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## Detection of pancreatic cancer using serum protein profiling

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#### ABSTRACT

#### Background

Currently, no suitable biomarkers for early detection of pancreatic cancer (PC) are available. Proteins present in the serum could reflect a state of disease. In this study we evaluated these profiles as a diagnostic marker for PC.

#### Methods

Serum samples were obtained from PC patients (n = 50 calibration set, n = 39 validation set) and healthy volunteers (n = 110 and n = 75 respectively) according to a uniform standardized collection and processing protocol. For peptide- and protein isolation, automated solid-phase extraction (SPE) with Weak Cation Exchange (WCX) magnetic beads was performed using a 96-channel liquid handling platform. Protein profiles were obtained by mass spectrometry and evaluated by linear discriminant analysis with double cross-validation.

#### Results

We have identified a discriminating profile for PC, with a sensitivity of 78% and a specificity of 89% in the calibration set with an area under the curve (AUC) of 90%. These results were validated with a sensitivity of 74% and a specificity of 91% (AUC 90%).

#### Conclusion

We can discriminate between serum profiles of healthy controls and PC. Further research is warranted to evaluate specificity and whether this biosignature can be used for early detection in a high risk population.

#### INTRODUCTION

Although pancreatic cancer (PC) has an annual incidence of only 8.2 cases per 100 000 males and of 5.4 cases per 100 000 females, it is the fifth (male) and fourth (female) leading cause of cancer death in developed countries.<sup>1</sup> Patients with PC have an extremely poor prognosis with an overall 5-year survival rate of less than 5%.<sup>2</sup> When surgical resection is possible, 5-year survival rates increase to approximately 25%, but unfortunately most tumors are at an advanced stage when diagnosed.<sup>3,4</sup> Delays in diagnosis are often caused by the lack of specific symptoms for early cancers, such as pain, jaundice and weight loss. Biomarkers might be an additional tool for diagnostics next to currently available imaging techniques. The mostly studied available clinical serum biomarker carbohydrate antigen 19-9 (CA19-9) has a sensitivity of 80% and a specificity of 90% but misses the appropriate sensitivity and specificity for small, resectable cancers.<sup>5</sup> Moreover, CA19-9, is often elevated in benign cholangitis, pancreatitis and other cancers, and therefore lacks the specificity for detecting potentially curable lesions. At this moment the use of CA19-9 is only recommended for follow up. Currently, only imaging techniques such as ultrasound (transcutaneous or endoscopic), CT scan, endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP) are used for diagnosis and staging of the pancreatic disease.

Chronic pancreatitis could mimic PC at diagnostics and hampers patient selection for a pancreaticoduodenectomy. For these patients a new biomarker that discriminates between pancreatitis and cancer could be of great value.

It has been estimated that 5% to 10% of PC cases are associated with an inherited predisposition. Tumor syndromes associated with an increased risk of PC include Peutz–Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM), hereditary breast cancer (BRCA2 mutation carriers), and possibly Lynch syndrome. The lifetime risk of PC varies between 5% in BRCA2 mutation carriers and 36% in patients with Peutz–Jeghers syndrome.<sup>6-9</sup> For this patient group, early detection is of paramount importance since prognosis is usually dismal when diagnosis follows symptoms. No studies for early detection in this specific high risk group using CA19-9 have been performed. At this time this is only possible through imaging surveillance.<sup>10</sup> Therefore, there is an urgent need for new and better biomarkers for PC.

A sensitive and specific option could be the use of proteomic serum biomarkers. During transformation of a normal cell into a neoplastic cell, distinct changes occur at the protein level which may affect cellular function.<sup>11</sup> Therefore, proteins are considered promising targets for biomarker discovery. Mass spectrometry (MS) has become the method of choice for protein analysis in serum.<sup>12,13</sup> Provided standardized sample workup, MS measurement, data processing and –evaluation, peptide- and protein pro-

