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New Onset of DiabetEs in aSsociacion with pancreatic cancer (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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New Onset of DiabetEs in aSsociacion with pancreatic cancer (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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Key words: new-onset diabetes mellitus, pancreatic cancer, biomarker, screening

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Strengths and limitations of this study:

Strength 1: As patients are included prospectively, the study will yield a cohort to examine the metabolic changes that coincide with the occurrence of pancreatic cancer at a very early stage before it is diagnosed.
Strength 2: The criteria for the diagnoses of diabetes and pancreatic cancer will be uniformly applied throughout the study period, moreover the diagnosis of pancreatic cancer will be confirmed with a high level of certainty in all subjects.
Strength 3: Taking part in the screening is connected to a very low burden, as the blood collection is only minimally invasive.
Strength 4: All patients will be monitored closely and frequently, which will increase the survival of all participants, especially the high-risk patients.
Limitation 1: It might be really difficult to include the required number of patients, consideing that PaC is a rare disease, and the elderly population has more comorbidities which not even make our observation more difficult, but also leads to a higher follow-up loss during the 36 months.

INTRODUCTION

Pancreatic cancer (PaC) is a rare disease with a lifetime prevalence of 1.39%, but its prevalence is continuously increasing (1-3). The prognosis is extremely poor: it has the lowest five-year survival of all cancers, only 6% (4), and this rate has not significantly changed in the last 40 years (5). PaC is projected to be the third leading cause of cancer-related death by 2030 (6). The high mortality rate is a consequence of delayed diagnosis: in the absence of specific symptoms, PaC is often diagnosed at an advanced stage. Surgery is the only curative treatment at this moment. Unfortunately, only 20% of the patients are eligible for curative resection at the time of the diagnosis because of the presence of metastases and locoregional infiltration (7). The success in reducing the mortality rate of PaC is related to a significant extent to the development of early detection and prevention programs. An effective screening programme is needed for the early diagnosis of PaC in the asymptomatic stage to improve the prognosis. Due to the low lifetime prevalence, the population-based screening is neither feasible nor cost-effective. It is recommended that subjects at high risk of PaC should be screened (8).

Pancreatic cancer and diabetes mellitus

Patients with diabetes mellitus (DM) have an eight-fold higher risk of developing PaC within 2–3 years after the diagnosis of diabetes relative to the general population (9). In a meta-analysis which included 36

studies, individuals in whom DM had only recently been diagnosed (<4 years) had a 50% increased risk of PaC as compared with individuals who had diabetes for >5 years (10). Another meta-analysis of 35 cohort studies showed that DM was associated with an increased risk of PaC (summary relative risks (RRs)=1.94; 95% CI, 1.66–2.27). Interestingly, the risk decreased with the duration of diabetes (5.38 for <1 year, 1.95 for 1–4 years, and 1.49 for 5–9 years, 1.47 for ≥10 years), thus providing evidence that several diabetes in PaC patients is caused by the cancer itself (11). In these cases, patients are actually suffering from diabetes type 3c (T3cDM). Diabetes is already prevalent in small PaCs (12), and what is more important, that diabetes occurs before the tumour is radiologically detectable (13). A population-based study found that approximately 1% of patients with new-onset diabetes at age 50 or older will be diagnosed with PaC within 3 years of first meeting criteria for diabetes, and 56% of these within 6 months of meeting the criteria for diabetes (9). Recognition of new-onset diabetes as an early manifestation of PaC could lead to diagnosis of asymptomatic, early-stage PaC (14). In our recent prospective study, the prevalence of PaC in patients with new-onset type 2 diabetes (T2DM) was significantly higher than in the general population (the value of the Standardised Incidence Ratio for PaC in new-onset type 2 diabetic patients was 198.6 (95% CI = 6.25-46.9)); therefore, screening seems to be beneficial for detecting PaC in this patient population (15). Weight loss in patients with pancreatic carcinoma-associated DM often precedes the onset of diabetes, while new-onset primary type 2 DM is typically associated with weight gain (16). The paradoxical development of diabetes in the face of ongoing weight loss may be an important clue to diagnose pancreatic carcinoma in patients with new onset of diabetes.

Screening modalities

The carbohydrate antigen 19-9 (CA19-9) is currently the only blood-based biomarker in clinical use for PaC. The sensitivity of this marker for PaC is 75%, the specificity is 90%, the positive predictive value is 69%, and the negative predictive value is 90% (17). These values fall below the required characteristics of a reliable screening test (10, 18); therefore, serum CA19-9 measurement is not suitable for screening for PaC. Imaging modalities represent the gold standard for diagnosing PaC. The first choice is transabdominal ultrasound. The sensitivity of transabdominal ultrasonography in PaC diagnosis is only 50–70%. Its accuracy is low in tumours <1 cm, which are usually operable and negatively influenced by obesity and meteorism (19). Computer tomography has a better accuracy in diagnosing PaC; however, the low prevalence of PaC and radiation exposure associated with the modality prevents it from being used as a screening test. The odds for a correct diagnosis are also high employing endoscopic ultrasound or endoscopic retrograde cholangiopancreatography (ERCP), but again the low prevalence of PaC in combination with the burden of the endoscopic intervention to the patient preclude the application of these

diagnostic methods for screening. Furthermore, it is not economically feasible to employ computer tomography or endoscopic imaging for screening as these methods are associated with high costs to the healthcare system.

The success of the strategy of using new-onset diabetes as a screening tool to identify subjects with a high likelihood of having asymptomatic PaC will depend on our ability to differentiate PaC-associated diabetes from the more common type 2 diabetes. PaC-induced diabetes is thought to be a paraneoplastic phenomenon involving the release of products from the tumour rather than a result of the destruction of the pancreas due to malignant infiltration (20, 21). Data on incidence of PaC in new onset of DM is rare, numbers of 0.25% (22), 0.85% (9), and 3.6% (23) have been reported. Therefore, to enable a diagnostic follow-up of new onset of diabetes, a further enrichment of this group is needed (14, 24, 25), e.g. elderly subjects (age is an independent risk factor for PaC), weight loss (26), or smoking.

A biomarker panel consisting of nine metabolites plus the established protein CA19-9 were recently identified by Mayerle and colleagues with 89.9% sensitivity, 91.3% specificity and 99.8% negative predictive value for differentiating PaC from chronic pancreatitis (27). Employing the same methods, a biomarker panel for differential diagnosis between PaC and non-cancer-related diabetes was identified. The metabolite signature needs validation in an independent test cohort, which will be enabled with the present study. Provided the biomarker is validated, the panel could be effective for screening of the high-risk group patients diagnosed with new-onset DM.

A screening test will be cost-effective if sensitivity exceeds 88% and specificity is given at 85%, if the costs for the test are below $400, and if we accept $16 889 per quality-adjusted life-year (10).

Aims of the project

a) Estimate the incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes
b) Diagnose pancreatic ductal adenocarcinoma in an early operable stage
c) Validate a biomarker that distinguishes patients with PaC-caused T3cDM from patients with T2DM.

METHODS AND ANALYSIS

Design

This is a prospective, multicentre, observational cohort study aiming to validate a biomarker panel in the early stage of PaC. The data collection is based on questionnaires and blood samples will be drawn from all patients. The questionnaires (Form A at recruitment, Form B at every follow up visit) will be filled by every included patinets. This study was structured as the SPIRIT 2013 guide recommended (Figure 1) (28).

Table 1. Diagnostic criteria of diabetes mellitus.

Parameter	Value and unit	Description
Fasting plasma glucose	≥126 mg/dL (7.0 mmol/L)	Fasting is defined as no caloric intake for at least 8 h.
2 h plasma glucose	≥200 mg/dL (11.1 mmol/L)	Oral glucose tolerance test. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
HbA1c	≥6.5% (48 mmol/mol)	The test should be performed in a laboratory using a method that is NGSP certified and standardised to the DCCT assay.
random plasma glucose	≥200 mg/dL (11.1 mmol/L)	In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis.

The inclusion criteria of this study are the following: (1) patients over 60 years of age; (2) diabetes diagnosed within six months (newly diagnosed) - diagnostical criteria are based on the Diabetes Control and Complications Trial (Table 1. (29)); (3) signed written informed consent.

Exclusion criteria are as follows: (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; and (5) present malignant disease.

Sample size

20 cases in which PaC developed during follow-up. Considering a 105 drop-out rate, this means 2522 samples from follow-up patients over 60 years of age with diabetes diagnosed within 6 months and 250 matched patients in the control group.

Duration:

The first recruiting centre will be initialized in 1 July 2019. The planned finishing date of the study is January 31 December 2022.

Clinical data and clinical endpoints:

Essential baseline clinical data: age, sex, body weight, BMI, date of DM diagnosis, date of sampling, comorbidities, antidiabetic medication, clinical symptoms, histology and stage of pancreatic carcinoma.

Primary clinical endpoints: incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes

Secondary endpoints:

(1) mortality of pancreatic ductal adenocarcinoma in new-onset diabetic patients; (2) the proportion of localised and resectable pancreatic ductal adenocarcinoma; (3) change in body weight before Visit 1 and during Visit 2-6; (4) change in fasting blood glucose and HbA1c before Visit 1 and during Visit 2-6; (5) antidiabetic medications and the risk of pancreatic ductal adenocarcinoma; (6) presence of concomitant diseases; (7) smoking and alcohol intake; (8) the sensitivity, specificity, positive and negative predictive values, and accuracy of the biomarker test; (9) cost-benefit analysis.

Study protocol:

Diabetic patients will be recruited by our diabetologist and collaborating family physicians based on a recent (< 6 months) laboratory test (Table 1). Visit 0 is scheduled within 2 weeks from the referral (Figure 2). Patients who meet study entry criteria and no exclusion, will be informed and offered to participate in the study, however signed informed consent will be necessary for inclusion. Clinical data, body weight and worrisome features (unintentional weight loss: 5% of body weight within 6 months without knowing the reason (30), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection) will be recorded at Visit 0, and a fasting blood sample will be taken for assessment of laboratory data and metabolomics. C-peptide and glutamic acid decarboxylase antibodies (GADA) will be determined to classify diabetes at Visit 0. If worrisome features are present at Visit 0, MRI or EUS is performed. Unambigous PaC lesions (>1 cm or seen also by magnetic resonance imaging) will be referred to surgery for resection. In case of ambigous lesions in the pancreas, EUS-fine needle aspiration will be performed. Visit 1-5 are scheduled every 6 months. Clinical symptoms, body weight, laboratory data (fasting blood glucose, HbA1c, liver and renal function, lipids, blood count) will be collected at each visit. Blood to biobank and CA 19-9 will be taken at every 12 months. The follow-up will be closed at 36 months.

Biochemical methods

After informed consent, fasted (overnight, at least 8 h) patients' blood samples will be drawn into an EDTA tube. 9 ml blood tubes are centrifuged within 2 h after blood draw using a swing-out rotor at 2000 xg for 10 minutes. The sample processing is done at room temperature and the centrifuge is temperature-controlled at 19-21°C. After centrifugation, the supernatant is carefully removed, transferred to a fresh 9 ml tube and gently mixed in order to homogenise any gradient that might have been generated in the plasma supernatant. After that, the plasma is transferred in 0.5 ml aliquots to tubes (either Eppendorf Safe-Lock-Tubes 2 ml or Sarstedt Screw cap micro tubes 2 ml) and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer). Biomarkers will be determined comparing metabolite levels in plasma samples from patients

diagnosed with PaC and diabetic cancer-free patients (26). CA19-9 determination is performed centralised at a certified clinical laboratory applying a cut-off of 37 U/ml as a classifier.

Cost of the biomarker test, quality-adjusted life-year (QALY) and incremental cost-effectiveness ratio (ICER) will be determined.

Metabolite profiling:

MxP® Global Profiling:

Two types of mass spectrometry analyses are applied. GC–MS (gas chromatography-mass spectrometry; Agilent 6890 GC coupled to an Agilent 5973 MS System, Agilent, Waldbronn, Germany) and LC–MS/MS (liquid chromatography-MS/MS; Agilent 1100 HPLC-System, Agilent, Waldbronn, Germany, coupled to an Applied Biosystems API4000 MS/MS-System, Applied Biosystems, Darmstadt, Germany) are used for a metabolite profiling approach (31). Fractionation and derivatisation of samples and detection technologies have been previously described (32-35). Proteins are removed from plasma samples (60 µl) by precipitation. Subsequently, polar and non-polar fractions are separated for both GC–MS and LC–MS/MS analyses by adding water and a mixture of ethanol and dichloromethane. For GC–MS analyses, the non-polar fraction is treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolysed complex lipids. The polar and non-polar fractions are further derivatised with O-methyl-hydroxylamine hydrochloride (20 mg/ml in pyridine) to convert oxo-groups to O-methyloximes and subsequently with a silylating agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) before GC–MS analysis. For LC–MS/MS analyses, both fractions are dried and subsequently reconstituted in appropriate solvent mixtures. HPLC (High performance LC) is performed by gradient elution using methanol/water/formic acid on reversed phase separation columns.

MxP® Lipids:

MxP® Lipids covers profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). The metabolites are analysed in a semi-quantitative approach (i.e. relative to a pool). Total lipids are extracted from plasma by liquid/liquid extraction using chloroform/methanol. The lipid extracts are subsequently fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to (32, 36). The fractions are analysed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with detection of specific multiple reaction monitoring (MRM) transitions for sphingomyelins (SM) and ceramides (CER) respectively.

Data normalization

Details of data normalization have been published (27). Metabolite profiling based on a semi-quantitative analytical platform results in relative metabolite levels ("ratio") to a defined reference. To support this concept and to allow an alignment of different analytical batches, two different reference sample types are run in parallel throughout the whole process. First, a project pool is generated from aliquots of all samples and measured with four replicates within each analytical sequence that comprised 24 samples. For all semi-quantitatively analysed metabolites, the results of each analyte from each sample are normalised against the median of the corresponding analyte in the pool reference samples within each analytical sequence to provide pool-normalised ratios. This process step compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences are analysed by different devices. Second, to allow for an experiment-to-experiment alignment of semi-quantitative data, MxPool™ (a large pool of a commercial human EDTA plasma suited for alignment of MxP® studies) is analysed with 12 replicated samples, and the pool-normalised ratios are further normalised to the median of the MxPool™ samples, i.e. ratios from this study are on the same level and therefore comparable with data from other studies normalised to other aliquots of the same MxPool™. A rigorous quality control is performed on peak, analyte and sample level and has been described previously (37).

