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Original research

Identification and validation of a multivariable prediction model based on blood plasma and serum metabolomics for the distinction of chronic pancreatitis subjects from non-pancreas disease control subjects

M Gordian Adam,[1] Georg Beyer,[2] Nicole Christiansen,[3] Beate Kamlage,[1] Christian Pilarsky,[4] Marius Distler,[5] Tim Fahlbusch,[6] Ansgar Chromik,[7] Fritz Klein,[8] Marcus Bahra,[8] Waldemar Uhl,[6] Robert Grützmann,[4] Ujjwal M Mahajan ,[2] Frank U Weiss ,[9] Julia Mayerle ,[2] Markus M Lerch[9]

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For numbered affiliations see end of article.

Correspondence to
Professor Markus M Lerch, Department of Medicine A, University Medicine Greifswald, Greifswald, Mecklenburg-Vorpommern, Germany; markus.lerch@med.uni-greifswald.de

MGA and GB contributed equally.
JM and MML contributed equally.

MGA and GB are joint first authors.
JM and MML are joint senior authors.

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ABSTRACT

Objective Chronic pancreatitis (CP) is a fibroinflammatory syndrome leading to organ dysfunction, chronic pain, an increased risk for pancreatic cancer and considerable morbidity. Due to a lack of specific biomarkers, diagnosis is based on symptoms and specific but insensitive imaging features, preventing an early diagnosis and appropriate management.

Design We conducted a type 3 study for multivariable prediction for individual prognosis according to the TRIPOD guidelines. A signature to distinguish CP from controls (n=160) was identified using gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry on ethylenediaminetetraacetic acid (EDTA)-plasma and validated in independent cohorts.

Results A Naive Bayes algorithm identified eight metabolites of six ontology classes. After algorithm training and computation of optimal cut-offs, classification according to the metabolic signature detected CP with an area under the curve (AUC) of 0.85 ((95% CI 0.79 to 0.91). External validation in two independent cohorts (total n=502) resulted in similar accuracy for detection of CP compared with non-pancreatic controls in EDTA-plasma (AUC 0.85 (95% CI 0.81 to 0.89)) and serum (AUC 0.87 (95% CI 0.81 to 0.95)).

Conclusions This is the first study that identifies and independently validates a metabolomic signature in plasma and serum for the diagnosis of CP in large, prospective cohorts. The results could provide the basis for the development of the first routine laboratory test for CP.

Significance of this study

What is already known on this subject?

- Chronic pancreatitis greatly increases the morbidity and mortality of affected patients.
- No blood-based, serum-based or plasma-based biomarkers exist for the diagnosis of chronic pancreatitis.

What are the new findings?

- In a two-step identification and validation study a biomarker signature for chronic pancreatitis was identified by mass spectrometry (gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry).
- In 670 patients and controls from three cohorts, and among 620 identified metabolites, a panel of 8 was found to distinguish chronic pancreatitis with high accuracy from non-pancreatic controls.
- This is the first study that identifies and independently validates a metabolic biomarker signature for the diagnosis of chronic pancreatitis in large, prospective cohorts.

How might it impact on clinical practice in the foreseeable future?

- The results of this study could provide the basis for the development of a first routine laboratory test for chronic pancreatitis.

INTRODUCTION

Chronic pancreatitis (CP) is an inflammatory syndrome[1] of the pancreas in which repetitive episodes result in fibrotic tissue replacement, organ dysfunction and chronic pain.[2] Multiple aetiologies and risk factors lead to the development of CP in humans, which include immoderate alcohol consumption,[3] tobacco smoke and variety of genetic predispositions.[4–6] Affected patients are at high risk for developing exocrine pancreatic insufficiency leading to maldigestion, as well as endocrine insufficiency, leading to diabetes mellitus type 3c and pancreatic cancer.[7] Chronic pain, maldigestion and brittle diabetes will often lead to a significant reduction in quality of life, increased healthcare utilisation and reduced life expectancy.[8–11]

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In spite of multiple aetiologies and pathogeneses most CP patients will develop a similar clinical and histopathological phenotype.[12] Following current guidelines, the diagnosis of CP is made when the following criteria are met: recurrent bouts of pancreatic pain with documented rise in amylase or lipase activity, and imaging evidence such as pancreatic calcifications, histological evidence of CP, unequivocal changes in pancreatic duct morphology or severely abnormal pancreatic function tests with maldigestion.[2 13–15] Sensitivity and specificity of imaging in CP is variable and stage dependent. Non-invasive biomarkers for diagnosis of CP have not been established and remain an unmet clinical need. Moreover, it is presently impossible to identify patients at risk, or at an early stage of CP, due to a lack of reliable biomarkers.[16] While numerous genomic studies in large cohorts have identified a growing number of genetic modifiers and risk factors,[17 18] transcriptome or proteome-based approaches have failed to produce robust diagnostic tools for CP.

