/2	1	41/42
P8	Leiden	PDAC	T3N0M0	M/66	*CDKN2A*	2/2	1	8/8
P9	Leiden	PDAC	T3N0M0	M/70	*CDKN2A*	2/2	1	51/54
P10	Leiden	PDAC	T3N1M0	F/67	*CDKN2A*	2/2	0	23/24
Benign surgery controls								
B1	FPC	PanIN2 + pNET <2 cm	T1N0M0	F/49	*CDKN2A*	5/10	2	49/50
B2	FPC	PanIN2	NA	M/47	FPC	4/11	4	0/17
B3	FPC	PanIN1	NA	M/46	FPC	2/11	2	0/5
B4	FPC	MD-PMN, IGD	NA	F/47	*BRCA2*	3/6	18	0/28
B5	FPC	MT-IPMN, LGD	NA	F/64	FPC	2/4	3	0/5
B6	Leiden	No precursor	NA	M/58	*CDKN2A*	2/2	14	23/26
B7	Leiden	No precursor	NA	M/55	*CDKN2A*	2/2	0	23/26
B8	FPC	No precursor	NA	M/50	*BRCA2*	2/3	2	14/16
Other malignancy controls								
M1	FPC	Melanoma	T1aN0M0	F/50	*CDKN2A*	1/11	6	6/6
M2	Leiden	Melanoma	T1aN0M0	F/47	*CDKN2A*	1/3	11	11/11
M3	Leiden	Melanoma	T1bN0M0	F/58	*CDKN2A*	2/2	3	55/55
M4	FPC	Breast cancer	T1N0M0	F/54	FPC	5/9	10	58/59
M5	FPC	Breast cancer	TxN0M0	F/59	FPC	7/7	0	83/87
M6	FPC	Breast cancer	TxNx	F/70	FPC	2/4	0	12/NA
M7	FPC	Esophageal cancer	TxN0M0	F/57	*CDKN2A*	2/2	6	18/22
M8	FPC	Lymphoma	NA	F/50	FPC	2/3	2	27/NA
M9	FPC	Duodenal NET <2 cm	T2N0M0	M/51	*CDKN2A*	1/2	9	0/9

PDAC = pancreatic ductal adenocarcinoma, MD-IPMN = main-duct intraductal papillary mucinous neoplasm, MT-IPMN = mixed-type intraductal papillary mucinous neoplasm, IGD = intermediate grade dysplasia, LGD = low-grade dysplasia, (p)NET = (pancreatic) neuroendocrine tumor, FPC = mutation-negative familial pancreatic cancer kindred.

3.3. Comparison of glycosylation traits between cases and controls

Selection of candidate glycosylation traits was based on published data from our group comparing protein glycosylation in sporadic PDAC cases and healthy controls [14]. In the current study, we evaluated 13 (1st quartile with lowest p-value) of the 51 glycosylation traits that differed between PDAC cases and controls ($\leq$0.05). Notably, all selected glycosylation traits consisted of 'complex-type' glycans (Fig. 1).

To evaluate the natural course of these glycosylation traits per investigated group, their mean relative abundance was plotted over time for cases (n = 10) and controls (N = 155; Fig. 2). In the case group, values of three glycosylation traits decreased over time (CA2, CB0, A3F0L; Fig. 2A, C, I; Supplemental Figs. S1A and C; Supplemental Fig. S2C), whereas ten increased (CA4, CFa, A3F, A3Fa, A2LF, A3LF, A3FE, A4FE, A2F0E, A3F0E; Fig. 2B, D-H, J-M; Supplemental Fig. S1B, D-F; Supplemental Figs. S2A and B, D-G; Table 3). These decreases and increases were in agreement with down- and upregulation, respectively, as previously objectified in cross-sectional analysis (sporadic PDAC *vs* healthy controls; Table 3) [14]. The majority of PDAC cases already showed differences in glycosylation traits 3–50 months prior to PDAC development (t_0; Table 3) as compared to controls. The glycosylation levels of two glycosylation traits were higher at the last measurement before PDAC diagnosis ($p \leq 0.004$; A2LF, A3Fa; data not shown). With regard to change over time, the difference between first and last measurement was calculated and normalized by the time in months. After correction for multiple testing, one glycosylation trait (A3F) showed a different change over time for cases, as compared to controls ($p \leq 0.004$; data not shown).