Data collection and follow-up

Data collection is based on questionnaires, and will be stored in a personalised electronic database (eCRF). Form A: contains all antropometric parameters, routine clinical chemistry tests, fasting blood glucose and HbA1c. Follow-up visits will be scheduled by the patient registration system every 6 months. Blood will be taken for biomarker identification with metabolomics and CA19-9 determination at every 12 months. The total follow-up period is three years.

Pancreas adenocarcinoma will be diagnosed by histological examination.

STATISTICS

Data set analysis and normalization

Descriptive statistics – mean, median, standard deviation, quartiles and relative frequency –relative risk (dichotomous variables), Independent Two-sample T test (continuous variable) in the case of normal distribution, furthermore Mann-Whitney test in lack of normal distribution will be performed. Logistic regression will be applied for the exploring of predictive factors. Affiliated statistical analyses will be performed with an error probability of 0.05 (type-I error probability).

Prior to statistical analysis, log10 transformation of ratios is conducted so that the data distribution becomes approximately normal. SIMCA-P version 14.0 (Umetrics AB, Umea, Sweden), TIBCO® Spotfire® 7.12.0

and R 3.3.4 are used for data analyses and visualizations. Initially, an exploratory multivariate analysis (Principal Component Analysis, PCA) is applied to log10-transformed ratios scaled to unit variance.

A simple linear model (ANOVA, package nlme) addressing additional clinical information and potentially confounding factors such as “disease”, “age”, “body mass index”, “gender” and “sample storage time” as fixed effects is fitted to the data. Significance level is set to 5%. The multiple test problem for the number of metabolites is addressed by calculating the false discovery rate (FDR) using the Benjamini & Hochberg method (38).

To classify patients depending on their metabolic profiles a penalised logistic regression is fitted via Elastic Net Algorithm using the R package glmnet (38). Equal penalties are used for both the L1 and the L2 norm. Afterwards the cutoff established previously on the biomarker identification dataset is applied on the test data without retraining, and the performance is measured in terms of area under the curve (AUC), sensitivity and specificity. Confidence levels for the AUC are calculated using the binormal model for the receiver operating characteristic (ROC) curve. When the sensitivity is fixed at a particular value, the positive and negative predictive values (PPV, NPV) and the accuracy become monotone functions of the specificity; and confidence intervals for these estimates are obtained by transformation of the confidence interval for the specificity. Confidence intervals for sensitivity, specificity and accuracy are obtained for the cutoff pre-specified in the training data by the method of Clopper and Pearson for the binomial distribution. For PPV and NPV the confidence intervals will be obtained by the method of Gart and Nam (39) for ratios of binomial parameters as implemented in the R package pairwise CI (40). When comparing the biomarker and CA19-9 on the test data, differences in sensitivity and specificity will be tested for with the McNemar test.

Centres

The study will start with the following centres (University of Szeged, University of Pécs, University of Semmelweis), however, other centres are welcome to participate as an open label study. Completion of the LETTER OF INTENT form will be mandatory for registering the participation of each institution.

Publication policy

Centres providing more than 50 patients can provide author to the authorship list.

Dissemination policy

We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for

professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

Patients and public involvement

Patients will be provided with informational material about the background and aims of the study.

Discussion

PaC has a dismal prognosis, which is due to its late diagnosis. The success in reducing the mortality rate of PaC is related to the development of early detection and prevention programs. Age and DM are known as risk factors of PaC (9-11, 14, 15).

The expected positive endpoint of this study is to validate a biomarker panel that is suitable for early stage diagnosis in a mostly incurable, high-mortality cancer, when surgery is still possible and the cancer can be cured. This test only requires one blood sample collection, which means that it is simple, repeatable, tolerable, minimally invasive, nearly painless, widely achievable and relatively cheap – it thus fulfils all the criteria set for a screening method. Identifying PaC in an earlier (still resectable) stage through surveillance of high-risk patients would increase surgical resection rate, cure rates and survival by 30–40%. It would save lives, maintain better well-being among the population and would have an enormous financial benefit: the increasing number of successful surgical interventions leads to a lower necessity of chemotherapy and palliative interventions (such as stent implantations or gastroenteroanastomosis operations), moreover lower the burden the healthcare cost.

Trial organization, committees and boards: The coordinator of the NODES study is LC with the support of the Hungarian Pancreatic Study Group (HPSG-coordinating society, https://tm-centre.org/en/study-groups/hungarian-pancreatic-study-group/). HPSG has been running high-quality international, multicentre clinical trials since 2014 and has published the relevant guidelines for pancreatic diseases to improve patient care in pancreatology (41-49).

The trial will be supported by the following committees:

Steering Committee (SC): This committee will be led by PH (gastroenterologist and internal medicine specialist). The members in Szeged (HU) will be: Dóra Illés, Emese Iványi.

International Translational Advisory Board (ITAB): This board will involve gastroenterologists. The ITAB will regularly monitor the progression of the trial and might give recommendations to the SC.

Data Monitoring Committee (DMC): DMC will handle all the data and ensure that the data in the eCRF is accurate, complete and legible. Data Management Plan (DMP) will describe the detailed data flow. The Data Manager will validate the data from completed eCRFs, according to a Data Cleaning Plan (DCP). Any missing, implausible or inconsistent recordings in the eCRFs will be referred back to the Investigator using a data query form (DQF), and be documented for each individual subject before clean file status is declared. All changes to eCRFs will be recorded.

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Authors' contributions

The study was designed by ID, LC, BK. ID, LC, MGA drafted the manuscript. All authors edited, read and approved the final manuscript. Literature search, statistical calculation and figures preparation will be done by JA, NZ, AS. During the study ID, EI, GH, KK, HV, GZ, MT, MS, VH, LC are going to collect the patients.

CONFLICT OF INTEREST STATEMENT

BK and MGA are employees of Metanomics Health GmbH, Germany.

The other authors have no competing interest.

ETHICS AND DISSEMINATION

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602)

Ethical approval: Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019).

Protocol Version: V1.0 08.01.2019.

Start of the patient recruitment: January 31, 2020.

Planned finish of the study: June 30, 2023

Abbreviations:

APCI - atmospheric pressure chemical ionization

AUC – area under the curve

CA 19-9 – carbohydrate antigen 19-9

CER – ceramides

DM – diabetes mellitus

DMC - Data Monitoring Committee

ERCP – endoscopic retrograde cholangio-pancreatography

ESI - electrospray ionization

EUS – endoscopis ultrasound

FDR - false discovery rate

GC-MS - gas chromatography-mass spectrometry

HbA1c – haemoglobin A1c

HPSG – Hungarian Pancreatic Study Group

ITAB - International Translational Advisory Board

LC- MS/MS - liquid chromatography-MS/MS

MRI – magnetic resonance imaging

MRM - multiple reaction monitoring

NPLC - normal phase liquid chromatography

NPV – negative predictive value

PaC – pancreatic cancer

PCA - principal component analysis

PPV – positive predictive value

ROC - receiver operating characteristic

SC - Steering Committee

SM – sphingomyelins

SP –Sponsor

T2DM – type 2 diabetes mellitus

T3cDM – type 3c diabetes mellitus

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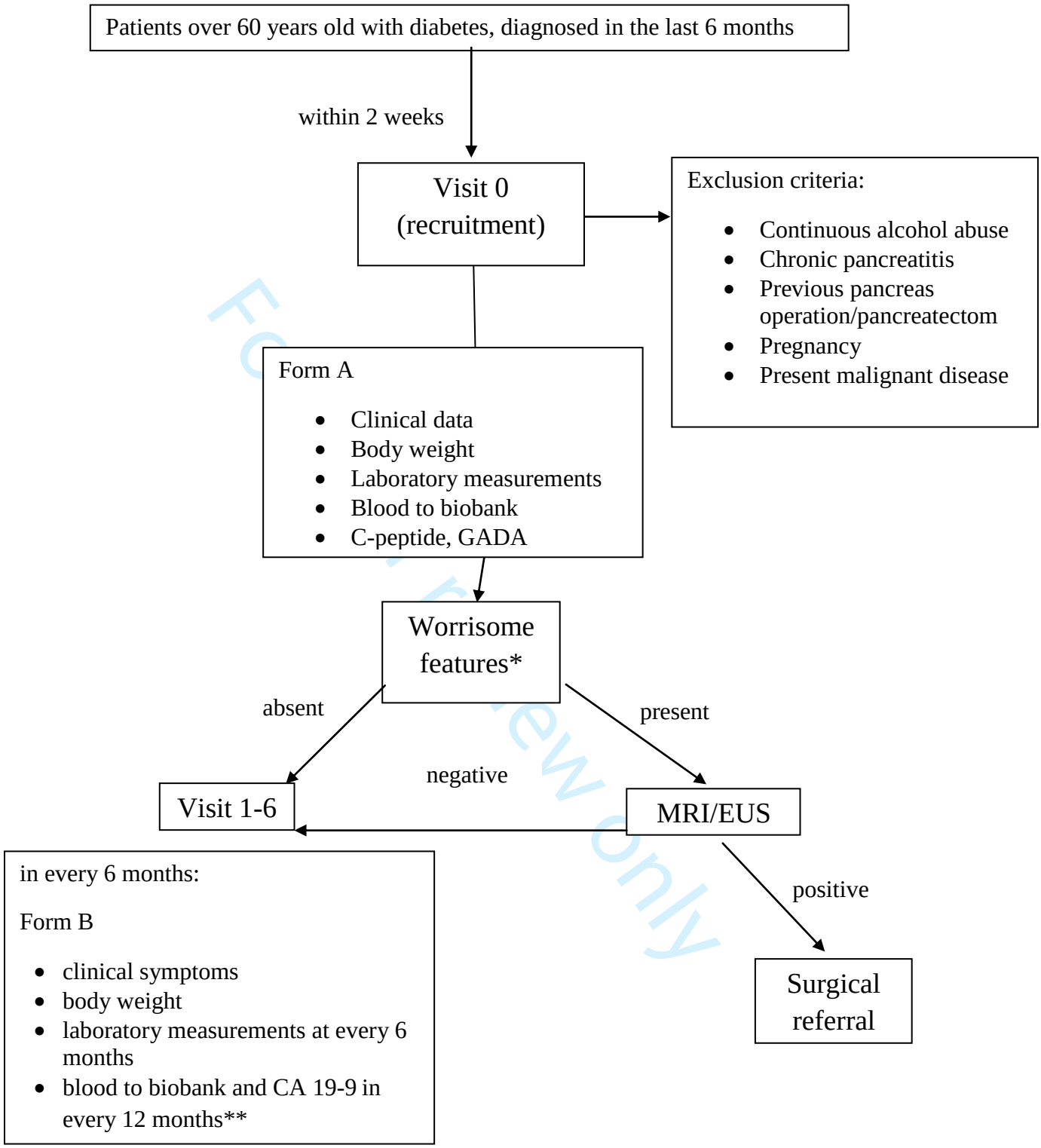
Figure 1. The schedule of enrolment and assessments according to the SPIRIT guideline

	STUDY PERIOD							
	Enrolment	*Post enrolment*						Close-out
TIMEPOINT	*0*	*Visit1* *0.5 y*	*Visit2* *1 y*	*Visit3* *1.5 y*	*Visit4* *2 y*	*Visit5* *2.5y*	*Visit6* *3y*	t_x
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
FORM A	X							
Blood samples to biobank	X							
VISITS:								
FORM B		X	X	X	X	X	X	
Laboratory test		X	X	X	X	X	X	
Blood samples to biobank*	X		X		X		X	
MRI/EUS if worrisome features are present	X	X	X	X	X	X	X	
DATA ANALYSIS:								X

*Fasted (overnight, at least 8 h) patients' blood samples at room temperature will be drawn into an EDTA tube. Within 2 h after blood draw samples will be at 19-21°C. After centrifugation, the supernatant is carefully removed. After that, the plasma is transferred in 0.5 ml aliquots to tubes and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer). Central laboratory: Metanomics Health GmbH, Tegeler Weg 33, 10589 Berlin, Germany

Figure 2. Flowchart of the study protocol



* weight loss (except at visit0), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection (except at visit0)
EUS: endoscopic ultrasound; MRI: magnetic resonance imaging

** Fasted (overnight, at least 8 h) patients' blood samples at room temperature will be drawn into an EDTA tube. Within 2 h after blood draw samples will be at 19-21°C. After centrifugation, the supernatant is carefully removed. After that, the plasma is transferred in 0.5 ml aliquots to tubes and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer).

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New Onset of DiabetEs in aSsociation with pancreatic ductal adenocarcinoma (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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Key words: new-onset diabetes mellitus, pancreatic cancer, biomarker, screening

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ABSTRACT

Introduction: Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with an overall 5-year survival of approximately 8%. The success in reducing the mortality rate of PDAC is related not only to the discovery of new therapeutic agents, but also to a significant extent to the development of early detection and prevention programs. Patients with new-onset diabetes mellitus represent a high-risk group for PDAC as they have an 8-fold higher risk of PDAC than the general population**.** The proposed screening program may allow the detection of PDAC in the early, operable stage. Diagnosing more patients in the curable stage might decrease the morbidity and mortality rates of PDAC and additionally reduce the burden of the healthcare.

Methods & Analysis: This is a prospective, multicentre observational cohort study. Patients ≥60 years old diagnosed with new-onset (≤ 6 months) diabetes will be included. Exclusion criteria are (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; (5) present malignant disease and (6) type-1 diabetes mellitus. Follow up visits are scheduled every 6 months for up to 36 months. Data collection is based on questionnaires. Clinical symptoms, body weight and fasting blood will be collected at each, CA 19-9 and blood to biobank at every second visit. The blood samples will be processed to plasma and analysed with mass spectrometry-based metabolomics. The metabolomic data will be used for biomarker validation for early detection of PDAC in the high-risk group new-onset diabetes patients. Patients with worrisome features will undergo MRI or EUS investigation, and surgical referral depending on the radiological findings. The primary endpoint is the incidence of PDAC in patients with newly diagnosed diabetes mellitus.