files are highly reproducible.<sup>14</sup> With respect to speed and automation strategies needed for high-throughput screening, matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) MS workflows remain unrivalled.<sup>12,15,16</sup> Since human body fluids such as serum are highly complex a suitable "clean-up" procedure is required.<sup>17</sup> Based on the physicochemical properties of protein separation techniques magnetic beads (mb's) have been functionalized accordingly (*e.g.* Weak Cation Exchange (WCX). These beads are not only suited for clean-up, but also enrich subsets of peptides and proteins and can thus contribute to the sensitivity of the assay.<sup>18</sup> Our serum peptide/protein capture procedure has been fully automated with a liquid handling robot, such as a 96-channel Hamilton STARplus<sup>®</sup> platform. This ensures reproducibility and allows high-throughput screening which is essential for large scale disease profiling studies.<sup>19,20</sup> In the last decade multiple studies have been carried out using a magnetic bead-based method for offline serum peptide/protein capture and MALDI-TOF-MS readout.<sup>14,21-25</sup>

For pancreatic cancer several proteomic studies have been performed since the introduction of protein profiling in 2002 by Petricoin et al<sup>26</sup>, all with different fractionation platforms and type of MSs.<sup>27-33</sup> For example, Koopman et al<sup>34</sup> used a SELDI-TOF approach combined with WCX and metal affinity protein chips as SPE method to discriminate between patient groups with a sensitivity of 78% and a specificity of 97%. No study has been published using the combination of WCX mb's and MALDI-TOF.

For this study a MALDI-TOF serum platform in combination with functionalized WCX mb's was used to generate serum protein profiles in a first attempt to differentiate between PC patients and healthy controls in a stringent sample handling and high throughput and automated processing protocol. The obtained discriminating profile was validated in a second case-control group.

#### MATERIAL AND METHODS

#### Patients

Blood samples were obtained from 50 patients with PC prior to surgery, and from 110 (age and sex matched) healthy volunteers at the outpatient clinic of the Leiden University Medical Center (LUMC), the Netherlands from October 2002 until December 2008. Healthy volunteers were partners or accompanying persons of included patients. For the validation set, blood samples were obtained from 39 patients and 75 healthy (age and sex matched) volunteers, included from January 2009 until July 2010. Patients were selected candidates for curative surgery; this meant that no patients with primary irresectable tumors were included. All surgical specimens were examined according to routine histological evaluation and the extent of the tumor spread was assessed by TNM

(TNM Classification of Malignant Tumors) classification. Furthermore, the tumor marker CA-19.9 was noted if determined pre-operative. An Elecsys CA 19-9 tumor marker assay based on the monoclonal 116-NS 19-9 antibody (Roche) was used. This tumor marker has a normal reference value of 0.0-27.9 U/ml (95<sup>th</sup> percentile). Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the LUMC.

#### **Blood collection**

Samples from both the calibration set and the validation set were collected and processed according a standardized protocol<sup>14</sup>: shortly, all blood samples were drawn by antecubital venapuncture while the individuals were seated and had not been fasting prior to any invasive procedure. The samples were collected in an 8.5 cc Serum Separator Vacutainer Tube (BD Diagnostics, Plymouth, UK) and within maximally 4 hours at room temperature centrifuged at 1000g for 10 minutes.<sup>14</sup> The samples were then distributed into sterile 500  $\mu$ l barcode labeled polypropylene aliquots (TrakMate, Matrix TechCorp.) and stored at -80°C.

#### Sample processing

#### Aliquotting and storage

An overview of the processing platform of the serum samples, MALDI-TOF profiles and data is given in figure 1. All serum samples were thawed on ice once and randomly placed in barcode labeled racks in an 8-channel Hamilton STAR® pipetting robot (Hamilton, Bonaduz, Switzerland) for automated aliquotting in 60 µl daughter tubes. The aliquots were stored at -80°C until further sample processing. The processing steps for the validation set were identical with those for the calibration set.

#### WCX-mb sample work up

The isolation of proteins from serum was performed using a commercially available kit based on magnetic bead purification (Bruker Daltonics, Bremen, Germany). The WCX mb's were applied according to the manufacturer's instructions with further optimization to allow implementation on a 96-channel Hamilton STARplus® pipetting robot (Hamilton). Binding- and washing buffers were used from the kit. In the final pipetting steps, peptides and proteins were first eluted from the beads using a 130 mM ammoniumhydroxide solution (J.T. Baker, Deventer, The Netherlands) and then stabilized with a 3% trifluoro acetic acid solution (Sigma, St Louis, USA).