As the majority of the cases harbored a *CDKN2A* germline mutation (9/10), we also visualized the course of glycosylation traits for all cases and controls with a *CDKN2A* germline mutation (Supplemental Fig. S3). This cohort showed a similar course as that of the total cohort.

3.4. Resected controls and other malignancies

To evaluate if changes in glycosylation levels (over time) are due to cancer (or malignant development) in general rather than PDAC specifically, the mean relative abundance was plotted over time for three subgroups (Fig. 3): 1. cases (n = 10); 2. pathology-proven benign surgery controls (n = 8); 3. other malignancy controls (n = 9; Table 2). Due to the limited sample size, we did not perform a statistical analysis, yet graphs indicate a difference between PDAC cases and benign surgery controls, as well as the other malignancy controls for six glycosylation traits (CFa, A3F, A3Fa, A2LF, A3LF, A4FE; Fig. 3D–H, K).

3.5. Diagnostic performance of change over time

For glycosylation traits that showed a significant difference between cases and controls over time, ROC curves were generated (Fig. 4A). Overall, A3F performed best (AUC 0.77; 95% CI 0.62–0.92). Per glycosylation trait, two cut-offs were selected, the first aiming at high sensitivity (with specificity $\geq$40%), the second aiming at

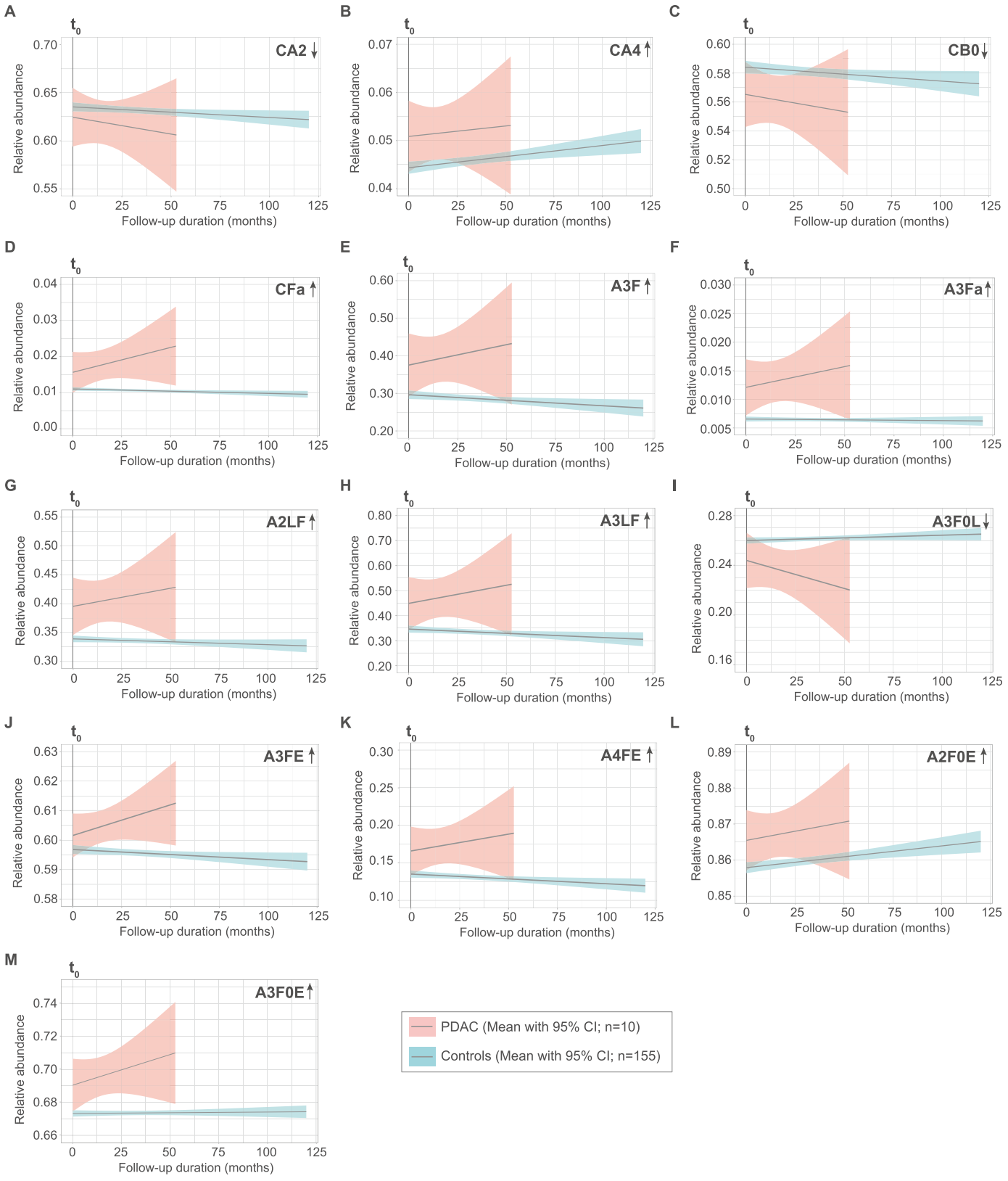


Fig. 2. Graphs showing the relative abundance over time for 13 candidate markers. The black lines indicate the mean per group (cases = pink, controls = blue) with 95% confidence interval (CI). Arrows indicate whether the glycosylation trait was up- or downregulated in PDAC cases in previous cross-sectional analysis[14]).

Table 3
Overview of odds-ratio (OR) at previous cross-sectional analysis[14] as well as the median glycosylation trait level for cases and controls at baseline and at most recent sample. Grey rows indicate a lower value when developing PDAC in cross sectional analysis[14]. Arrows in the glycosylation in last sample column indicate whether the glycosylation value increased or decreased compared to baseline.