Ethical approval: Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019).

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602).

Strengths and limitations of this study:

Strength 1: As patients are included prospectively, the study will yield a cohort to examine the metabolic changes that coincide with the occurrence of PDAC at a very early stage before it is diagnosed.
Strength 2: The criteria for the diagnoses of diabetes and PDAC will be uniformly applied throughout the study period, moreover the diagnosis of pancreatic cancer will be confirmed with a high level of certainty in all subjects.
Strength 3: Taking part in the screening is connected to a very low burden, as the blood collection is only minimally invasive.
Strength 4: All patients will be monitored closely and frequently, which will increase the survival of all participants, especially the high-risk patients.
Limitation 1: It might be really difficult to include the required number of patients, consideing that PDAC is a rare disease, and the elderly population has more comorbidities which not even make our observation more difficult, but also leads to a higher follow-up loss during the 36 months.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a rare disease with a lifetime prevalence of 1.39%, but its prevalence is continuously increasing (1-3). The prognosis is extremely poor: it has a five-year survival rate of only 7-8% (4), and this rate has barely improved in the last 40 years (5). PDAC will be the second leading cause of cancer-related death by 2030 (6). The high mortality rate is a consequence of delayed diagnosis: in the absence of specific symptoms, PDAC is often diagnosed at an advanced stage. Surgery is the only curative treatment at this moment. Unfortunately, only 20% of the patients are eligible for curative resection at the time of the diagnosis because of the presence of metastases and locoregional infiltration (7). The success in reducing the mortality rate of PDAC is related to a significant extent to the development of early detection and prevention programs. An effective screening programme is needed for the early diagnosis of PDAC in the asymptomatic stage to improve the prognosis. Due to the low lifetime prevalence, the population-based screening is neither feasible nor cost-effective. It is recommended that subjects at high risk of PDAC should be screened (8).

Pancreatic ductal adenocarcinoma and diabetes mellitus

Patients with diabetes mellitus (DM) have an eight-fold higher risk of developing PDAC within 2–3 years after the diagnosis of diabetes relative to the general population (9). In a meta-analysis which included 36

studies, individuals in whom DM had only recently been diagnosed (<4 years) had a 50% increased risk of PDAC as compared with individuals who had diabetes for >5 years (10). Another meta-analysis of 35 cohort studies showed that DM was associated with an increased risk of PDAC (summary relative risks (RRs)=1.94; 95% CI, 1.66–2.27). Interestingly, the risk decreased with the duration of diabetes (5.38 for <1 year, 1.95 for 1–4 years, and 1.49 for 5–9 years, 1.47 for ≥10 years), thus providing evidence that several diabetes in PDAC patients is caused by the cancer itself (11). In these cases, patients are actually suffering from diabetes type 3c (T3cDM). Diabetes is already prevalent in small PDACs (12), and what is more important, that diabetes occurs before the tumour is radiologically detectable (13). A population-based study found that approximately 1% of patients with new-onset diabetes at age 50 or older will be diagnosed with PDAC within 3 years of first meeting criteria for diabetes, and 56% of these within 6 months of meeting the criteria for diabetes (9). Recognition of new-onset diabetes as an early manifestation of PDAC could lead to diagnosis of asymptomatic, early-stage PDAC (14). In our recent prospective study, the prevalence of PDAC in patients with new-onset type 2 diabetes (T2DM) was significantly higher than in the general population (the value of the Standardised Incidence Ratio for PDAC in new-onset type 2 diabetic patients was 198.6 (95% CI = 6.25-46.9)); therefore, screening seems to be beneficial for detecting PDAC in this patient population (15). Weight loss in patients with pancreatic carcinoma-associated DM often precedes the onset of diabetes, while new-onset primary type 2 DM is typically associated with weight gain (16). The paradoxical development of diabetes in the face of ongoing weight loss may be an important clue to diagnose PDAC in patients with new onset of diabetes.

Screening modalities

The carbohydrate antigen 19-9 (CA19-9) is currently the only blood-based biomarker in clinical use for PDAC. The sensitivity of this marker for PDAC is 75%, the specificity is 90%, the positive predictive value is 69%, and the negative predictive value is 90% (17). These values fall below the required characteristics of a reliable screening test (10, 18); therefore, serum CA19-9 measurement is not suitable for screening for PDAC. Imaging modalities represent the gold standard for diagnosing PDAC. The first choice is transabdominal ultrasound. The sensitivity of transabdominal ultrasonography in PDAC diagnosis is only 50–70%. Its accuracy is low in tumours <1 cm, which are usually operable and negatively influenced by obesity and meteorism (19). Computer tomography has a better accuracy in diagnosing PDAC; however, the low prevalence of PDAC and radiation exposure associated with the modality prevents it from being used as a screening test. The odds for a correct diagnosis are also high employing endoscopic ultrasound or endoscopic retrograde cholangiopancreatography (ERCP), but again the low prevalence of PDAC in combination with the burden of the endoscopic intervention to the patient preclude the application of these

diagnostic methods for screening. Furthermore, it is not economically feasible to employ computer tomography or endoscopic imaging for screening as these methods are associated with high costs to the healthcare system.

The success of the strategy of using new-onset diabetes as a screening tool to identify subjects with a high likelihood of having asymptomatic PDAC will depend on our ability to differentiate PDAC-associated diabetes from the more common type 2 diabetes. PDAC-induced diabetes is thought to be a paraneoplastic phenomenon involving the release of products from the tumour rather than a result of the destruction of the pancreas due to malignant infiltration (20, 21). Data on incidence of PDAC in new onset of DM is rare, numbers of 0.25% (22), 0.85% (9), and 3.6% (23) have been reported. Therefore, to enable a diagnostic follow-up of new onset of diabetes, a further enrichment of this group is needed (14, 24, 25), e.g. elderly subjects (age is an independent risk factor for PDAC), weight loss (26), or smoking.

A biomarker panel consisting of nine metabolites plus the established protein CA19-9 were recently identified by Mayerle and colleagues with 89.9% sensitivity, 91.3% specificity and 99.8% negative predictive value for differentiating PDAC from chronic pancreatitis (27). Employing the same methods, a biomarker panel for differential diagnosis between PDAC and non-cancer-related diabetes was identified. The metabolite signature needs validation in an independent test cohort, which will be enabled with the present study. Provided the biomarker is validated, the panel could be effective for screening of the high-risk group patients diagnosed with new-onset DM.

A screening test will be cost-effective if sensitivity exceeds 88% and specificity is given at 85%, if the costs for the test are below $400, and if we accept $16 889 per quality-adjusted life-year (10).

Aims of the project

a) Estimate the incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes
b) Diagnose pancreatic ductal adenocarcinoma in an early operable stage
c) Validate a biomarker that distinguishes patients with PDAC-caused T3cDM from patients with T2DM.

METHODS AND ANALYSIS

Design

This is a prospective, multicentre, observational cohort study aiming to validate a biomarker panel in the early stage of PDAC. The data collection is based on questionnaires and blood samples will be drawn from all patients. The questionnaires (Form A at recruitment, Form B at every follow up visit) will be filled by every included patients.

Parameter	Value and unit	Description
Fasting plasma glucose	≥126 mg/dL (7.0 mmol/L)	Fasting is defined as no caloric intake for at least 8 h.
2 h plasma glucose	≥200 mg/dL (11.1 mmol/L)	Oral glucose tolerance test. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
HbA1c	≥6.5% (48 mmol/mol)	The test should be performed in a laboratory using a method that is NGSP certified and standardised to the DCCT assay.

Table 1. Diagnostic criteria of diabetes mellitus.

The inclusion criteria of this study are the following: (1) patients over 60 years of age; (2) diabetes diagnosed within six months (newly diagnosed) - diagnostical criteria are based on the Diabetes Control and Complications Trial (Table 1. (28)); (3) signed written informed consent.

Exclusion criteria are as follows: (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; and (5) present malignant disease; (6) type-1 diabetes mellitus.

Sample size

Chari et al. concluded that about 1% of elderly subjects with new-onset DM has 8 times higher risk for pancreatic cancer than for a person of similar age and sex without DM (9). Based on these results, we have an assumption with respect to cancerous cases of the distribution in the case and the control groups (Case: PDAC 1%, non-PDAC: 99%; Control: PDAC 0.125%, non-PDAC 99.875%). Sample size calculation suggests that 2552 patients (1:1) will need to be enrolled in order to confirm or reject the hypothesis for the primary endpoint with a 10% dropout, 80% power and 95% significance level. 250 patients with non-pancreatic, non-malignant gastrointestinal diseases without diabetes serve as control group. The recruitment period is planned to last 36 months, and all included patients will be followed for 36 months.

Duration:

The first recruiting centre will be initialized in 1 July 2019. Start of the patient recruitment: January 31, 2020. Planned finish of the study-recruitment: January 30, 2023.

Clinical data and clinical endpoints:

Essential baseline clinical data: age, sex, body weight, BMI, date of DM diagnosis, date of sampling, comorbidities, antidiabetic medication, clinical symptoms, histology and stage of PDAC.

Primary clinical endpoints: incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes

Secondary endpoints:

(1) mortality of pancreatic ductal adenocarcinoma in new-onset diabetic patients; (2) the proportion of localised and resectable pancreatic ductal adenocarcinoma; (3) change in body weight before Visit 1 and during Visit 2-6; (4) change in fasting blood glucose and HbA1c before Visit 1 and during Visit 2-6; (5) antidiabetic medications and the risk of pancreatic ductal adenocarcinoma; (6) presence of concomitant diseases; (7) smoking and alcohol intake; (8) the sensitivity, specificity, positive and negative predictive values, and accuracy of the biomarker test; (9) cost-benefit analysis.

Study protocol:

Diabetic patients will be recruited by our diabetologist and collaborating family physicians based on a recent (< 6 months) laboratory test (Table 1). Visit 0 is scheduled within 2 weeks from the referral (Figure 1). Patients who meet study entry criteria and no exclusion, will be informed and offered to participate in the study, however signed informed consent will be necessary for inclusion. Clinical data, body weight and worrisome features (unintentional weight loss: 5% of body weight within 6 months without knowing the reason (29), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection) will be recorded at Visit 0, and a fasting blood sample will be taken for assessment of laboratory data and metabolomics. C-peptide and glutamic acid decarboxylase antibodies (GADA) will be determined to classify diabetes at Visit 0. Patients with type-1 diabetes mellitus will be excluded. If worrisome features are present at Visit 0, MRI or EUS is performed. Unambiguous PaC lesions (>1 cm or seen also by magnetic resonance imaging) will be referred to surgery for resection. In case of ambiguous lesions in the pancreas, EUS-fine needle aspiration will be performed. Visit 1-5 are scheduled every 6 months. Clinical symptoms, body weight, laboratory data (fasting blood glucose, HbA1c, liver and renal function, lipids, blood count) will be collected at each visit. Blood to biobank and CA 19-9 will be taken at every 12 months. The follow-up will be closed at 36 months.

Biochemical methods

After informed consent, fasted (overnight, at least 8 h) patients' blood samples will be drawn into an EDTA tube. 9 ml blood tubes are centrifuged within 2 h after blood draw using a swing-out rotor at 2000 xg for 10 minutes. The sample processing is done at room temperature and the centrifuge is temperature-controlled at 19-21°C. After centrifugation, the supernatant is carefully removed, transferred to a fresh 9 ml tube and

gently mixed in order to homogenise any gradient that might have been generated in the plasma supernatant. After that, the plasma is transferred in 0.5 ml aliquots to tubes (either Eppendorf Safe-Lock-Tubes 2 ml or Sarstedt Screw cap micro tubes 2 ml) and stored at –80°C, in a dedicated freezer (≤6 h from centrifuge to freezer). Biomarkers will be determined comparing metabolite levels in plasma samples from patients diagnosed with PDAC and diabetic cancer-free patients (26). CA19-9 determination is performed centralised at a certified clinical laboratory applying a cut-off of 37 U/ml as a classifier.

Cost of the biomarker test, quality-adjusted life-year (QALY) and incremental cost-effectiveness ratio (ICER) will be determined.

Metabolite profiling:

MxP® Global Profiling:

Two types of mass spectrometry analyses are applied. GC–MS (gas chromatography-mass spectrometry; Agilent 6890 GC coupled to an Agilent 5973 MS System, Agilent, Waldbronn, Germany) and LC–MS/MS (liquid chromatography-MS/MS; Agilent 1100 HPLC-System, Agilent, Waldbronn, Germany, coupled to an Applied Biosystems API4000 MS/MS-System, Applied Biosystems, Darmstadt, Germany) are used for a metabolite profiling approach (30). Fractionation and derivatisation of samples and detection technologies have been previously described (31-34). Proteins are removed from plasma samples (60 µl) by precipitation. Subsequently, polar and non-polar fractions are separated for both GC–MS and LC–MS/MS analyses by adding water and a mixture of ethanol and dichloromethane. For GC–MS analyses, the non-polar fraction is treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolysed complex lipids. The polar and non-polar fractions are further derivatised with O-methyl-hydroxylamine hydrochloride (20 mg/ml in pyridine) to convert oxo-groups to O-methyloximes and subsequently with a silylating agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) before GC–MS analysis. For LC–MS/MS analyses, both fractions are dried and subsequently reconstituted in appropriate solvent mixtures. HPLC (High performance LC) is performed by gradient elution using methanol/water/formic acid on reversed phase separation columns.

MxP® Lipids:

MxP® Lipids covers profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). The metabolites are analysed in a semi-quantitative approach (i.e. relative to a pool). Total lipids are extracted from plasma by liquid/liquid extraction using chloroform/methanol. The lipid extracts are subsequently fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to (31, 35). The fractions are analysed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure

chemical ionization (APCI) with detection of specific multiple reaction monitoring (MRM) transitions for sphingomyelins (SM) and ceramides (CER) respectively.