**Figure 1.** Overview of the used workflow model of sample, profile, data processing and statistics. Sample processing:

- 1. Aliquotting and storage of the serum at -80 degrees Celsius until further processing.
- 2. Automated fractionation of samples with WCX mb's. Storage until measurement at -80 degrees Celsius.
- 3. Automated spotting in quadruplicate on maldiplate.

Profile processing

- 1. Measurements of maldiplates in MALDI-TOF.
- 2. Baseline correction & alignment of four profiles per samples, removal of spectra without signal due to spotting failure.
- 3. Selection of interesting peaks and extraction of this data with Xtractor.
- Data processing & statistics
- 1. Data-analysis on mean of remaining profiles.
- 2. Analysis of the profiles with linear discriminant analysis with double cross validation.

#### MALDI spotting

Two microliter of each stabilized eluate was transferred into a 384-well microtitrationplate to carry out mixing with MALDI matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid from Bruker Daltonics, 3 mg/mL in acetone/ethanol 1:2). One microliter of this mixture was spotted in quadruplicate onto a MALDI 600  $\mu$ m AnchorChip<sup>TM</sup> plate (Bruker Daltonics).

#### **Profile processing**

#### MALDI-TOF measurement

MALDI-TOF mass spectra (profiles) of the peptides and proteins were obtained using a positive-ion linear mode acquisition on an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics) equipped with a SCOUT ion source and controlled by the Flexcontrol 3.0 software package (Bruker Daltonics). Ions generated by the Smartbeam<sup>™</sup> laser were accelerated to 25 kV and mass analyzed from 960 to 11,024 Da. Each mass spectrum represents the sum of 20 mass spectra obtained from 60 laser shots. All unprocessed spectra were exported from the Ultraflex II in standard 8-bit binary ASCII format.

#### Baseline correction & alignment of profiles

For optimal data analysis, all profiles generated after sample workup with WCX magnetic beads (further referred to as WCX-profiles) required baseline correction followed by alignment. First, a baseline subtraction of all profiles was performed using the baseline subtraction tool of FlexAnalysis 3.0. Second, to perform the alignment of WCX profiles from one MALDI target plate at least three peptides at different *m*/*z*-values were essential for internal calibration. In order to compensate for the possible absence of one or two peptides in a spectrum, the following seven peptides were selected based on a manual inspection of a few spectra, namely at *m*/*z* 1866.1, 3158.0, 4643.6, 5903.7, 6631.1, 7765.5 and 9290.9.<sup>22,35</sup>

#### Peak selection and -quantification

Protein and/or peptide signals in WCX-profiles were quantified as follows. First, based on visual inspection of the profiles, 113 peaks in WCX were selected for further analysis. To this end, a so-called reference file was compiled for both types of profiles including a certain m/z-window for each signal or peak. In the WCX-profiles, this m/z-window reflected the peak width and varied from 5 – 30 Da. Three examples of the selected peaks are shown in figure 2. Then, the in-house developed Xtractor tool was used to determine the intensity of each user-defined peak. This open source tool generates uniform data (peak) arrays regardless of spectral content (www.msutils.org/Xtractor).

MALDI-TOF profiles were exported as DAT (.dat) files, all containing *m*/*z*-values with corresponding intensities.

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**Figure 2.** Example of three of the selected peaks from WCX fractionated sampling. On the x-axis m/z values are shown, on the y-axis intensities. The peaks with a m/z of 2084, 2770 and 8393 were highlighted.

#### **Data processing and statistics**

#### Mean of replicate spectra

The peakfiles generated by Xtractor were used for data analysis. The mean of the remaining profiles of the quadruplicate spots for the WCX purified samples was used. These processed profiles will be further referred to as WCX dataset.