Little is known about the potential role of metabolomic signatures including lipidomics of body fluids as a diagnostic tool for CP. We have recently identified and validated a metabolomic signature to distinguish between pancreatic cancer and CP.[19] This prompted the search for a biomarker signature that can discriminate between CP and controls including patients with non-pancreatic conditions.

MATERIALS AND METHODS

Study design

We conducted a type 3 study for multivariable prediction for individual prognosis according to the TRIPOD guidelines.[20] A total of 670 patients and controls were prospectively enrolled in the three cohorts and the analysis was done retrospectively. The diagnosis 'CP' was made if one or more of the clinical and imaging criteria described by Mayerle *et al*[7] were met and no other diagnosis was more likely. Similar distribution of age and gender between patients and controls was attempted. Figure 1 and table 1 comprise an overview over the identification and validation cohorts.

The identification study was performed in a case control cohort at a university referral centre and included 80 CP patients and 80 non-pancreatic disease controls who underwent small, non-pancreas-related surgical procedures under general anaesthesia (table 1 and online supplemental methods).

For the first validation study, 144 CP patients and 204 non-pancreatic controls were consecutively recruited from three different university referral centres.

For the second validation study, conducted to validate the robustness of the method using serum instead of plasma samples, CP, liver cirrhosis patients and healthy blood donors were enrolled at a fourth referral centre. In this cohort, some blood samples had low sample quality, identified by their extremely low glucose levels (<2800 µmol/L), and were consecutively excluded from analysis. The samples from 49 CP patients, 57 liver cirrhosis patients and 56 healthy blood donors were included in the final analysis.

Clinical information on gender, age and body mass index (BMI) were recorded. In addition, information on the disease aetiology, disease duration, calcifications, endocrine insufficiency and enzyme supplementation were recorded if available. All cohort characteristics can be found in table 1.

All participants gave their written informed consent prior to inclusion. Further information on study details and sample processing can be found in online supplemental material and methods.

Metabolite profiling

All samples were analysed with MxP Global Profiling and MxP Lipids. MxP Global Profiling was performed employing (1) gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass-selective detector and (2) liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1100 high-performance liquid chromatography system coupled to an Applied Biosystems API 4000 triple quadrupole mass spectrometer, as has been described in detail before.[21–23]

Up to 1449 metabolites were detected within the studies depending on the sample matrix and the analytical technique. The metabolites originated from 10 different ontology classes and comprised 838 known metabolites and 611 unknown spectral features. Only those metabolites that met specific quality criteria as described in[24] were included in further statistical analyses. Furthermore, quality assessment of plasma samples was performed using the MxP Biofluids Quality Control assay (see our patent application WO2015145387A1).[25]

Statistics

All metabolite profiling data were log10-transformed before further analysis to achieve an approximate normal distribution. R V.3.3.4 was used for data analyses, see online supplemental methods for a list of R packages used (online supplemental table S1).

For an exploratory multivariate analysis (principal component analysis, PCA), the log10-transformed data of the identification and first validation study were centred and scaled to unit

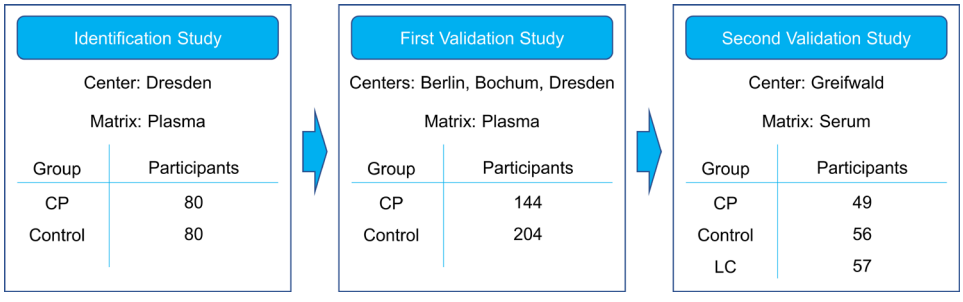

Figure 1 Study design. The biomarker signature was identified on the metabolomic data from the identification study, comparing chronic pancreatitis patients (CP) with control patients. These data were used as a training set for the algorithm. Participants of the first validation study were recruited independently and their sample data served as a test set. For the second validation study, participants were recruited independently as well. In this study, liver cirrhosis patients (LC) were included as an additional control group.