Data normalization

Details of data normalization have been published (27). Metabolite profiling based on a semi-quantitative analytical platform results in relative metabolite levels ("ratio") to a defined reference. To support this concept and to allow an alignment of different analytical batches, two different reference sample types are run in parallel throughout the whole process. First, a project pool is generated from aliquots of all samples and measured with four replicates within each analytical sequence that comprised 24 samples. For all semi-quantitatively analysed metabolites, the results of each analyte from each sample are normalised against the median of the corresponding analyte in the pool reference samples within each analytical sequence to provide pool-normalised ratios. This process step compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences are analysed by different devices. Second, to allow for an experiment-to-experiment alignment of semi-quantitative data, MxPool™ (a large pool of a commercial human EDTA plasma suited for alignment of MxP® studies) is analysed with 12 replicated samples, and the pool-normalised ratios are further normalised to the median of the MxPool™ samples, i.e. ratios from this study are on the same level and therefore comparable with data from other studies normalised to other aliquots of the same MxPool™. A rigorous quality control is performed on peak, analyte and sample level and has been described previously (36).

Data collection and follow-up

Data collection is based on questionnaires, and will be stored in a personalised electronic database (eCRF). Form A: contains all antropometric parameters, routine clinical chemistry tests, fasting blood glucose and HbA1c. Follow-up visits will be scheduled by the patient registration system every 6 months. Blood will be taken for biomarker identification with metabolomics and CA19-9 determination at every 12 months. The total follow-up period is three years.

Pancreas adenocarcinoma will be diagnosed by histological examination.

Data set analysis and normalization

Descriptive statistics – mean, median, standard deviation, quartiles and relative frequency –relative risk (dichotomous variables), Independent Two-sample T test (continuous variable) in the case of normal distribution, furthermore Mann-Whitney test in lack of normal distribution will be performed. Logistic regression will be applied for the exploring of predictive factors. Affiliated statistical analyses will be performed with an error probability of 0.05 (type-I error probability).

Prior to statistical analysis, log10 transformation of ratios is conducted so that the data distribution becomes approximately normal. SIMCA-P version 14.0 (Umetrics AB, Umea, Sweden), TIBCO® Spotfire® 7.12.0 and R 3.3.4 are used for data analyses and visualizations. Initially, an exploratory multivariate analysis (Principal Component Analysis, PCA) is applied to log10-transformed ratios scaled to unit variance.

A simple linear model (ANOVA, package nlme) addressing additional clinical information and potentially confounding factors such as “disease”, “age”, “body mass index”, “gender” and “sample storage time” as fixed effects is fitted to the data. Significance level is set to 5%. The multiple test problem for the number of metabolites is addressed by calculating the false discovery rate (FDR) using the Benjamini & Hochberg method (37).

To classify patients depending on their metabolic profiles a penalised logistic regression is fitted via Elastic Net Algorithm using the R package glmnet (37). Equal penalties are used for both the L1 and the L2 norm. Afterwards the cutoff established previously on the biomarker identification dataset is applied on the test data without retraining, and the performance is measured in terms of area under the curve (AUC), sensitivity and specificity. Confidence levels for the AUC are calculated using the binormal model for the receiver operating characteristic (ROC) curve. When the sensitivity is fixed at a particular value, the positive and negative predictive values (PPV, NPV) and the accuracy become monotone functions of the specificity; and confidence intervals for these estimates are obtained by transformation of the confidence interval for the specificity. Confidence intervals for sensitivity, specificity and accuracy are obtained for the cutoff pre-specified in the training data by the method of Clopper and Pearson for the binomial distribution. For PPV and NPV the confidence intervals will be obtained by the method of Gart and Nam (38) for ratios of binomial parameters as implemented in the R package pairwise CI (39). When comparing the biomarker and CA19-9 on the test data, differences in sensitivity and specificity will be tested for with the McNemar test.

Centres

The study will start with the following centres (University of Szeged, University of Pécs, University of Semmelweis), however, other centres are welcome to participate as an open label study. Completion of the LETTER OF INTENT form will be mandatory for registering the participation of each institution.

PATIENTS AND PUBLIC INVOLVEMENT

If possible, PPI will be included although the study is very specific and the actual scientific protocol can only be done in one way, leaving little room for public involvement in the design.

ETHICS AND DISSEMINATION

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602)

Ethical approval: Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019). Protocol Version: V1.0 08.01.2019.

Publication policy

Centres providing more than 50 patients can provide author to the authorship list.

Dissemination policy

We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

DISCUSSION

PDAC has a dismal prognosis, which is due to its late diagnosis. The success in reducing the mortality rate of PDAC is related to the development of early detection and prevention programs. Age and DM are known as risk factors of PDAC (9-11, 14, 15).

The expected positive endpoint of this study is to validate a biomarker panel that is suitable for early stage diagnosis in a mostly incurable, high-mortality cancer, when surgery is still possible and the cancer can be cured. This test only requires one blood sample collection, which means that it is simple, repeatable, tolerable, minimally invasive, nearly painless, widely achievable and relatively cheap – it thus fulfils all the criteria set for a screening method. Identifying PDAC in an earlier (still resectable) stage through surveillance of high-risk patients would increase surgical resection rate, cure rates and survival by 30–40%. It would save lives, maintain better well-being among the population and would have an enormous financial benefit: the increasing number of successful surgical interventions leads to a lower necessity of chemotherapy and palliative interventions (such as stent implantations or gastroenteroanastomosis operations), moreover lower the burden the healthcare cost.

Trial organization, committees and boards: The coordinator of the NODES study is LC with the support of the Hungarian Pancreatic Study Group (HPSG-coordinating society, https://tm-centre.org/en/study-groups/hungarian-pancreatic-study-group/). HPSG has been running high-quality international, multicentre clinical trials since 2014 and has published the relevant guidelines for pancreatic diseases to improve patient care in pancreatology (40-48).

The trial will be supported by the following committees:

Steering Committee (SC): This committee will be led by PH (gastroenterologist and internal medicine specialist). The members in Szeged (HU) will be: DI, EI.

International Translational Advisory Board (ITAB): This board will involve gastroenterologists. The ITAB will regularly monitor the progression of the trial and might give recommendations to the SC.

Data Monitoring Committee (DMC): DMC will handle all the data and ensure that the data in the eCRF is accurate, complete and legible. Data Management Plan (DMP) will describe the detailed data flow. The Data Manager will validate the data from completed eCRFs, according to a Data Cleaning Plan (DCP). Any missing, implausible or inconsistent recordings in the eCRFs will be referred back to the Investigator using a data query form (DQF), and be documented for each individual subject before clean file status is declared. All changes to eCRFs will be recorded.

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Authors' contributions

The study was designed by ID, LC, BK, PH. ID, LC, MGA drafted the manuscript. All authors edited, read and approved the final manuscript. Literature search, statistical calculation and figures preparation will be done by SV, NZ, AS, KM. During the study ID, EI, GH, KK, HV, GZ, MT, MS, VH, IO, LC are going to collect the patients.

CONFLICT OF INTEREST STATEMENT

BK and MGA are employees of Metanomics Health GmbH, Germany.

The other authors have no competing interest.

Abbreviations:

APCI - atmospheric pressure chemical ionization

AUC – area under the curve

CA 19-9 – carbohydrate antigen 19-9

CER – ceramides

DM – diabetes mellitus

DMC - Data Monitoring Committee

ERCP – endoscopic retrograde cholangio-pancreatography

ESI - electrospray ionization

EUS – endoscopis ultrasound

FDR - false discovery rate

GC-MS - gas chromatography-mass spectrometry

HbA1c – haemoglobin A1c

HPSG – Hungarian Pancreatic Study Group

ITAB - International Translational Advisory Board

LC- MS/MS - liquid chromatography-MS/MS

MRI – magnetic resonance imaging

MRM - multiple reaction monitoring

NPLC - normal phase liquid chromatography

NPV – negative predictive value

PaC – pancreatic cancer

PCA - principal component analysis

PPV – positive predictive value

ROC - receiver operating characteristic

SC - Steering Committee

SM – sphingomyelins

SP –Sponsor

T2DM – type 2 diabetes mellitus

T3cDM – type 3c diabetes mellitus

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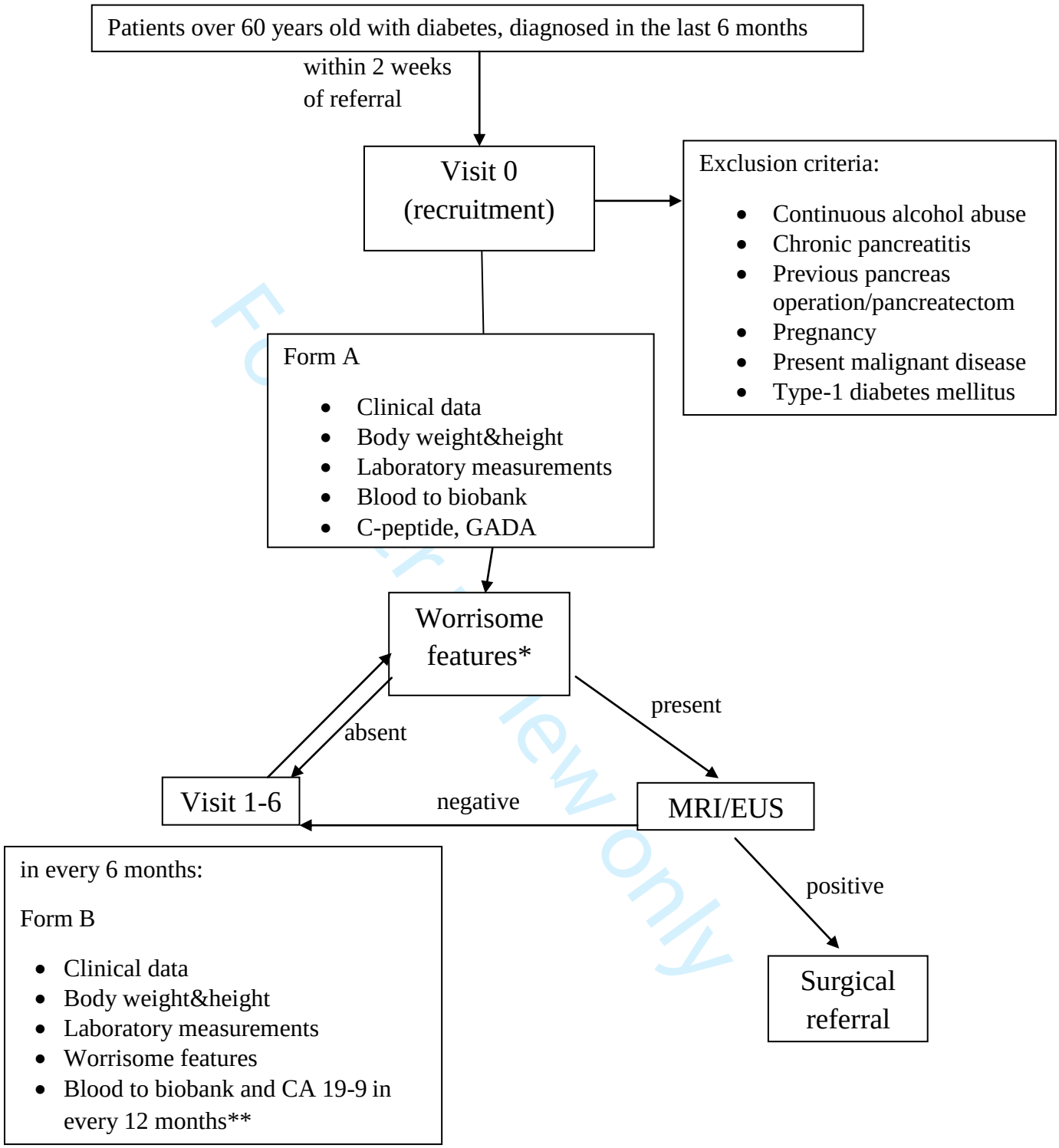
Figure 1. Flowchart of the study protocol



* weight loss (except at visit0), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection (except at visit0)
EUS: endoscopic ultrasound; MRI: magnetic resonance imaging

** Fasted (overnight, at least 8 h) patients' blood samples at room temperature will be drawn into an EDTA tube. Within 2 h after blood draw samples will be at 19-21°C. After centrifugation, the supernatant is carefully removed. After that, the plasma is transferred in 0.5 ml aliquots to tubes and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer).

BMJ Open

New Onset of DiabetEs in aSsociation with pancreatic ductal adenocarcinoma (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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Keywords:	General diabetes < DIABETES & ENDOCRINOLOGY, Pancreatic disease < GASTROENTEROLOGY, PREVENTIVE MEDICINE, Protocols & guidelines < HEALTH SERVICES ADMINISTRATION & MANAGEMENT

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New Onset of DiabetEs in aSsociation with pancreatic ductal adenocarcinoma (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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Key words: new-onset diabetes mellitus, pancreatic cancer, biomarker, screening

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ABSTRACT

Introduction: Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with an overall 5-year survival of approximately 8%. The success in reducing the mortality rate of PDAC is related not only to the discovery of new therapeutic agents, but also to a significant extent to the development of early detection and prevention programs. Patients with new-onset diabetes mellitus represent a high-risk group for PDAC as they have an 8-fold higher risk of PDAC than the general population**.** The proposed screening program may allow the detection of PDAC in the early, operable stage. Diagnosing more patients in the curable stage might decrease the morbidity and mortality rates of PDAC and additionally reduce the burden of the healthcare.

Methods & Analysis: This is a prospective, multicentre observational cohort study. Patients ≥60 years old diagnosed with new-onset (≤ 6 months) diabetes will be included. Exclusion criteria are (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; (5) present malignant disease and (6) type-1 diabetes mellitus. Follow up visits are scheduled every 6 months for up to 36 months. Data collection is based on questionnaires. Clinical symptoms, body weight and fasting blood will be collected at each, CA 19-9 and blood to biobank at every second visit. The blood samples will be processed to plasma and analysed with mass spectrometry-based metabolomics. The metabolomic data will be used for biomarker validation for early detection of PDAC in the high-risk group new-onset diabetes patients. Patients with worrisome features will undergo MRI or EUS investigation, and surgical referral depending on the radiological findings. The primary endpoint is the incidence of PDAC in patients with newly diagnosed diabetes mellitus.