For each  $i^{th}$  patient, i = 1, ..., n a set of spectral measurements  $x^{1}_{i} = (x^{1}_{i1}, ..., x^{1}_{il})$  is collected from the WCX bead processed samples such that the complete data may be represented by the matrix

$$X = \begin{pmatrix} X_1 \\ \vdots \\ \vdots \\ X_n \end{pmatrix}$$

with n = 160 and l = 113 (calibration set) represent the dimensionality of the peak list. To complete the observed information on individuals, we have the binary case-control outcome *Y* which equals 1 for cases or 0 for controls<sup>36</sup> (n = 50 cases, n = 110 controls). The same procedure is performed for the validation set with n = 114 and l = 113 (n = 39 cases, n = 75 controls).

#### Linear discriminant analysis

As previously described by Mertens et al<sup>35</sup> and de Noo et al<sup>25</sup> a double cross-validatory implementation of linear discriminant analysis for the calibration of a diagnostic rule based on a single (mean) spectrum per patient was performed. Each sample was assigned to the group for which the probability was highest. For each analysis error rate (Error), sensitivity, specificity and area under the curve (AUC) were calculated. The error rates are based on sensitivity and specificity values, assuming a prior class probability of

0.5 for each group. The validation data was subsequently predicted from a single calibration of the discriminant on the calibration data (based on the observed optima from the double-cross-validatory analysis) and results were compared with known disease status.

Receiver operating characteristic (ROC) curves with the true positive rate (sensitivity) are plotted in function of the false positive rate (1-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal).

An Independent Student's T-test on the case-control calibration data was performed on a selection of the most discriminating peaks observed in the calibrated discriminant model on the calibration data. A P-value of < 0.05 was considered as significant.

Subsequently, all analyzes were repeated after an additional internal standardization performed on the calibration and validation sets separately, by using the mean of all spectra and standard deviation (SD) such that the new data within each set is obtained as

$$X_{i}^{*} = \frac{X_{i} - \overline{X}_{i}}{SD_{i}}$$

to minimize differences in peak intensities between the calibration set and the validation set which may be caused by batch effects. It should be noted that the above standardization does not affect calibrations or results on the calibration data (but it does for any predictive analysis, such as in double cross-validation or when predicting the validation set), as linear discrimination is invariant to the above standardization.

#### RESULTS

#### Patients

Patient characteristics for each set are shown in table 1. All patients were diagnosed with adenocarcinoma of the pancreas. The calibration set consisted of 50 pre-operative PC patients (n = 23 male, n = 27 female) with a median age of 66 years (range 41-80). The validation set consisted of 39 pre-operative PC patients (17 male, 22 female), included with the same criteria as the calibration set. The median age of this group was 63 years (range 38-81). The median age of the control group in the calibration set (n = 50 male, n = 60 female) was 63 years (range 44-80) and the median age of the control group in the validation set (n = 27 male, n = 48 female) was 46 years (range 21-75). No significant difference in median ages of the cases and the controls in the calibration set (p = 0.073) was seen. In the validation set a difference was observed with a p-value of < 0.001.

	Calibration set		Correct	Validation set		Correct
	Cases	Controls	classification	Cases	Controls	classification
Ν	50	110		39	75	
Age median (min - max)	65.8 (41-80)	63.0 (44-80) <sup>~</sup>		63.4 (38-81)	45.9 (21-75) <sup>^</sup>	
Male	23	50		17	27	
Female	27	60		22	48	
Diagnosis						
Adenocarcinoma	50		38 (76%)	39		29 (74%)
Stage						
IA	7		6 (86%)	2		0 (0%)
IB	5		5 (100%)	2		1 (50%)
IIA	3		2 (67%)	3		2 (75%)
IIB	21		16 (76%)*	21		18 (86%)
III	5		3 (60%)	2		2 (100%)
IV	9		6 (67%)	9		6 (67%)
Ca 19-9	26		19 (73%)	20		14 (70%)
Pos	17		12 (71%) *	13		11 (85%)
Neg	9		7 (78%)	7		3 (43%)

**Table 1.** Patient characteristics for the calibration set and the validation set. p = 0.073, p = 0.000, n = 1 missing due to low quality profile

In the calibration set twelve (24%) patients had stage I, twenty-four (48%) had stage II, five (10%) had stage III and nine (18%) had stage IV. In the validation set four (10.3%) patients were classified as stage I, twenty-four (61.5%) as stage II, two (5.1%) as stage III and nine (23.1%) as stage IV.