	Cross-sectional analysis[14]		Glycosylation at baseline			Glycosylation in last sample		
	OR[14] (95% CI)	**P-value[14]**	**Cases** Median (IQR)	**Controls** Median (IQR)	**Ratio** Cases/controls	**Cases** Median (IQR)	**Controls** Median (IQR)	**Ratio** Cases/controls
Complex type glycans								
CA2	0.35 (0.25–0.50)	1.052E-08	0.632 (0.094)	0.633 (0.056)	0.998	0.630 (0.050) ↓	0.631 (0.057) ↓	0.998
CA4	6.19 (3.57–10.75)	9.209E-11	0.052 (0.031)	0.043 (0.013)	1.209	0.049 (0.015) ↓	0.045 (0.015) ↑	1.089
CB0	0.39 (0.27–0.54)	5.12E-08	0.565 (0.062)	0.579 (0.057)	0.976	0.571 (0.038) ↑	0.582 (0.055) ↑	0.981
CFa	13.27 (5.68–30.98)	2.306E-09	0.013 (0.008)	0.011 (0.005)	1.182	0.019 (0.019) ↑	0.011 (0.006) =	1.727
Fucosylation (F)								
A3F	2.34 (1.70–3.23)	2.066E-07	0.363 (0.208)	0.282 (0.158)	1.287	0.460 (0.327) ↑	0.281 (0.154) ↓	1.637
A3Fa	5.35 (3.01–9.52)	1.115E-08	0.011 (0.007)	0.006 (0.005)	1.833	0.015 (0.021) ↑	0.006 (0.005) =	2.500
A2LF	2.67 (1.88–3.78)	3.854E-08	0.389 (0.140)	0.337 (0.088)	1.154	0.457 (0.203) ↑	0.332 (0.083) ↓	1.377
A3LF	2.68 (1.91–3.75)	9.323E-09	0.425 (0.265)	0.324 (0.199)	1.312	0.565 (0.421) ↑	0.333 (0.191) ↑	1.697
A2,3-linked sialylation (L)								
A3F0L	0.34 (0.23–0.49)	8.342E-09	0.246 (0.041)	0.260 (0.035)	0.946	0.221 (0.098) ↓	0.260 (0.032) =	0.850
A2,6-linked sialylation (E)								
A3FE	2.44 (1.73–3.43)	3.184E-07	0.601 (0.015)	0.595 (0.019)	1.010	0.610 (0.024) ↑	0.598 (0.018) ↑	1.020
A4FE	3.5 (2.32–5.30)	2.622E-09	0.157 (0.075)	0.133 (0.073)	1.180	0.201 (0.126) ↑	0.132 (0.070) ↓	1.523
A2F0E	3.99 (2.55–6.24)	1.203E-09	0.869 (0.024)	0.858 (0.021)	1.013	0.872 (0.032) ↑	0.859 (0.021) ↑	1.015
A3F0E	2.63 (1.88–3.69)	2.019E-08	0.686 (0.018)	0.672 (0.027)	1.021	0.707 (0.065) ↑	0.674 (0.027) ↑	1.051