Ethics and dissemination: the study has been approved by the Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019). We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602).

Strengths and limitations of this study:

Strength 1: As patients are included prospectively, the study will yield a cohort to examine the metabolic changes that coincide with the occurrence of PDAC at a very early stage before it is diagnosed.

Strength 2: The criteria for the diagnoses of diabetes and PDAC will be uniformly applied throughout the study period, moreover the diagnosis of pancreatic cancer will be confirmed with a high level of certainty in all subjects.

Strength 3: Taking part in the screening is connected to a very low burden, as the blood collection is only minimally invasive.

Strength 4: All patients will be monitored closely and frequently, which will increase the survival of all participants, especially the high-risk patients.

Limitation 1: It might be really difficult to include the required number of patients, consideing that PDAC is a rare disease, and the elderly population has more comorbidities which not even make our observation more difficult, but also leads to a higher follow-up loss during the 36 months.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a rare disease with a lifetime prevalence of 1.39%, but its prevalence is continuously increasing (1-3). The prognosis is extremely poor: it has a five-year survival rate of only 7-8% (4), and this rate has barely improved in the last 40 years (5). PDAC will be the second leading cause of cancer-related death by 2030 (6). The high mortality rate is a consequence of delayed diagnosis: in the absence of specific symptoms, PDAC is often diagnosed at an advanced stage. Surgery is the only curative treatment at this moment. Unfortunately, only 20% of the patients are eligible for curative resection at the time of the diagnosis because of the presence of metastases and locoregional infiltration (7). The success in reducing the mortality rate of PDAC is related to a significant extent to the development of early detection and prevention programs. An effective screening programme is needed for the early diagnosis of PDAC in the asymptomatic stage to improve the prognosis. Due to the low lifetime prevalence, the population-based screening is neither feasible nor cost-effective. It is recommended that subjects at high risk of PDAC should be screened (8).

Pancreatic ductal adenocarcinoma and diabetes mellitus

Patients with diabetes mellitus (DM) have an eight-fold higher risk of developing PDAC within 2–3 years after the diagnosis of diabetes relative to the general population (9). In a meta-analysis which included 36

studies, individuals in whom DM had only recently been diagnosed (<4 years) had a 50% increased risk of PDAC as compared with individuals who had diabetes for >5 years (10). Another meta-analysis of 35 cohort studies showed that DM was associated with an increased risk of PDAC (summary relative risks (RRs)=1.94; 95% CI, 1.66–2.27). Interestingly, the risk decreased with the duration of diabetes (5.38 for <1 year, 1.95 for 1–4 years, and 1.49 for 5–9 years, 1.47 for ≥10 years), thus providing evidence that diabetes in PDAC patients is caused by the cancer itself (11). In these cases, patients are actually suffering from diabetes type 3c (T3cDM). Diabetes is already prevalent in small PDACs (12), and what is more important, that diabetes occurs before the tumour is radiologically detectable (13). A population-based study found that approximately 1% of patients with new-onset diabetes at age 50 or older will be diagnosed with PDAC within 3 years of first meeting criteria for diabetes, and 56% of these within 6 months of meeting the criteria for diabetes (9). Recognition of new-onset diabetes as an early manifestation of PDAC could lead to diagnosis of asymptomatic, early-stage PDAC (14). In our recent prospective study, the prevalence of PDAC in patients with new-onset diabetes was significantly higher than in the general population (the value of the Standardised Incidence Ratio for PDAC in new-onset type 2 diabetic patients was 198.6 (95% CI = 6.25-46.9)); therefore, screening seems to be beneficial for detecting PDAC in this patient population (15). Weight loss in patients with pancreatic carcinoma-associated DM often precedes the onset of diabetes, while new-onset primary type 2 DM is typically associated with weight gain (16). The paradoxical development of diabetes in the face of ongoing weight loss may be an important clue to diagnose PDAC in patients with new onset of diabetes.

Screening modalities

The carbohydrate antigen 19-9 (CA19-9) is currently the only blood-based biomarker in clinical use for PDAC. The sensitivity of this marker for PDAC is 75%, the specificity is 90%, the positive predictive value is 69%, and the negative predictive value is 90% (17). These values fall below the required characteristics of a reliable screening test (10, 18); therefore, serum CA19-9 measurement is not suitable for screening for PDAC. Imaging modalities represent the gold standard for diagnosing PDAC. The first choice is transabdominal ultrasound. The sensitivity of transabdominal ultrasonography in PDAC diagnosis is only 50–70%. Its accuracy is low in tumours <1 cm, which are usually operable and negatively influenced by obesity and meteorism (19). Computer tomography has a better accuracy in diagnosing PDAC; however, the low prevalence of PDAC and radiation exposure associated with the modality prevents it from being used as a screening test. The odds for a correct diagnosis are also high employing endoscopic ultrasound or endoscopic retrograde cholangiopancreatography (ERCP), but again the low prevalence of PDAC in combination with the burden of the endoscopic intervention to the patient

preclude the application of these diagnostic methods for screening. Furthermore, it is not economically feasible to employ computer tomography or endoscopic imaging for screening as these methods are associated with high costs to the healthcare system.

The success of the strategy of using new-onset diabetes as a screening tool to identify subjects with a high likelihood of having asymptomatic PDAC will depend on our ability to differentiate PDAC-associated diabetes from the more common type 2 diabetes. PDAC-induced diabetes is thought to be a paraneoplastic phenomenon involving the release of products from the tumour rather than a result of the destruction of the pancreas due to malignant infiltration (20, 21). Data on incidence of PDAC in new onset of DM is rare, numbers of 0.25% (22), 0.85% (9), and 3.6% (23) have been reported. Therefore, to enable a diagnostic follow-up of new onset of diabetes, a further enrichment of this group is needed (14, 24, 25), e.g. elderly subjects (age is an independent risk factor for PDAC), weight loss (26), or smoking.

A biomarker panel consisting of nine metabolites plus the established protein CA19-9 were recently identified by Mayerle and colleagues with 89.9% sensitivity, 91.3% specificity and 99.8% negative predictive value for differentiating PDAC from chronic pancreatitis (27). Employing the same methods, a biomarker panel for differential diagnosis between PDAC and non-cancer-related diabetes was identified. The metabolite signature needs validation in an independent test cohort, which will be enabled with the present study. Provided the biomarker is validated, the panel could be effective for screening of the high-risk group patients diagnosed with new-onset DM.

Aims of the project

a) Estimate the incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes
b) Diagnose pancreatic ductal adenocarcinoma in an early operable stage
c) Validate a biomarker that distinguishes patients with PDAC-caused T3cDM from patients with T2DM.

METHODS AND ANALYSIS

Design

This is a prospective, multicentre, observational cohort study aiming to validate a biomarker panel in the early stage of PDAC. The data collection is based on questionnaires and blood samples will be drawn from all patients. The questionnaires (Form A at recruitment, Form B at every follow up visit) will be filled by every included patients.

Parameter	Value and unit	Description
Fasting plasma glucose	≥126 mg/dL (7.0 mmol/L)	Fasting is defined as no caloric intake for at least 8 h.
2 h plasma glucose	≥200 mg/dL (11.1 mmol/L)	Oral glucose tolerance test. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
HbA1c	≥6.5% (48 mmol/mol)	The test should be performed in a laboratory using a method that is NGSP certified and standardised to the DCCT assay.

Table 1. Diagnostic criteria of diabetes mellitus.

The inclusion criteria of this study are the following: (1) patients over 60 years of age; (2) diabetes diagnosed within six months (newly diagnosed) - diagnostical criteria are based on the Diabetes Control and Complications Trial (Table 1. (28)); (3) signed written informed consent.

Exclusion criteria are as follows: (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; (5) present malignant disease; and (6) type-1 diabetes mellitus. Patients with chronic pancreatitis were excluded because a metabolic signature differentiating between chronic pancreatitis and PDAC patients has already been published (27) and is currently further evaluated by the META-PAC consortium, while the present study aims to differentiate between patients with PDAC-caused new onset diabetes and new onset diabetes due to other causes.

Sample size

Mayerle et al. found that the biomarker signature in question could distinguish patients with PDAC from those without with an 89.9% sensitivity (marginal error 8.9%) and 81.3% specificity (marginal error 10.3%)(27). Chari et al. concluded that elderly subjects with new-onset DM has 8 times higher risk for pancreatic cancer than for a person of similar age and sex without DM (9), and also considering the epidemiologic data suggest that in Hungary the prevalence of PDAC is considerably higher than that compared to other contries' (29), we assumed a 4% prevalence for PDAC. In reference to these data, sample size calculation suggests that 1241 patients will need to be enrolled in order to confirm or reject the hypothesis for the primary endpoint with a 10% dropout, 80% power and 95% significance level. The recruitment period is planned to last 36 months, and all included patients will be followed for 36 months.

Duration:

The first recruiting centre will be initialized in 1 July 2019. Start of the patient recruitment: January 31, 2020. Planned finish of the study-recruitment: January 30, 2023.

Clinical data and clinical endpoints:

Essential baseline clinical data: age, sex, body weight, BMI, date of DM diagnosis, date of sampling, comorbidities, antidiabetic medication, clinical symptoms, histology and stage of PDAC.

Primary clinical endpoints: the sensitivity, specificity, positive and negative predictive values, and accuracy of the biomarker test

Secondary endpoints:

(1) mortality of pancreatic ductal adenocarcinoma in new-onset diabetic patients; (2) the proportion of localised and resectable pancreatic ductal adenocarcinoma; (3) change in body weight before Visit 1 and during Visit 2-6; (4) change in fasting blood glucose and HbA1c before Visit 1 and during Visit 2-6; (5) antidiabetic medications and the risk of pancreatic ductal adenocarcinoma; (6) presence of concomitant diseases; (7) smoking and alcohol intake; (8) incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes; (9) cost-benefit analysis.

Study protocol:

Diabetic patients will be recruited by our diabetologist and collaborating family physicians based on a recent (< 6 months) laboratory test (Table 1). Visit 0 is scheduled within 2 weeks from the referral (Figure 1). Patients who meet study entry criteria and no exclusion, will be informed and offered to participate in the study, however signed informed consent will be necessary for inclusion. Clinical data, body weight and worrisome features (unintentional weight loss: 5% of body weight within 6 months without knowing the reason (30), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection) will be recorded at Visit 0, and a fasting blood sample will be taken for assessment of laboratory data and metabolomics. C-peptide and glutamic acid decarboxylase antibodies (GADA) will be determined to classify diabetes at Visit 0. Patients with type-1 diabetes mellitus will be excluded. If worrisome features are present at Visit 0, MRI or EUS is performed. Unambiguous PaC lesions (>1 cm or seen also by magnetic resonance imaging) will be referred to surgery for resection. In case of ambiguous lesions in the pancreas, EUS-fine needle aspiration will be performed. Visit 1-5 are scheduled every 6 months. Clinical symptoms, body weight, laboratory data (fasting blood glucose, HbA1c, liver and renal function, lipids, blood count) will be collected at each visit. Blood to biobank and CA 19-9 will be taken at every 12 months. The follow-up will be closed at 36 months.

Biochemical methods

After informed consent, fasted (overnight, at least 8 h) patients' blood samples will be drawn into an EDTA tube. 9 ml blood tubes are centrifuged within 2 h after blood draw using a swing-out rotor at 2000 xg for 10 minutes. The sample processing is done at room temperature and the centrifuge is temperature-controlled at 19-21°C. After centrifugation, the supernatant is carefully removed, transferred to a fresh 9 ml tube and gently mixed in order to homogenise any gradient that might have been generated in the plasma supernatant. After that, the plasma is transferred in 0.5 ml aliquots to tubes (either Eppendorf Safe-Lock-Tubes 2 ml or Sarstedt Screw cap micro tubes 2 ml) and stored at –80°C, in a dedicated freezer (≤6 h from centrifuge to freezer). Biomarkers will be determined comparing metabolite levels in plasma samples from patients diagnosed with PDAC and diabetic cancer-free patients (26). CA19-9 determination is performed centralised at a certified clinical laboratory applying a cut-off of 37 U/ml as a classifier.

Cost of the biomarker test, quality-adjusted life-year (QALY) and incremental cost-effectiveness ratio (ICER) will be determined.

Metabolite profiling:

MxP® Global Profiling:

Two types of mass spectrometry analyses are applied. GC–MS (gas chromatography-mass spectrometry; Agilent 6890 GC coupled to an Agilent 5973 MS System, Agilent, Waldbronn, Germany) and LC–MS/MS (liquid chromatography-MS/MS; Agilent 1100 HPLC-System, Agilent, Waldbronn, Germany, coupled to an Applied Biosystems API4000 MS/MS-System, Applied Biosystems, Darmstadt, Germany) are used for a metabolite profiling approach (31). Fractionation and derivatisation of samples and detection technologies have been previously described (32-35). Proteins are removed from plasma samples (60 µl) by precipitation. Subsequently, polar and non-polar fractions are separated for both GC–MS and LC–MS/MS analyses by adding water and a mixture of ethanol and dichloromethane. For GC–MS analyses, the non-polar fraction is treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolysed complex lipids. The polar and non-polar fractions are further derivatised with O-methyl-hydroxylamine hydrochloride (20 mg/ml in pyridine) to convert oxo-groups to O-methyloximes and subsequently with a silylating agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) before GC–MS analysis. For LC–MS/MS analyses, both fractions are dried and subsequently reconstituted in appropriate solvent mixtures. HPLC (High performance LC) is performed by gradient elution using methanol/water/formic acid on reversed phase separation columns.

MxP® Lipids:

MxP® Lipids covers profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). The metabolites are analysed in a semi-quantitative approach (i.e. relative to a pool). Total lipids are extracted from plasma by liquid/liquid extraction using chloroform/methanol. The lipid extracts are subsequently fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to (32, 36). The fractions are analysed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with detection of specific multiple reaction monitoring (MRM) transitions for sphingomyelins (SM) and ceramides (CER) respectively.