In 29 patients (32.6%) tumor appeared irresectable during surgery. In all other cases (n = 60) a pancreaticoduodenectomy (65.2%) or a pancreatic tail resection (2.2%) was performed.

In total, five patients (5.7%) had an increased inherited risk for developing PC. In four patients a P16-Leiden mutation (also annotated as a 19-base pair deletion of exon 2 of the CDKN2A gene) was found. One patient was a BRCA2 mutation carrier.

#### Statistics

In total, 274 serum samples were processed with WCX Mb's and MALDI-TOF profiles were obtained in quadruplicate, yielding 1096 WCX-profiles. Only one sample was excluded from further statistical analysis because of low-quality profiles (as a result of failed sample workup or not optimal MALDI spotting).

Linear discriminant analysis with double cross-validation resulted in an error of 0.2054, a sensitivity of 73% and a specificity of 85% with an AUC of 0.90 (table 2)

**Table 2.** Error, sensitivity, specificity and AUC data in percentages from various analyzes for the calibration and the validation set. The first column represents the double-cross validation results of the analysis on 113 peaks. In the second column the results from the standardized data are shown without double cross validation. The third column shows the most reliable results after standardization and double cross validation. The last two columns represent the validation data with and without standardization. In blue highlighted are the two columns representing the definitive data of the calibration set and the validation set. NA = not applicable.

	Cal	ibration set	Validation set		
Data	Original data (113 peaks)	Selected 7 peaks	Selected 7 peaks	Original data (113 peaks)	Selected 7 peaks
Double-cross validation	Yes	No	Yes	NA	NA
Standardized	No	Yes	Yes	No	Yes
Error %	21	12	17	23	17
Sensitivity %	73	84	78	59	74
Specificity %	85	92	89	95	91
AUC	0.895	0.932	0.897	0.94	0.893



Figure 3. ROC-curves of the calibration set and validation set after peak selection with standardization.

Next, we selected a number of most discriminating peaks, based on the fitted discriminant weights from a single recalibration of the linear discriminant rule, based on the observed optima from the previous double cross-validatory analysis. We selected seven peaks with m/z values of 2084, 2178, 2770, 2899, 3096, 8760, and 8939. In table 3 these peaks are shown together with a two-sample t-test, the corresponding pooled estimate of the population standard deviation, p-value and confidence interval.

Subsequently, calculations after internal standardization were repeated as previously described and using the above set of selected peaks in the calibration set which gave an

m/z value	t-test	SD	P-value	Confidence Interval	
2084	-3,7054	0,9674	0,00029 *	-0,9438	-0,2875
2178	-0,5519	0,8925	0,58179	-0,3874	0,2182
2770	-5,5234	0,8236	0,00014 *	-1,0607	-0,5019
2899	0,1172	0,7440	0,90683	-0,2374	0,2674
3096	-2,7116	0,7581	0,00744 *	-0,6103	-0,0959
8760	-1,2342	1,1477	0,21898	-0,6327	0,1461
8939	0,5841	1,0086	0,56002	-0,2410	0,4433

**Table 3.** The seven most discriminating peaks in Dalton with corresponding t-test value, standard deviation (SD), P-value and 95%- confidence interval. Significant peaks with a p-value < 0.05 are marked with a \*.</th>

error rate of 0.12, a sensitivity of 84%, a specificity of 92% and an AUC of 0.932. This result is however likely strongly biased due to the absence of any validatory analysis and the preliminary peak selection. Adding double-cross validation to this analysis to reduce the bias resulted in the final results of an error rate of 0.17, a sensitivity of 78% and a specificity of 89% with an AUC of 0.897. Next, the standardized validation data was predicted, using the peak-selected calibration classification rule on standardized calibration data, confirming our first findings; which gave an error rate of 0.17, a sensitivity of 74%, a specificity of 91% and an AUC of 0.893 (table 2). ROC-curves of both the calibration as the validation set are shown in figure 3.