OR = odds ratio; 95% CI = 95% confidence interval; IQR = interquartile range.

high specificity (sensitivity ≥40%; Fig. 4B). When aiming high sensitivity, A3F had a sensitivity 90.0% (95% CI 55.5–99.8%) and specificity of 49.0 (95% CI 42.8–59.1%); when aiming high specificity, the sensitivity was 40.0% (95% CI 12.2–73.8%) and specificity 96.1 (95% CI 91.8–96.6%).

4. Discussion

After our explorative study among symptomatic PDAC patients, we now evaluated the longitudinal performance of glycosylation traits in a PDAC surveillance cohort consisting of hereditarily predisposed high-risk individuals. Our results demonstrate that a change of distinct serum glycosylation traits is related to the development of PDAC in carriers of a *CDKN2A* germline mutation (as 9/10 cases were). Of the 13 investigated glycosylation traits, A3F showed significant deviations during the course of PDAC development (after correction for multiple testing). Interestingly, biomarker differences seem to have been present at baseline and increasingly changed during the course of PDAC development. This may indicate a long window of opportunity to capture malignant progression. This finding is consistent with previous data showing that development of PDAC takes multiple years [17] and potentially makes these biomarkers useful for surveillance purposes.Imaging-based surveillance programs have not yet convincingly shown improved survival in individuals undergoing pancreas surveillance for hereditary risk [18]. Diagnosis of PDAC in surveillance programs for high-risk individuals rely on imaging and FNA/FNB (in case of suspected PDAC). However, both modalities have a limited discriminative performance for small lesions and the use of FNA/FNB is limited due to potential adverse events in case of repetitive sampling [19,20]. Due to high rates of false positive findings (and therefore potential harm), repetitive CA19-9 monitoring in individuals at risk of developing PDAC is not recommended by guidelines [3]. Thus, there is an urgent need for more sensitive and specific biomarkers to complement imaging in surveillance programs [21].

Implementation of a (panel of) biomarkers in a surveillance program could guide risk stratification and select individuals who should undergo intensified surveillance (in case of a test with high sensitivity), yet could also support in decision-making for surgery or additional diagnostic procedures (in case of a test with high specificity). For this reason, we selected two cut-offs for each glycosylation trait aiming at high sensitivity or high specificity. The performance of changes of glycosylation traits over time was promising (accuracy 87.9–92.7%). For surveillance purposes, a potent biomarker is expected to vary over time, yet, in current literature, data on longitudinal sampling of biomarkers is lacking. This well-defined surveillance cohort offers the unique opportunity to perform this longitudinal analysis as it has a relatively high incidence of PDAC and surveillance was performed in a standardized fashion [22]. At the same time, due to the time-consuming set-up of a surveillance study (median follow-up 35 months) and the relatively low prevalence of PDAC, the absolute number of cases in this cohort was limited and only a first indication of potential differences over time can be extracted. For this reason, we have decided to show our data in a descriptive fashion without extensive statistical analysis. Additionally, as studies have previously shown distinct molecular and transcriptomic subtypes, protein glycosylation may also be heterogeneous between patients. Therefore, it is likely that a panel of biomarkers that complements each other is required [23]. Unfortunately, the low number of cases in this study did not allow analysis of combined biomarkers. Furthermore, we were not able to correct for clinical variables, as these may also influence glycosylation level. One example is the presence of diabetes mellitus, Dotz et al. (2018) [24] has previously shown that alpha-2,6-linked sialylation is increased (odds-ratio 1.4; p = 5.48E-07) in plasma samples of patients with diabetes mellitus. Another example is the presence of a germline mutation. The majority of the cases (9/10) were *CDKN2A* germline mutation carriers. Therefore, we cannot formally exclude the possibility that the results of this study show the differences between a subgroup of high-risk *CDKN2A* mutation carriers and controls, rather than PDAC cases and controls, *per se*. However, a similar course of markers is observed when only *CDKN2A* mutation carriers are considered (Supplemental Fig. S3). External validation in other cohorts with more variety in high-risk cases would allow assessment of generalizability of these markers beyond this subgroup. Lastly, another limitation is that the sample collection of one cohort was less standardized, leading to a lower number of consecutive samples per individual. Thus, external validation with a larger cohort (preferably including more longitudinal data points) is required to evaluate if these biomarkers really enable both risk stratification