Data normalization

Details of data normalization have been published (27). Metabolite profiling based on a semi-quantitative analytical platform results in relative metabolite levels ("ratio") to a defined reference. To support this concept and to allow an alignment of different analytical batches, two different reference sample types are run in parallel throughout the whole process. First, a project pool is generated from aliquots of all samples and measured with four replicates within each analytical sequence that comprised 24 samples. For all semi-quantitatively analysed metabolites, the results of each analyte from each sample are normalised against the median of the corresponding analyte in the pool reference samples within each analytical sequence to provide pool-normalised ratios. This process step compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences are analysed by different devices. Second, to allow for an experiment-to-experiment alignment of semi-quantitative data, MxPool™ (a large pool of a commercial human EDTA plasma suited for alignment of MxP® studies) is analysed with 12 replicated samples, and the pool-normalised ratios are further normalised to the median of the MxPool™ samples, i.e. ratios from this study are on the same level and therefore comparable with data from other studies normalised to other aliquots of the same MxPool™. A rigorous quality control is performed on peak, analyte and sample level and has been described previously (37).

Data collection and follow-up

Data collection is based on questionnaires, and will be stored in a personalised electronic database (eCRF). Form A: contains all antropometric parameters, routine clinical chemistry tests, fasting blood glucose and HbA1c. Follow-up visits will be scheduled by the patient registration system every 6 months. Blood will be taken for biomarker identification with metabolomics and CA19-9 determination at every 12 months. The total follow-up period is three years.

Pancreas adenocarcinoma will be diagnosed by histological examination.

Data set analysis and normalization

Descriptive statistics – mean, median, standard deviation, quartiles and relative frequency –relative risk (dichotomous variables), Independent Two-sample T test (continuous variable) in the case of normal distribution, furthermore Mann-Whitney test in lack of normal distribution will be performed. Logistic regression will be applied for the exploring of predictive factors. Affiliated statistical analyses will be performed with an error probability of 0.05 (type-I error probability).

Prior to statistical analysis, log10 transformation of ratios is conducted so that the data distribution becomes approximately normal. SIMCA-P version 14.0 (Umetrics AB, Umea, Sweden), TIBCO® Spotfire® 7.12.0 and R 3.3.4 are used for data analyses and visualizations. Initially, an exploratory multivariate analysis (Principal Component Analysis, PCA) is applied to log10-transformed ratios scaled to unit variance.

A simple linear model (ANOVA, package nlme) addressing additional clinical information and potentially confounding factors such as “disease”, “age”, “body mass index”, “gender” and “sample storage time” as fixed effects is fitted to the data. Significance level is set to 5%. The multiple test problem for the number of metabolites is addressed by calculating the false discovery rate (FDR) using the Benjamini & Hochberg method (38).

To classify patients depending on their metabolic profiles a penalised logistic regression is fitted via Elastic Net Algorithm using the R package glmnet (38). Equal penalties are used for both the L1 and the L2 norm. Afterwards the cutoff established previously on the biomarker identification dataset is applied on the test data without retraining, and the performance is measured in terms of area under the curve (AUC), sensitivity and specificity. Confidence levels for the AUC are calculated using the binormal model for the receiver operating characteristic (ROC) curve. When the sensitivity is fixed at a particular value, the positive and negative predictive values (PPV, NPV) and the accuracy become monotone functions of the specificity; and confidence intervals for these estimates are obtained by transformation of the confidence interval for the specificity. Confidence intervals for sensitivity, specificity and accuracy are obtained for the cutoff pre-specified in the training data by the method of Clopper and Pearson for the binomial distribution. For PPV and NPV the confidence intervals will be obtained by the method of Gart and Nam (39) for ratios of binomial parameters as implemented in the R package pairwise CI (40). When comparing the biomarker and CA19-9 on the test data, differences in sensitivity and specificity will be tested for with the McNemar test.

Centres

The study will start with the following centres (University of Szeged, University of Pécs, University of Semmelweis), however, other centres are welcome to participate as an open label study. Completion of the LETTER OF INTENT form will be mandatory for registering the participation of each institution.

PATIENT AND PUBLIC INVOLVEMENT

The scientific protocol can only be done in one way, leaving little room for public involvement in the design, therefore the patients have not been involved in the design and conception of this study.

ETHICS AND DISSEMINATION

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602)

Ethical approval: the study has been approved by the Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019). Protocol Version: V1.0 08.01.2019.

Publication policy

Centres providing more than 50 patients can provide author to the authorship list.

Dissemination policy

We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

DISCUSSION

PDAC has a dismal prognosis, which is due to its late diagnosis. The success in reducing the mortality rate of PDAC is related to the development of early detection and prevention programs. Age and DM are known as risk factors of PDAC (9-11, 14, 15).

The expected positive endpoint of this study is to validate a biomarker panel in elderly patients diagnosed with diabetes; whether it is suitable for early stage diagnosis of a mostly incurable, high-mortality cancer, when surgery is still possible and the cancer can be cured. PDAC-induced diabetes belongs to the group T3cDM and in parallel, T3cDM means the highest-high risk group for PDAC. Unfortunately, it is still underdiagnosed in the clinical practice – maybe because its symptoms are very similar to T2DM's and its diagnosis is based on complex, expensive tests that are not routinely available (41). To diagnose T3cDM patients based on these criteria would lead to enormous difficulties and it would not be a cost-effective screening method, which is unfavorable. While there are several pancreatic diseases that can cause T3cDM, this study focuses on the differences between PDAC-T3cDM and T2DM only. In that manner, this biomarker panel could be a diagnostic tool for the T3cDM-subgroup PDAC-T3cDM. The test only requires one blood sample collection, which means that it is simple, repeatable, tolerable, minimally invasive, nearly painless, widely achievable and relatively cheap – it fulfils all the criteria set for a screening method. Identifying PDAC in an earlier (still resectable) stage through surveillance of high-risk

patients would increase surgical resection rate, cure rates and survival by 30–40%. It would save lives, maintain better well-being among the population and would have an enormous financial benefit: the increasing number of successful surgical interventions leads to a lower necessity of chemotherapy and palliative interventions (such as stent implantations or gastroenteroanastomosis operations), moreover lower the burden the healthcare cost.

Trial organization, committees and boards: The coordinator of the NODES study is LC with the support of the Hungarian Pancreatic Study Group (HPSG-coordinating society, https://tm-centre.org/en/study-groups/hungarian-pancreatic-study-group/). HPSG has been running high-quality international, multicentre clinical trials since 2014 and has published the relevant guidelines for pancreatic diseases to improve patient care in pancreatology (42-50).

The trial will be supported by the following committees:

Steering Committee (SC): This committee will be led by PH (gastroenterologist and internal medicine specialist). The members in Szeged (HU) will be: DI, EI.

International Translational Advisory Board (ITAB): This board will involve gastroenterologists. The ITAB will regularly monitor the progression of the trial and might give recommendations to the SC.

Data Monitoring Committee (DMC): DMC will handle all the data and ensure that the data in the eCRF is accurate, complete and legible. Data Management Plan (DMP) will describe the detailed data flow. The Data Manager will validate the data from completed eCRFs, according to a Data Cleaning Plan (DCP). Any missing, implausible or inconsistent recordings in the eCRFs will be referred back to the Investigator using a data query form (DQF), and be documented for each individual subject before clean file status is declared. All changes to eCRFs will be recorded.

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Authors' contributions

The study was designed by ID, LC, BK. ID, LC, MGA drafted the manuscript. All authors edited, read and approved the final manuscript. Literature search, statistical calculation and figures preparation will be done by SV, NZ, AS, BC, KM. Steering Committee will be led by PH. During the study ID, EI, GH, KK, IO, GZ, MT, MS, VH, LC are going to collect the patients.

CONFLICT OF INTEREST STATEMENT

BK and MGA are employees of Metanomics Health GmbH, Germany.

The other authors have no competing interest.

Abbreviations:

APCI - atmospheric pressure chemical ionization
AUC – area under the curve
CA 19-9 – carbohydrate antigen 19-9
CER – ceramides
DM – diabetes mellitus
DMC - Data Monitoring Committee
ERCP – endoscopic retrograde cholangio-pancreatography
ESI - electrospray ionization
EUS – endoscopis ultrasound
FDR - false discovery rate
GC-MS - gas chromatography-mass spectrometry
HbA1c – haemoglobin A1c
HPSG – Hungarian Pancreatic Study Group
ITAB - International Translational Advisory Board
LC- MS/MS - liquid chromatography-MS/MS
MRI – magnetic resonance imaging
MRM - multiple reaction monitoring
NPLC - normal phase liquid chromatography
NPV – negative predictive value
PaC – pancreatic cancer
PCA - principal component analysis
PPV – positive predictive value

ROC - receiver operating characteristic

SC - Steering Committee

SM – sphingomyelins

SP –Sponsor

T2DM – type 2 diabetes mellitus

T3cDM – type 3c diabetes mellitus

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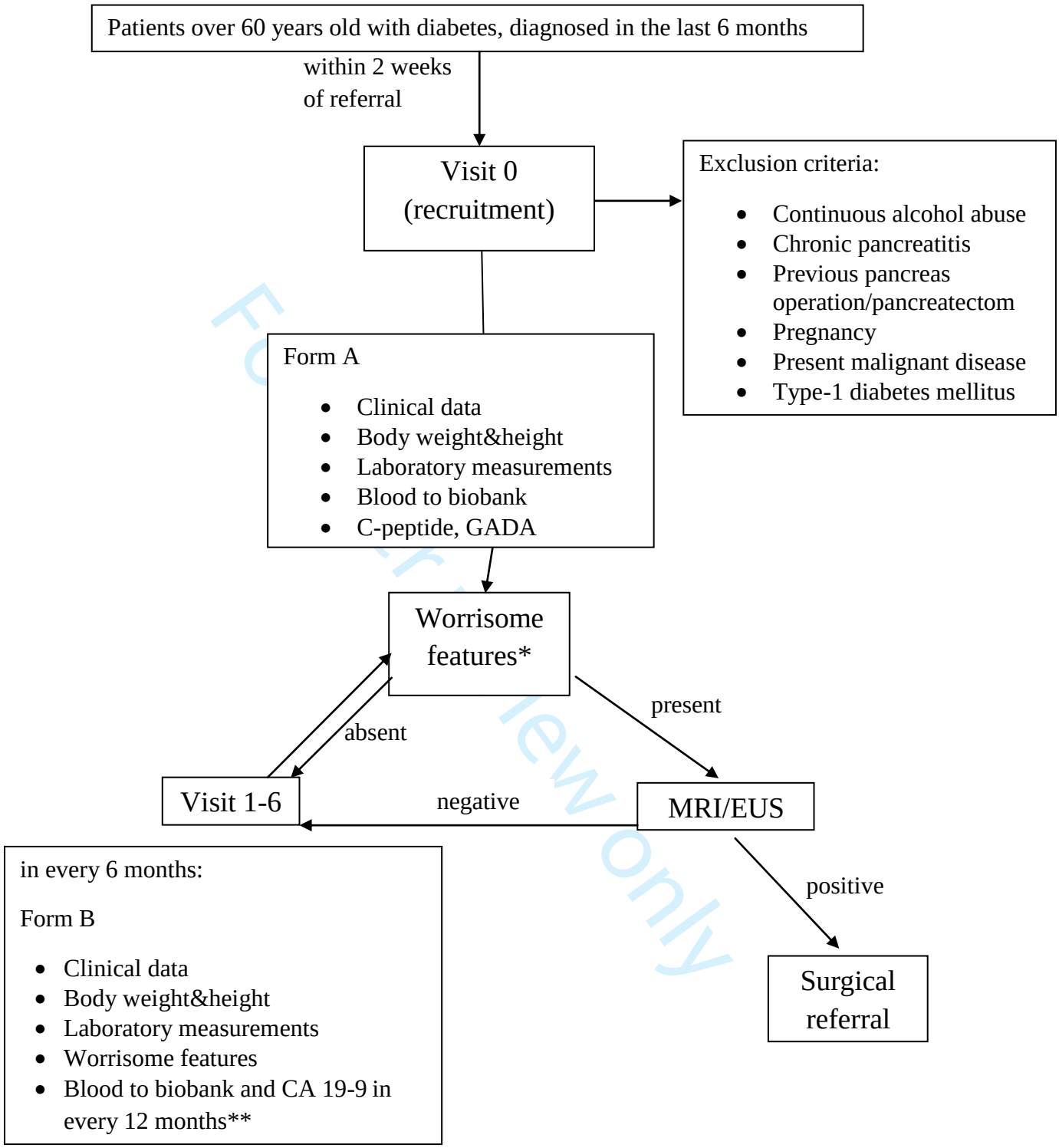
Figure 1. Flowchart of the study protocol



* weight loss (except at visit0), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection (except at visit0)
EUS: endoscopic ultrasound; MRI: magnetic resonance imaging

** Fasted (overnight, at least 8 h) patients' blood samples at room temperature will be drawn into an EDTA tube. Within 2 h after blood draw samples will be at 19-21°C. After centrifugation, the supernatant is carefully removed. After that, the plasma is transferred in 0.5 ml aliquots to tubes and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer).

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New Onset of DiabetEs in aSsociation with pancreatic ductal adenocarcinoma (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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New Onset of DiabetEs in aSsociation with pancreatic ductal adenocarcinoma (NODES trial): Protocol of a Prospective, Multicentre Observational trial

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Key words: new-onset diabetes mellitus, pancreatic cancer, biomarker, screening

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ABSTRACT

Introduction: Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with an overall 5-year survival of approximately 8%. The success in reducing the mortality rate of PDAC is related not only to the discovery of new therapeutic agents, but also to a significant extent to the development of early detection and prevention programs. Patients with new-onset diabetes mellitus represent a high-risk group for PDAC as they have an 8-fold higher risk of PDAC than the general population**.** The proposed screening program may allow the detection of PDAC in the early, operable stage. Diagnosing more patients in the curable stage might decrease the morbidity and mortality rates of PDAC and additionally reduce the burden of the healthcare.