A correct classification was obtained for 38 of 50 (76%) PC patients in the calibration set. In table 1 these classifications are displayed per diagnosis and stage. Seventy-six percent of the correctly classified cases were diagnosed with an early stage of PC (stage I and II). On the other hand, 29 (81%) of the 36 patients with an early stage were correctly classified. In the validation set, a correct classification was achieved for 29 of 39 (74%) patients. Twenty-one of these patients (72%) were operated upon in an early stage and 75% of the patients with an early stage were correctly classified. None of the two patients with stage 1A in the validation set were correctly classified. This concerns one male, age 60, diagnosed with a grade 2, small, 17 mm adenocarcinoma of the pancreas and one female, age 63, diagnosed with a grade 1, small, 5 mm adenocarcinoma of the pancreas.

#### Serum biomarker

As shown in table 1, in the calibration set, for 26 (52%) patients a pre-operative CA19-9 value was determined. This compared to 20 (51%) in the validation set. In the calibration set 17 (65.4%) patients had an elevated CA19-9 value. Nineteen (73%) patients were correctly classified with protein profiling. When comparing protein profiling with CA19-9, protein profiling was correctly classifying two (19 versus 17) more than CA19-9. In nine (34.6%) patients CA19-9 was not increased. Seven (78%) of these patients were correctly

classified by protein profiling. Totally, protein profiling gives additional information in nine (two and seven) cases. In the validation set, 65% (n = 13) of the patients had an elevated CA19-9 value. Fourteen (70%) of the twenty patients with a known Ca19-9 value were correctly classified. Protein profiling was correctly classifying one (14 versus 13) more than CA19-9. In seven (35%) patients CA19-9 was not increased. Three of these patients (43%) were correctly classified. In total, in the validation set, protein profiling gives additional information in four (one and three) cases.

#### Patients with increased risk

Three of the five (60%) patients with an increased risk were correctly classified with protein profiling (two with a P16-Leiden mutation, one with a BRCA2 mutation). In three cases Ca19-9 was known, two with elevated Ca19-9 values. One of these patients was correctly classified with protein profiling. One patient had no increased value of Ca19-9. This patient was incorrectly classified by protein profiling. Thus, protein profiling gives additional information in one case.

#### DISCUSSION

Accurate and early detection of PC may result in more patients that could benefit from a pancreaticoduodenectomy and may increase survival time. Clinical proteomics has emerged as a promising strategy to develop novel tools for biomarker strategies.<sup>37-42</sup>

In this study, a high-resolution MS profiling of human serum was used, aiming at detecting specific patterns present in patients with PC. Using this method, we found a set of 7 peptides that differentiated PC from healthy volunteers with a sensitivity of 78% and a specificity of 89%. These results were successfully validated in an independent case-control group.

Although in several studies serum protein patterns were found to show high sensitivity and specificity as an early diagnostic tool, critical notes have been made on biological variation, pre-analytical conditions and analytical reproducibility of serum protein profiles.<sup>43</sup> Thus, application of proteomic spectra can only be applied in a routine clinical setting when the collection and processing of the data is subjected to stringent quality control procedures.<sup>14,21,43</sup> In the Leiden University Medical Center a We have spent much effort in optimizing the protocol for high throughput analysis. We use a completely robotized and automated procedure for sample handling. This protocol was tested with respect to the number of freeze–thaw cycles, the period between collection and serum centrifugation and reproducibility.<sup>44</sup>

In principle a high-end mass spectrometry setup is sensitive enough to detect almost any protein, but the true sensitivity of MS is modulated by the nature of the sample. Biological samples are typically characterized by a wide range of protein abundances, and mass spectrometers are not well equipped to deal with this wide dynamic range. Peptides do not ionize with equal efficiency, potentially putting some proteins at a disadvantage in terms of detection. This issue is further complicated by low-molecular-weight proteins and those expressed at low abundance. Sample fractionation overcomes these issues, reduces the impact of undersampling and improves reproducibility between analyzes.<sup>13</sup> In our study we used WCX magnetic beads with specific binding characteristics. These magnetic beads can well be implemented in a robotic platform for fully automated use and this automation ensures the control of each step in the extraction protocol and thus minimizes technical variability.<sup>20</sup> In addition, this automation in combination with the high speed of data acquisition of MALDI MS allows high-throughput analysis of thousands of samples.