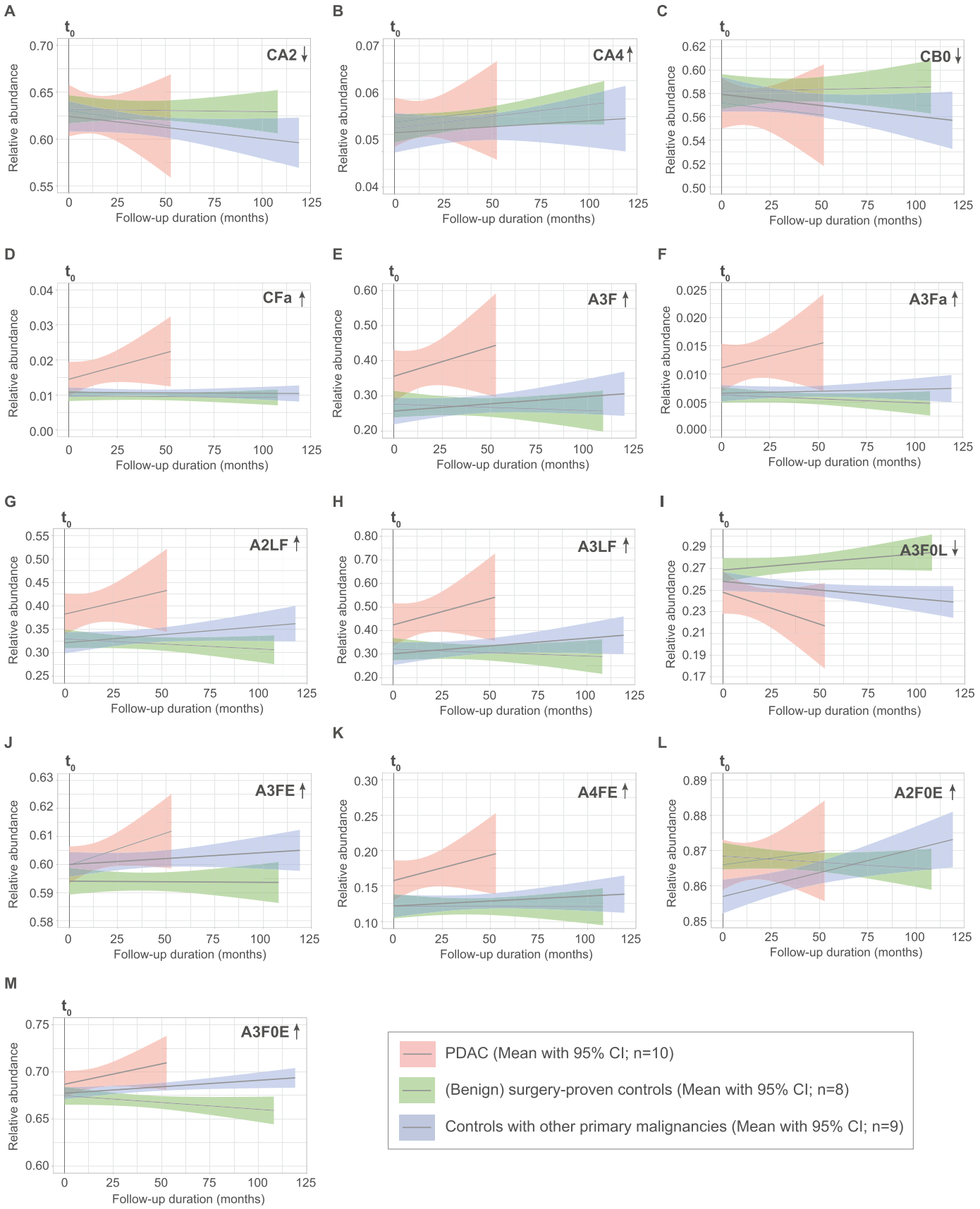
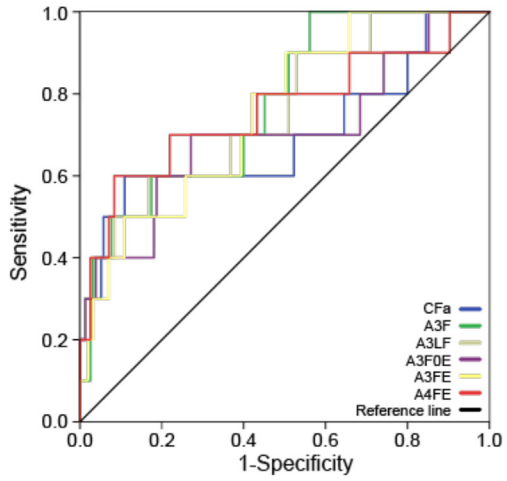