Methods & Analysis: This is a prospective, multicentre observational cohort study. Patients ≥60 years old diagnosed with new-onset (≤ 6 months) diabetes will be included. Exclusion criteria are (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; (5) present malignant disease and (6) type-1 diabetes mellitus. Follow up visits are scheduled every 6 months for up to 36 months. Data collection is based on questionnaires. Clinical symptoms, body weight and fasting blood will be collected at each, CA 19-9 and blood to biobank at every second visit. The blood samples will be processed to plasma and analysed with mass spectrometry-based metabolomics. The metabolomic data will be used for biomarker validation for early detection of PDAC in the high-risk group new-onset diabetes patients. Patients with worrisome features will undergo MRI or EUS investigation, and surgical referral depending on the radiological findings. One of the secondary endpoints is the incidence of PDAC in patients with newly diagnosed diabetes mellitus.

Ethics and dissemination: the study has been approved by the Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019). We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602).

Strengths and limitations of this study:

Strength 1: As patients are included prospectively, the study will yield a cohort to examine the metabolic changes that coincide with the occurrence of PDAC at a very early stage before it is diagnosed.

Strength 2: The criteria for the diagnoses of diabetes and PDAC will be uniformly applied throughout the study period, moreover the diagnosis of pancreatic cancer will be confirmed with a high level of certainty in all subjects.

Strength 3: Taking part in the screening is connected to a very low burden, as the blood collection is only minimally invasive.

Strength 4: All patients will be monitored closely and frequently, which will increase the survival of all participants, especially the high-risk patients.

Limitation 1: It might be really difficult to include the required number of patients, considering that PDAC is a rare disease, and the elderly population has more comorbidities which not just makes our observations more difficult, but also leads to a higher follow-up loss during the 36 months.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a rare disease with a lifetime prevalence of 1.39%, but its prevalence is continuously increasing (1-3). The prognosis is extremely poor: it has a five-year survival rate of only 7-8% (4), and this rate has barely improved in the last 40 years (5). PDAC will be the second leading cause of cancer-related death by 2030 (6). The high mortality rate is a consequence of delayed diagnosis: in the absence of specific symptoms, PDAC is often diagnosed at an advanced stage. Surgery is the only curative treatment at this moment. Unfortunately, only 20% of the patients are eligible for curative resection at the time of the diagnosis because of the presence of metastases and locoregional infiltration (7). The success in reducing the mortality rate of PDAC is related to a significant extent to the development of early detection and prevention programs. An effective screening programme is needed for the early diagnosis of PDAC in the asymptomatic stage to improve the prognosis. Due to the low lifetime prevalence, the population-based screening is neither feasible nor cost-effective. It is recommended that subjects at high risk of PDAC should be screened (8).

Pancreatic ductal adenocarcinoma and diabetes mellitus

Patients with diabetes mellitus (DM) have an eight-fold higher risk of developing PDAC within 2–3 years after the diagnosis of diabetes relative to the general population (9). In a meta-analysis which included 36

studies, individuals in whom DM had only recently been diagnosed (<4 years) had a 50% increased risk of PDAC as compared with individuals who had diabetes for >5 years (10). Another meta-analysis of 35 cohort studies showed that DM was associated with an increased risk of PDAC (summary relative risks (RRs)=1.94; 95% CI, 1.66–2.27). Interestingly, the risk decreased with the duration of diabetes (5.38 for <1 year, 1.95 for 1–4 years, and 1.49 for 5–9 years, 1.47 for ≥10 years), thus providing evidence that diabetes in PDAC patients is caused by the cancer itself (11). In these cases, patients are actually suffering from diabetes type 3c (T3cDM). Diabetes is already prevalent in small PDACs (12), and what is more important, that diabetes occurs before the tumour is radiologically detectable (13). A population-based study found that approximately 1% of patients with new-onset diabetes at age 50 or older will be diagnosed with PDAC within 3 years of first meeting criteria for diabetes, and 56% of these within 6 months of meeting the criteria for diabetes (9). Recognition of new-onset diabetes as an early manifestation of PDAC could lead to diagnosis of asymptomatic, early-stage PDAC (14). In our recent prospective study, the prevalence of PDAC in patients with new-onset diabetes was significantly higher than in the general population (the value of the Standardised Incidence Ratio for PDAC in new-onset type 2 diabetic patients was 198.6 (95% CI = 6.25-46.9)); therefore, screening seems to be beneficial for detecting PDAC in this patient population (15). Weight loss in patients with pancreatic carcinoma-associated DM often preceeds the onset of diabetes, while new-onset primary type 2 DM is typically associated with weight gain (16). The paradoxical development of diabetes in the face of ongoing weight loss may be an important clue to diagnose PDAC in patients with new onset of diabetes.

Screening modalities

The carbohydrate antigen 19-9 (CA19-9) is currently the only blood-based biomarker in clinical use for PDAC. The sensitivity of this marker for PDAC is 75%, the specificity is 90%, the positive predictive value is 69%, and the negative predictive value is 90% (17). These values fall below the required characteristics of a reliable screening test (10, 18); therefore, serum CA19-9 measurement is not suitable for screening for PDAC. Imaging modalities represent the gold standard for diagnosing PDAC. The first choice is transabdominal ultrasonography, however, its sensitivity in PDAC diagnosis is only 50–70%. Its accuracy is low in tumours <1 cm, which are usually operable and negatively influenced by obesity and meteorism (19). Computer tomography has a better accuracy in diagnosing PDAC; however, the low prevalence of PDAC and radiation exposure associated with the modality prevents it from being used as a screening test. The odds for a correct diagnosis are also high employing endoscopic ultrasound or endoscopic retrograde cholangiopancreatography (ERCP), but again the low prevalence of PDAC in combination with the burden of the endoscopic intervention to the patient preclude the application of

these diagnostic methods for screening. Furthermore, it is not economically feasible to employ computer tomography or endoscopic imaging for screening as these methods are associated with high costs to the healthcare system.

The success of the strategy of using new-onset diabetes as a screening tool to identify subjects with a high likelihood of having asymptomatic PDAC will depend on our ability to differentiate PDAC-associated diabetes from the more common type 2 diabetes. PDAC-induced diabetes is thought to be a paraneoplastic phenomenon involving the release of products from the tumour rather than a result of the destruction of the pancreas due to malignant infiltration (20, 21). Data on incidence of PDAC in new onset DM is rare, numbers of 0.25% (22), 0.85% (9), and 3.6% (23) have been reported. Therefore, to enable a diagnostic follow-up of new onset of diabetes, a further enrichment of this group is needed (14, 24, 25), e.g. elderly subjects (age is an independent risk factor for PDAC), weight loss (26), or smoking.

A biomarker panel consisting of nine metabolites plus the established protein CA19-9 were recently identified by Mayerle and colleagues with 89.9% sensitivity, 91.3% specificity and 99.8% negative predictive value for differentiating PDAC from chronic pancreatitis (27). Employing the same methods, a biomarker panel for differential diagnosis between PDAC and non-cancer-related diabetes was identified. The metabolite signature needs validation in an independent test cohort, which will be enabled with the present study. Provided the biomarker is validated, the panel could be effective for screening of the high-risk group patients diagnosed with new-onset DM.

Aims of the project

a) Estimate the incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes
b) Diagnose pancreatic ductal adenocarcinoma in an early operable stage
c) Validate a biomarker that distinguishes patients with PDAC-caused T3cDM from patients with T2DM.

METHODS AND ANALYSIS

Design

This is a prospective, multicentre, observational cohort study aiming to validate a biomarker panel in the early stage of PDAC. The data collection is based on questionnaires and blood samples will be drawn from all patients. The questionnaires (Form A at recruitment, Form B at every follow up visit) will be filled by every included patients.

Table 1. Diagnostic criteria of diabetes mellitus.

Parameter	Value and unit	Description
Fasting plasma glucose	≥126 mg/dL (7.0 mmol/L)	Fasting is defined as no caloric intake for at least 8 h.
2 h plasma glucose	≥200 mg/dL (11.1 mmol/L)	Oral glucose tolerance test. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
HbA1c	≥6.5% (48 mmol/mol)	The test should be performed in a laboratory using a method that is NGSP certified and standardised to the DCCT assay.

The inclusion criteria of this study are the following: (1) patients over 60 years of age; (2) diabetes diagnosed within six months (newly diagnosed) - diagnostical criteria are based on the Diabetes Control and Complications Trial (Table 1. (28)); (3) signed written informed consent.

Exclusion criteria are as follows: (1) continous alcohol abuse; (2) chronic pancreatitis; (3) previous pancreas operation/pancreatectomy; (4) pregnancy; (5) present malignant disease; and (6) type-1 diabetes mellitus. Patients with chronic pancreatitis were excluded because a metabolic signature differentiating between chronic pancreatitis and PDAC patients has already been published (27) and is currently further evaluated by the META-PAC consortium, while the present study aims to differentiate between patients with PDAC-caused new onset diabetes and new onset diabetes due to other causes.

Sample size

Mayerle et al. found that the biomarker signature in question could distinguish patients with PDAC from those without with an 89.9% sensitivity (marginal error 8.9%) and 81.3% specificity (marginal error 10.3%)(27). Chari et al. concluded that elderly subjects with new-onset DM has 8 times higher risk for pancreatic cancer than for a person of similar age and sex without DM (9). In the light of the epidemiologic data suggest that in Hungary the prevalence of PDAC is considerably higher than that compared to other countries` (29), we assumed a 2% prevalence for PDAC. In reference to these data, sample size calculation suggests that 2661 patients will need to be enrolled in order to confirm or reject the hypothesis for the primary endpoint with a 10% dropout, 80% power and 95% significance level. The recruitment period is planned to last 36 months, and all included patients will be followed for 36 months.

Duration:

The first recruiting centre will be initialized in 1 July 2019. Start of the patient recruitment: January 31, 2020. Planned finish of the study-recruitment: January 30, 2023.

Clinical data and clinical endpoints:

Essential baseline clinical data: age, sex, body weight, BMI, date of DM diagnosis, date of sampling, comorbidities, antidiabetic medication, clinical symptoms, histology and stage of PDAC.

Primary clinical endpoints: the sensitivity, specificity, positive and negative predictive values, and accuracy of the biomarker test.

Secondary endpoints:

(1) mortality of pancreatic ductal adenocarcinoma in new-onset diabetic patients; (2) the proportion of localised and resectable pancreatic ductal adenocarcinoma; (3) change in body weight before Visit 1 and during Visit 2-6; (4) change in fasting blood glucose and HbA1c before Visit 1 and during Visit 2-6; (5) antidiabetic medications and the risk of pancreatic ductal adenocarcinoma; (6) presence of concomitant diseases; (7) smoking and alcohol intake; (8) incidence of pancreatic ductal adenocarcinoma in patients with new-onset diabetes; (9) cost-benefit analysis.

Study protocol:

Diabetic patients will be recruited by our diabetologist and collaborating family physicians based on a recent (< 6 months) laboratory test (Table 1). Visit 0 is scheduled within 2 weeks from the referral (Figure 1). Patients who meet study entry criteria and no exclusion, will be informed and offered to participate in the study, however signed informed consent will be necessary for inclusion. Clinical data, body weight and worrisome features (unintentional weight loss: 5% of body weight within 6 months without knowing the reason (30), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection) will be recorded at Visit 0, and a fasting blood sample will be taken for assessment of laboratory data and metabolomics. C-peptide and glutamic acid decarboxylase antibodies (GADA) will be determined to classify diabetes at Visit 0. Patients with type-1 diabetes mellitus will be excluded. If worrisome features are present at Visit 0, MRI or EUS is performed. Unambiguous PaC lesions (>1 cm or seen also by magnetic resonance imaging) will be referred to surgery for resection. In case of ambiguous lesions in the pancreas, EUS-fine needle aspiration will be performed. Visit 1-5 are scheduled every 6 months. Clinical symptoms, body weight, laboratory data (fasting blood glucose, HbA1c, liver and renal function, lipids, blood count) will be collected at each visit. Blood to biobank and CA 19-9 will be taken at every 12 months. The follow-up will be closed at 36 months.

Biochemical methods

After informed consent, fasted (overnight, at least 8 h) patients' blood samples will be drawn into an EDTA tube. 9 ml blood tubes are centrifuged within 2 h after blood draw using a swing-out rotor at

2000 xg for 10 minutes. The sample processing is done at room temperature and the centrifuge is temperature-controlled at 19-21°C. After centrifugation, the supernatant is carefully removed, transferred to a fresh 9 ml tube and gently mixed in order to homogenise any gradient that might have been generated in the plasma supernatant. After that, the plasma is transferred in 0.5 ml aliquots to tubes (either Eppendorf Safe-Lock-Tubes 2 ml or Sarstedt Screw cap micro tubes 2 ml) and stored at –80°C, in a dedicated freezer (≤6 h from centrifuge to freezer). Biomarkers will be determined comparing metabolite levels in plasma samples from patients diagnosed with PDAC and diabetic cancer-free patients (26). CA19-9 determination is performed centralised at a certified clinical laboratory applying a cut-off of 37 U/ml as a classifier.

Cost of the biomarker test, quality-adjusted life-year (QALY) and incremental cost-effectiveness ratio (ICER) will be determined.

Metabolite profiling:

MxP® Global Profiling:

Two types of mass spectrometry analyses are applied. GC–MS (gas chromatography-mass spectrometry; Agilent 6890 GC coupled to an Agilent 5973 MS System, Agilent, Waldbronn, Germany) and LC–MS/MS (liquid chromatography-MS/MS; Agilent 1100 HPLC-System, Agilent, Waldbronn, Germany, coupled to an Applied Biosystems API4000 MS/MS-System, Applied Biosystems, Darmstadt, Germany) are used for a metabolite profiling approach (31). Fractionation and derivatisation of samples and detection technologies have been previously described (32-35). Proteins are removed from plasma samples (60 µl) by precipitation. Subsequently, polar and non-polar fractions are separated for both GC–MS and LC–MS/MS analyses by adding water and a mixture of ethanol and dichloromethane. For GC–MS analyses, the non-polar fraction is treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolysed complex lipids. The polar and non-polar fractions are further derivatised with O-methyl-hydroxylamine hydrochloride (20 mg/ml in pyridine) to convert oxo-groups to O-methyloximes and subsequently with a silylating agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) before GC–MS analysis. For LC–MS/MS analyses, both fractions are dried and subsequently reconstituted in appropriate solvent mixtures. HPLC (High performance LC) is performed by gradient elution using methanol/water/formic acid on reversed phase separation columns.