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Table 1 Cohort characteristics

Identification study (plasma)	Chronic pancreatitis	Controls	
Male	76%	52%	
Age, years (mean±SD, range)	51 (±9.9, 20–73)	64 (±14.5, 22–88)	
BMI, kg/m² (mean±SD, range)	23.1 (±4.82, 14.5–50.0)	26.8 (±4.80, 17.6–42.3)	
Diabetes	44% (unknown in six patients)	23%	
	% alcoholic CP: 75% % calcifications: 45% % PEI: 46% % PERT: 43% Mean disease duration, years: 13±16.91	Vascular surgery: 25% Hernia repair: 22% Thyroid surgery: 4% Other: 49 %	
First Validation Study (plasma)			
Male	83%	66%	
Age, years (mean±SD, range)	51 (±11.5, 22–79)	52 (±14.9, 24–90)	
BMI, kg/m² (mean±SD, range)	23.6 (±3.70, 15.7–34.8)	27.3 (±5.16, 16.4–48.9)	
Diabetes	28%	13%	
	% alcoholic CP: 43% % calcifications: 68% (unknown in 29) % PEI: 60% (unknown in 17) % PERT: 60% (unknown in 17) Mean disease duration, years: 7.3±8.55 (unknown in 36)	Vascular surgery: - Hernia repair: 80% Thyroid surgery: 20% Other: -	
Second Validation Study (serum)		**Blood donors**	**Liver cirrhosis**
Male	86%	68%	81%
Age, years (mean±SD, range)	47 (±11.0, 25–71)	54.6 (±11.3, 25–68)	59.4 (±11.3, 28–82)
BMI, kg/m² (mean±SD, range)	24.7 (±14.1, 17–34, unknown in 37)	27.6 (±4.1, 20–36)	28.6 (±4.1, 19–38, unknown in 32)
Diabetes	12%	0 %	11%
	% alcoholic CP: 100% % calcifications: 16% (unknown in 34) % PEI: 18% (unknown in 28) % PERT: 16% (unknown in 32) Mean disease duration, years: 1.2±1.85 (unknown in 35)	NA	% alcoholic LC: 100%

BMI, body mass index; CP, chronic pancreatitis; LC, liver cirrhosis; NA, not applicable; PEI, pancreatic enzyme insufficiency; PERT, pancreatic enzyme replacement therapy.

variance, and missing values were imputed before the analysis (see online supplemental methods).

The log10-transformed, scaled and imputed ratios from the second validation study were also used for a PCA, which was visualised separately because of the different sample matrix. TIBCO Spotfire V.7.12.0 was used to visualise the PCAs.

To differentiate between CP patients and controls depending on their metabolic profiles, a Naive Bayes model was fitted using the log10-transformed, median-imputed, centred and scaled data from the identification study. Based on biomedical expertise a panel was nominated (see online supplemental methods). An algorithm was trained with the data on the selected panel metabolites. The fitted model was evaluated with 10-fold cross-validation. Optimal coefficients were determined, and an optimal cut-off based on the criteria of a sensitivity of 0.8 was calculated in order to classify the patients.

To validate the generated model for patient classification, the algorithm was applied to log-10-transformed, centred and scaled data from the first validation set. For scaling of the first validation dataset, the mean and SD of the identification dataset were applied. A prediction score was calculated for each patient and patients were classified according to their score being above or below the cut-off value. The cut-off established previously on the biomarker identification dataset was applied on the data from the first validation set without retraining, and the performance was measured in terms of area under the curve (AUC), sensitivity and specificity. Confidence levels for the AUC were calculated using the binormal model for the receiver operating characteristic (ROC) curve. Further information on the prediction model and more details on the statistical analysis can be found in online supplemental file.

To test the validity of this classification algorithm and the corresponding cut-off when the sample matrix is serum and not plasma, it was also applied separately to the log10-transformed, centred and scaled data from the second validation dataset in the same manner.

Independently, a simple linear model (multivariate analysis of variance (MANOVA)) was calculated using 'disease', 'age', 'BMI' and 'gender' as independent variables and log10-transformed metabolite profiling data as dependent variable from all three studies separately. Each numerical independent variable was scaled prior to the analysis.

In the second validation study, BMI values were not available for all participants. For inclusion of BMI as confounding factor in MANOVA, missing BMI values were imputed. Significance level was set to 5%. The multiple test problem for the number of metabolites was addressed by calculating the false discovery rate (FDR) using the Benjamini and Hochberg method.[26] The cut-off for the FDR was set at 20%.