Fig. 3. Graphs showing the relative abundance over time for 13 glycosylation traits, comparing three groups: 1. Patients with PDAC (pink); 2. Patients who underwent pancreatic resection, which appeared to be no/low-grade dysplasia ('benign surgery controls'; green); 3. Patients who develop other types of malignancies during the course of surveillance (blue). The black lines indicate the mean per group, the colored area indicates the 95% confidence interval (CI) per group. Arrows indicate whether the glycosylation trait was up- or downregulated in previous cross-sectional analysis [14]).

A



B

Derived trait	AUC (95% CI)	Cut-off	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)
CFa	0.70 (0.48-0.91)	0.000003	70.0 (34.8-93.3)	47.7 (39.7-55.9)	49.1 (41.2-57.0)
		0.000141	50.0 (18.7-81.3)	94.2 (89.3-97.3)	91.5 (86.2-95.3)
A3F	0.77 (0.64-0.91)	-0.000108	90.0 (55.5-99.8)	49.0 (44.0-60.8)	54.7 (46.5-62.7)
		0.001986	50.0 (18.7-86.8)	92.3 (86.9-95.9)	89.7 (84.0-93.9)
A3LF	0.76 (0.60-0.92)	-0.000082	90.0 (55.5-99.8)	47.1 (39.0-55.3)	49.7 (41.8-57.6)
		0.002235	50.0 (18.7-81.3)	90.3 (84.5-94.5)	87.9 (81.9-92.4)
A3F0E	0.70 (0.50-0.90)	0.000193	70.0 (34.8-93.3)	72.3 (64.5-79.1)	72.1 (64.6-78.8)
		0.000807	40.0 (12.2-73.8)	96.1 (91.8-98.6)	92.7 (87.6-96.2)
A3FE	0.75 (0.61-0.90)	-0.000006	90.0 (55.5-99.8)	49.7 (41.6-57.8)	52.1 (44.2-50.0)
		0.000341	40.0 (12.2-73.8)	92.9 (87.7-96.4)	89.7 (84.0-93.9)
A4FE	0.76 (0.57-0.95)	0.000039	80.0 (44.4-97.5)	56.8 (48.6-64.7)	58.2 (50.3-65.8)
		0.000594	60.0 (26.2-87.8)	91.6 (86.1-95.5)	89.7 (84.0-93.9)

Fig. 4. Diagnostic performance of change over time (between baseline and most recent measurement) for the differentiation between cases and controls. Only those glycosylation traits that were significantly different change (cases *vs* controls; p<0.05) were shown. A. ROC-curves **B.** Table showing the sensitivity, specificity and accuracy of individual glycosylation traits. Two cut-offs were selected aiming at: 1. high sensitivity (specificity ≥40%); 2. high specificity (sensitivity ≥40%). AUC = area under the curve; CI = confidence interval.

and early detection of PDAC (independent of clinical characteristics and how they perform when combined in a panel) [25].

Technical advances in high-throughput protein analysis have enabled identification of several post-translational changes, including glycosylation [26]. These protein alterations are of interest, as such modifications are be expected to be more disease-specific than solely a protein concentration. In our previous case-control study [14], we have identified a PDAC signature that is able to differentiate PDAC from controls. This current study shows that a change of A3F is indicative for PDAC development in a high-risk population. However, at this point, our markers did not outperform the accuracy commonly reported for CA19-9 [9,27]. In order to be successfully implemented in surveillance programs, sensitivity and specificity need improvement. Moreover, future in-depth glycoproteomic analyses are needed to give more detailed information on the protein origin and further specify a PDAC signature [28–31]. It is foreseen that the determination of these glycosylation changes in a protein-specific manner (i.e. glycoproteomics) will further increase the diagnostic potential (Fig. 1) [32]. In conclusion, this longitudinal study evaluated the relative abundance of 13 candidate glycosylation markers in consecutively collected serum samples. Our findings demonstrate the potential of specific glycosylation traits that change in the course of PDAC development. Future serum glycoproteomic analyses, which reveal glycoproteins driving these changes, are necessary and may result in more specific disease markers. Additionally, evaluating these markers in larger prospective studies is warranted to replicate these findings, assess heterogeneity between PDAC cases (e.g., hereditary *vs* sporadic) and allow correction for clinical variables to evaluate whether these markers are independently associated with PDAC development.