MxP® Lipids:

MxP® Lipids covers profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). The metabolites are analysed in a semi-quantitative approach (i.e. relative to a pool). Total lipids are extracted from plasma by liquid/liquid extraction using chloroform/methanol. The lipid extracts are subsequently

fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to (32, 36). The fractions are analysed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with detection of specific multiple reaction monitoring (MRM) transitions for sphingomyelins (SM) and ceramides (CER) respectively.

Data normalization

Details of data normalization have been published (27). Metabolite profiling based on a semi-quantitative analytical platform results in relative metabolite levels ("ratio") to a defined reference. To support this concept and to allow an alignment of different analytical batches, two different reference sample types are run in parallel throughout the whole process. First, a project pool is generated from aliquots of all samples and measured with four replicates within each analytical sequence that comprised 24 samples. For all semi-quantitatively analysed metabolites, the results of each analyte from each sample are normalised against the median of the corresponding analyte in the pool reference samples within each analytical sequence to provide pool-normalised ratios. This process step compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences are analysed by different devices. Second, to allow for an experiment-to-experiment alignment of semi-quantitative data, MxPool™ (a large pool of a commercial human EDTA plasma suited for alignment of MxP® studies) is analysed with 12 replicated samples, and the pool-normalised ratios are further normalised to the median of the MxPool™ samples, i.e. ratios from this study are on the same level and therefore comparable with data from other studies normalised to other aliquots of the same MxPool™. A rigorous quality control is performed on peak, analyte and sample level and has been described previously (37).

Data collection and follow-up

Data collection is based on questionnaires, and will be stored in a personalised electronic database (eCRF). Form A: contains all antropometric parameters, routine clinical chemistry tests, fasting blood glucose and HbA1c. Follow-up visits will be scheduled by the patient registration system every 6 months. Blood will be taken for biomarker identification with metabolomics and CA19-9 determination at every 12 months. The total follow-up period is three years.

Pancreas adenocarcinoma will be diagnosed by histological examination.

Data set analysis and normalization

Descriptive statistics – mean, median, standard deviation, quartiles and relative frequency –relative risk (dichotomous variables), Independent Two-sample T test (continuous variable) in the case of normal distribution, furthermore Mann-Whitney test in lack of normal distribution will be performed. Logistic

regression will be applied for the exploring of predictive factors. Affiliated statistical analyses will be performed with an error probability of 0.05 (type-I error probability).

Prior to statistical analysis, log10 transformation of ratios is conducted so that the data distribution becomes approximately normal. SIMCA-P version 14.0 (Umetrics AB, Umea, Sweden), TIBCO® Spotfire® 7.12.0 and R 3.3.4 are used for data analyses and visualizations. Initially, an exploratory multivariate analysis (Principal Component Analysis, PCA) is applied to log10-transformed ratios scaled to unit variance.

A simple linear model (ANOVA, package nlme) addressing additional clinical information and potentially confounding factors such as “disease”, “age”, “body mass index”, “gender” and “sample storage time” as fixed effects is fitted to the data. Significance level is set to 5%. The multiple test problem for the number of metabolites is addressed by calculating the false discovery rate (FDR) using the Benjamini & Hochberg method (38).

To classify patients depending on their metabolic profiles a penalised logistic regression is fitted via Elastic Net Algorithm using the R package glmnet (38). Equal penalties are used for both the L1 and the L2 norm. Afterwards the cutoff established previously on the biomarker identification dataset is applied on the test data without retraining, and the performance is measured in terms of area under the curve (AUC), sensitivity and specificity. Confidence levels for the AUC are calculated using the binormal model for the receiver operating characteristic (ROC) curve. When the sensitivity is fixed at a particular value, the positive and negative predictive values (PPV, NPV) and the accuracy become monotone functions of the specificity; and confidence intervals for these estimates are obtained by transformation of the confidence interval for the specificity. Confidence intervals for sensitivity, specificity and accuracy are obtained for the cutoff pre-specified in the training data by the method of Clopper and Pearson for the binomial distribution. For PPV and NPV the confidence intervals will be obtained by the method of Gart and Nam (39) for ratios of binomial parameters as implemented in the R package pairwise CI (40). When comparing the biomarker and CA19-9 on the test data, differences in sensitivity and specificity will be tested for with the McNemar test.

Centres

The study will start with the following centres (University of Szeged, University of Pécs, University of Semmelweis), however, other centres are welcome to participate as an open label study. Completion of the LETTER OF INTENT form will be mandatory for registering the participation of each institution.

PATIENT AND PUBLIC INVOLVEMENT

No patients involved.

ETHICS AND DISSEMINATION

Trial registration: The trial has been registered at the ClinicalTrials.gov (NCT04164602)

Ethical approval: the study has been approved by the Scientific and Research Ethics Committee of the Hungarian Medical Research Council (41085-6/2019). Protocol Version: V1.0 08.01.2019.

Publication policy

Centres providing more than 50 patients can provide author to the authorship list.

Dissemination policy

We plan to disseminate the results to several members of the healthcare system includining medical doctors, dietitians, nurses, patients etc. We plan to publish the results in a peer-reviewed high quality journal for professionals. In addition, we also plan to publish it for lay readers in order to maximalize the dissemination and benefits of this trial.

DISCUSSION

PDAC has a dismal prognosis, which is due to its late diagnosis. The success in reducing the mortality rate of PDAC is related to the development of early detection and prevention programs. Age and DM are known as risk factors of PDAC (9-11, 14, 15).

The expected positive endpoint of this study is to validate a biomarker panel in elderly patients diagnosed with diabetes; whether it is suitable for early stage diagnosis of a mostly incurable, high-mortality cancer, when surgery is still possible and the cancer can be cured. PDAC-induced diabetes belongs to the group T3cDM and in parallel, T3cDM means the highest-high risk group for PDAC. Unfortunately, it is still underdiagnosed in the clinical practice – maybe because its symptoms are very similar to T2DM's and its diagnosis is based on complex, expensive tests that are not routinely available (41). To diagnose T3cDM patients based on these criteria would lead to enormous difficulties and it would not be a cost-effective screening method, which is unfavorable. While there are several pancreatic diseases that can cause T3cDM, this study focuses on the differences between PDAC-T3cDM and T2DM only. In that manner, this biomarker panel could be a diagnostic tool for the T3cDM-subgroup PDAC-T3cDM. The test only requires one blood sample collection, which means that it is simple, repeatable, tolerable, minimally invasive, nearly painless, widely achievable and relatively cheap – it fulfils all the criteria set for a screening method. Identifying PDAC in an earlier (still resectable) stage through surveillance of high-risk patients would increase surgical resection rate, cure rates and survival by 30–40%. It would save lives, maintain better well-being among the population and would have an enormous financial benefit: the increasing number of successful surgical interventions leads to a lower necessity of chemotherapy and

palliative interventions (such as stent implantations or gastroenteroanastomosis operations), moreover lower the burden the healthcare cost.

Trial organization, committees and boards: The coordinator of the NODES study is LC with the support of the Hungarian Pancreatic Study Group (HPSG-coordinating society, https://tm-centre.org/en/study-groups/hungarian-pancreatic-study-group/). HPSG has been running high-quality international, multicentre clinical trials since 2014 and has published the relevant guidelines for pancreatic diseases to improve patient care in pancreatology (42-50).

The trial will be supported by the following committees:

Steering Committee (SC): This committee will be led by PH (gastroenterologist and internal medicine specialist). The members in Szeged (HU) will be: DI, EI.

International Translational Advisory Board (ITAB): This board will involve gastroenterologists. The ITAB will regularly monitor the progression of the trial and might give recommendations to the SC.

Data Monitoring Committee (DMC): DMC will handle all the data and ensure that the data in the eCRF is accurate, complete and legible. Data Management Plan (DMP) will describe the detailed data flow. The Data Manager will validate the data from completed eCRFs, according to a Data Cleaning Plan (DCP). Any missing, implausible or inconsistent recordings in the eCRFs will be referred back to the Investigator using a data query form (DQF), and be documented for each individual subject before clean file status is declared. All changes to eCRFs will be recorded.

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Authors' contributions

The study was designed by ID, LC, BK. ID, LC, MGA drafted the manuscript. All authors edited, read and approved the final manuscript. Literature search, statistical calculation and figures preparation will be

done by SV, NZ, AS, BC, KM. Steering Committee will be led by PH. During the study ID, EI, GH, KK, IO, GZ, MT, MS, VH, LC are going to collect the patients.

CONFLICT OF INTEREST STATEMENT

BK and MGA are employees of Metanomics Health GmbH, Germany.

The other authors have no competing interest.

Abbreviations:

APCI - atmospheric pressure chemical ionization
AUC – area under the curve
CA 19-9 – carbohydrate antigen 19-9
CER – ceramides
DM – diabetes mellitus
DMC - Data Monitoring Committee
ERCP – endoscopic retrograde cholangio-pancreatography
ESI - electrospray ionization
EUS – endoscopis ultrasound
FDR - false discovery rate
GC-MS - gas chromatography-mass spectrometry
HbA1c – haemoglobin A1c
HPSG – Hungarian Pancreatic Study Group
ITAB - International Translational Advisory Board
LC- MS/MS - liquid chromatography-MS/MS
MRI – magnetic resonance imaging
MRM - multiple reaction monitoring
NPLC - normal phase liquid chromatography
NPV – negative predictive value
PaC – pancreatic cancer
PCA - principal component analysis
PPV – positive predictive value
ROC - receiver operating characteristic
SC - Steering Committee
SM – sphingomyelins

SP –Sponsor

T2DM – type 2 diabetes mellitus

T3cDM – type 3c diabetes mellitus

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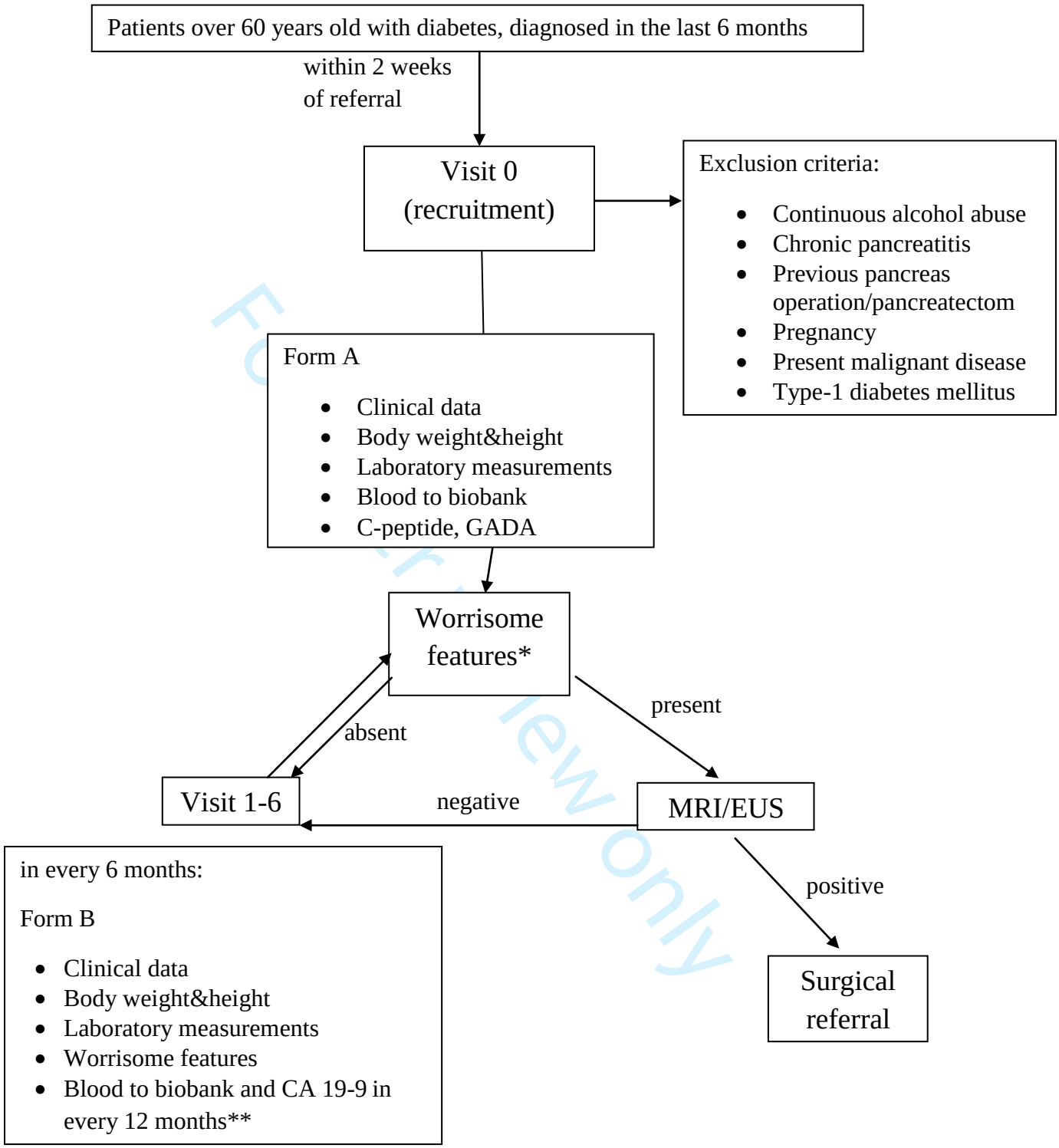
Figure 1. Flowchart of the study protocol



* weight loss (except at visit0), abdominal pain/discomfort, abnormal laboratory data, unstable glucose metabolism despite the adequate diet and medical treatment and without intercurrent infection (except at visit0)
EUS: endoscopic ultrasound; MRI: magnetic resonance imaging

** Fasted (overnight, at least 8 h) patients' blood samples at room temperature will be drawn into an EDTA tube. Within 2 h after blood draw samples will be at 19-21°C. After centrifugation, the supernatant is carefully removed. After that, the plasma is transferred in 0.5 ml aliquots to tubes and stored at -80°C, in a dedicated freezer (≤6 h from centrifuge to freezer).