Goonetilleke et al published in 2007 a systematic review about CA19-9 as a diagnostic serum marker for PC. They calculated a median sensitivity of 79% and a median specificity of 82%.<sup>45</sup> With our protein profiling a comparable sensitivity but a higher specificity was achieved. In 13 reports, CEA shows a mean sensitivity of 54% and a specificity of 79%. Other newer serum biomarkers described by Goonetilleke et al are carbohydrate antigen 242 (CA 242), carbohydrate antigen 50 (CA 50), SPAN-1 and sialyl-lcat-N-tetraose (DUPAN-2). These newer biomarkers perform with sensitivities of 65-83% and specificities of 80-93%. Until now, none of these biomarkers are clinically used.

In this study, CA19-9 was semi-routinely assessed with an overall sensitivity of 65% and 63%, respectively. Although this marker is not clinically used for detection of early PC, we choose to compare our profile with this 'best currently available' marker. Comparing the protein profiling classification with CA 19-9 values resulted in an improvement for thirteen patients. In the calibration set, 29 of 36 patients (81%) with stage I and II PC were correctly classified, whereas in the validation set early PC was correctly classified in 21 out of 28 patients (75%). Stage IA and IB differentiate between the tumor size of 2 cm. Also for higher stages, the test showed a high sensitivity and specificity. In total, this gives a sensitivity of 78% for detection of early PC with our profile.

Nowadays, emphasis in biomarker research has moved towards peak identification, *i.e.* biomarker characterization. It is convenient to first determine the diagnostic power of candidate markers before performing identification studies and further investigations into their biological role in disease mechanisms, since identification of peptide- or protein signals in a profile is not straight forward. Such efforts require specific separation- or enrichment strategies and a high MS/MS-data quality for identification of endogenous species, i.e. large coverage of fragment ions. Until now, most reported identifications of serum peptides in profiles were based on SELDI enrichment chips (i.e. Immobilized Metal Affinity Capture (IMAC)<sup>34</sup>) or on Reversed Phase (RP)C18 solid-phase extraction

(SPE) procedures<sup>44-46</sup> and can therefore not directly be overlaid with WCX profiles. Only a few peptide/protein identifications from WCX fractionation have been reported.<sup>23,34</sup> Previously, peaks in MALDI-TOF profiles at m/z's of 2084, 2178, 2770, 2899, 8769, 8939 have been characterized as fragments of FGA-chain, Apo-CIII and Apo-CII<sup>44</sup> Although these m/z values correspond with the seven most discriminating peaks of this study, these characterization have been performed after RPC18 purification and are therefore not completely transposable with our data. The latter peak of 8.9 kDa is also described in literature as Human Complement C3 (P01024, Unitprot.org), but identified as a discriminating peptide after fractionation with IMAC beads. Recently, Albrethsen et al<sup>47</sup> published an overview of the MALDI and SELDI characterization of peptides and proteins, but these m/z values did not correspond exactly with the seven discriminating peaks of this study.

Our profile is not yet tested on individuals with an increased risk (smoking, chronic pancreatitis, diabetes) in developing PC or an increased inherited risk (carriers of a p16-Leiden mutation, Peutz-Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM) or BRCA2 mutation carriers). Especially this group might benefit with a highly sensitive en specific new biomarker. Retrospective series will be analyzed and collection of serum during surveillance of this high risk group has been set up. Furthermore the method should be further improved before it is clinical applicable. The first step, which was presented in this manuscript, is that we can identify patients with pancreatic cancer from healthy controls. However, the value of the test for high-risk patients as patients with diabetes, smoking, pancreatitis and/or other contributing factors is still unknown. A next step in our research program is to analyze our serum proteomics parameter in a group of patients with an increased risk for developing pancreatic cancer (smoking, chronic pancreatitis, diabetes). Furthermore our research continues improving the cleaning up procedure using two different magnetic beads, with promising preliminary results. This could mean that we can improve sensitivity and specificity.

In conclusion, MS technology allows high throughput analysis of peptides and proteins, with accurate results and, when properly applied, with high reproducibility. Protein profiling can classify pancreatic patients from healthy volunteers based on the SPE fractionation with WCX magnetic beads. This promising new biomarker is a simple, additional test for the diagnosis of PC in clinical practice. Further research is necessary to evaluate its specificity.

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