Acknowledgements

We would like to acknowledge R.L.P van Vlierberghe. He does not fulfill the requirements for authorship, yet substantially attributed to the project. The current study was supported by ZonNW project number 531002011 and Genootschap Bollenstreek – Bollenstreekfonds, Lisse-Hillegom, The Netherlands.

I.J.M. Levink, D.C.F. Klatte, R.G. Hanna-Sawires, G.C.M. Vreeker, I.S. Ibrahim, Y.E.M. van der Burgt, K.A. Overbeek, B.D.M. Koopmann, D.L. Cahen, G.M. Fuhler, M. Wuhrer, B.A. Bonsing, R.A.E.M. Tollenaar, H.F.A. Vasen, W.E. Mesker, and M.E. van Leerdam have no conflicts of interest. F.P. Vleggaar is a Boston Scientific consultant. M.J Bruno has the following conflicts of interest: Boston Scientific (Consultant, support for industry and investigator-initiated studies), Cook Medical (Consultant, support for industry and investigator-initiated studies), Pentax Medical (Consultant, support for investigator-initiated studies), Mylan (Support for investigator-initiated studies), ChiRoStim (Support for investigator-initiated studies).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2022.03.021.

References

[1] Versteijne E, Suker M, Groothuis K, et al. Preoperative chemoradiotherapy versus immediate surgery for resectable and borderline resectable pancreatic cancer: results of the Dutch Randomized Phase III PREOPANC trial. J Clin Oncol 2020;38:1763–73.

[2] Vasen H, Ibrahim I, Ponce CG, et al. Benefit of surveillance for pancreatic cancer in high-risk individuals: outcome of long-term prospective follow-up studies from three European expert centers. J Clin Oncol 2016;34:2010–9.

[3] Goggins M, Overbeek KA, Brand R, et al. Management of patients with increased risk for familial pancreatic cancer: updated recommendations from the International Cancer of the Pancreas Screening (CAPS) Consortium. Gut 2020;69:7–17.

[4] Overbeek KA, Levink IJM, Koopmann BDM, Harinck F, Konings ICAW, Ausems MGEM, Wagner A, Fockens P, van Eijck CH, Koerkamp B Groot, Busch OR, MGBesselink BAJ Bastiaansen, van Driel LMJW, Erler NS, Vleggaar FP, Poley JW, Dl Cahen JE van Hooft, Bruno MJ. On behalf of the Dutch Familial Pancreatic Cancer Surveillance Study Group. Long-term yield of pancreatic cancer surveillance in high-risk individuals. Gut 2021.

[5] Corral JE, Mareth KF, Riegert-Johnson DL, et al. Diagnostic yield from screening asymptomatic individuals at high risk for pancreatic cancer: a meta-analysis of cohort studies. Clin Gastroenterol Hepatol 2019;17:41–53.

[6] Cheng B, Zhang Y, Chen Q, et al. Analysis of fine-needle biopsy vs fine-needle aspiration in diagnosis of pancreatic and abdominal masses: a prospective, multicenter, Randomized controlled trial. Clin Gastroenterol Hepatol 2018;16:1314–21.

[7] Sugiura R, Kuwatani M, Hirata K, et al. Effect of pancreatic mass size on clinical outcomes of endoscopic ultrasound-guided fine-needle aspiration. Dig Dis Sci 2019;64:2006–13.

[8] Kromrey M-L, Bülow R, Hübner J, et al. Prospective study on the incidence, prevalence and 5-year pancreatic-related mortality of pancreatic cysts in a population-based study. Gut 2018;67:138.

[9] Fahrmann JF, Schmidt CM, Mao X, et al. Lead-time Trajectory of CA19-9 as an

anchor marker for pancreatic cancer early detection. Gastroenterology 2021;160:1373–1383.e6.
[10] Balmana M, Gimenez E, Puerta A, et al. Increased alpha1-3 fucosylation of alpha-1-acid glycoprotein (AGP) in pancreatic cancer. J Proteonomics 2016;132:144–54.
[11] Krishnan S, Whitwell HJ, Cuenco J, et al. Evidence of altered glycosylation of serum proteins prior to pancreatic cancer diagnosis. Int J Mol Sci 2017;18.
[12] Munkley J. The glycosylation landscape of pancreatic cancer. Oncol Lett 2019;17:2569–75.
[13] Hanna-Sawires RG, Schiphuis JH, Wuhrer M, et al. Clinical Perspective on Proteomic and glycomic biomarkers for diagnosis, Prognosis, and prediction of pancreatic cancer. Int J Mol Sci 2021:22.
[14] Vreeker GCM, Hanna-Sawires RG, Mohammed Y, et al. Serum N-Glycome analysis reveals pancreatic cancer disease signatures. Cancer Med 2020;9:8519–29.
[15] Vasen HF, Gruis NA, Frants RR, et al. Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). Int J Cancer 2000;87:809–11.
[16] Vreeker GCM, Nicolardi S, Bladergroen MR, et al. Automated plasma glycomics with linkage-specific sialic acid esterification and ultrahigh Resolution MS. Anal Chem 2018;90:11955–61.
[17] Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 2010;467:1114–7.
[18] Lu C, Xu CF, Wan XY, et al. Screening for pancreatic cancer in familial high-risk individuals: a systematic review. World J Gastroenterol 2015;21:8678–86.
[19] Sugiura R, Kuwatani M, Hirata K, et al. Effect of pancreatic mass size on clinical outcomes of endoscopic ultrasound-guided fine-needle aspiration. Dig Dis Sci 2019;64:2006–13.
[20] Walsh RM, Zuccaro G, Dumot JA, et al. Predicting success of endoscopic aspiration for suspected pancreatic cystic neoplasms. J Oper Manag 2008;9:612–7.
[21] Pereira SP, Oldfield L, Ney A, et al. Early detection of pancreatic cancer. Lancet Gastroenterol Hepatol 2020;5:698–710.
[22] Saad AM, Turk T, Al-Husseini MJ, et al. Trends in pancreatic adenocarcinoma incidence and mortality in the United States in the last four decades; a SEER-based study. BMC Cancer 2018;18:688.
[23] Llop E, Eg P, Duran A, et al. Glycoprotein biomarkers for the detection of pancreatic ductal adenocarcinoma. World J Gastroenterol 2018;24:2537–54.
[24] Dotz V, Lemmers RFH, Reiding KR, et al. Plasma protein N-glycan signatures of type 2 diabetes. Biochim Biophys Acta Gen Subj 2018;1862:2613–22.
[25] Capello M, Bantis LE, Scelo G, et al. Sequential validation of blood-based protein biomarker candidates for early-stage pancreatic cancer. J Natl Cancer Inst 2017;109.
[26] Wang M, Zhu J, Lubman DM, et al. Aberrant glycosylation and cancer biomarker discovery: a promising and thorny journey. Clin Chem Lab Med 2019;57:407–16.
[27] Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. Eur J Surg Oncol 2007;33:266–70.
[28] Pan S, Chen R, Tamura Y, et al. Quantitative glycoproteomics analysis reveals changes in N-glycosylation level associated with pancreatic ductal adenocarcinoma. J Proteome Res 2014;13:1293–306.
[29] Kontro H, Joenvaara S, Haglund C, et al. Comparison of sialylated N-glycopeptide levels in serum of pancreatic cancer patients, acute pancreatitis patients, and healthy controls. Proteomics 2014;14:1713–23.
[30] Singh S, Pal K, Yadav J, et al. Upregulation of glycans containing 3' fucose in a subset of pancreatic cancers uncovered using fusion-tagged lectins. J Proteome Res 2015;14:2594–605.
[31] Holst S, Belo AI, Giovannetti E, et al. Profiling of different pancreatic cancer cells used as models for metastatic behaviour shows large variation in their N-glycosylation. Sci Rep 2017;7:16623.
[32] Clerc F, Reiding KR, Jansen BC, et al. Human plasma protein N-glycosylation. Glycoconj J 2016;33:309–43.