Advancement criteria

The goal of our study was to design a biomarker that can discriminate between CP patients and controls with an AUC of at least 0.8. In addition, the specificity needed to be higher than the sensitivity because guidelines emphasise that diagnosis of CP comes with great burden and may induce stigmatisation for the patient. For a successful validation, we expected the confidence intervals for the performance (AUC) to overlap between the training and the test set.

RESULTS

Study cohorts and patient characteristics

Clinical characteristics of all cohorts are shown in table 1. All studies were unbalanced between male and female participants

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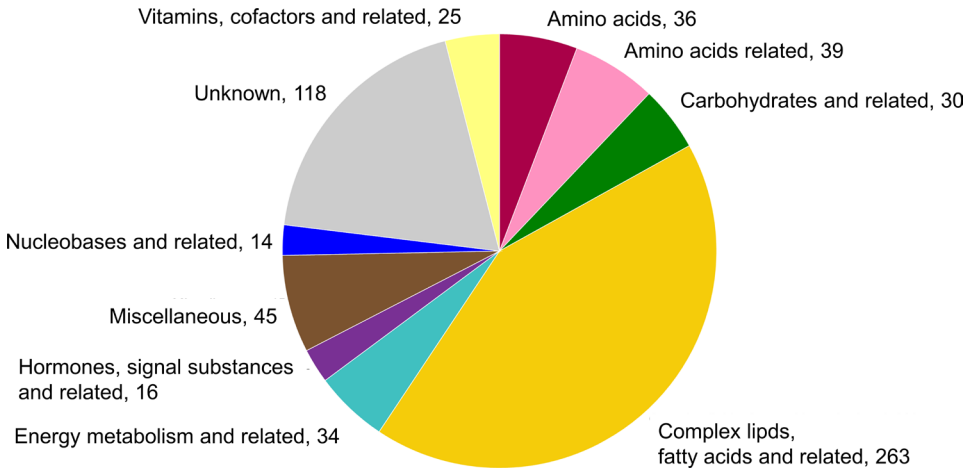

Figure 2 Metabolite distribution over ontology classes and number within class as analysed in the identification study and the first validation study.

due to the nature of CP, which is much more frequent in men than in women.[27]

Metabolomic analyses in samples from CP patients and non-pancreatic controls

The metabolomics data underwent a strict quality control after which 505 known and 115 unknown metabolites from plasma samples, and 498 known and 118 unknown metabolites from serum samples remained for statistical analysis. Most of the metabolites could be detected in both plasma and serum samples. Their distribution over the ontology classes is shown in figure 2.

Discrimination of CP and control patients by multivariate statistics

We investigated whether metabolic profiles of CP and control patients could generally be separated in an unsupervised multivariate statistical approach.

A PCA was performed on all plasma sample data. The best separation of groups was obtained in the principal components (PC) 1 and 2, which accounted for 12% and 7% of the whole variance of the dataset, respectively (figure 3A). The two groups showed a major overlap but samples from CP patients had a tendency towards lower scores in PC1, which was remarkable for a heterogeneous cohort with high interindividual variability due to diverse lifestyles, medications and comorbidities.

In the PCA obtained in the second validation study, liver cirrhosis patients were added as a third diagnosis group in addition to CP and controls (figure 3B). The best separation between the groups was again observed in PC1 and PC2 (21% and 13% of the observed variance). Remarkably, an almost complete separation of the control group from the other two could be observed. The CP patients tended to have higher scores in the PC2 than the liver cirrhosis patients, resulting in a visible separation between these groups.

While it is not common to perform a PCA in the validation cohorts, it was done in this case to show that the metabolomic profiles and the distribution of the CP versus control group patients in the identification study and the first validation study are very similar, proving that the two studies, although conducted independently, are actually comparable. The PCA of the second validation study yields a good overview on how the metabolic profile of the liver cirrhosis patients relate to the metabolic profiles of CP patients and controls.

Biomarker discovery and training

The observed separation tendencies in our multivariate approach indicated the possibility to compile a biomarker signature that allowed differentiation between CP and control patients.

As result of the Naive Bayes algorithm and biomedical expertise applied on the identification study a panel of eight metabolites (table 2) was nominated. Using the optimal calculated cut-off of 0.479 of the prediction score, the biomarker signature detected CP in comparison to control patients with an AUC of 0.85 (95% CI 0.79 to 0.91) as explained in online supplemental

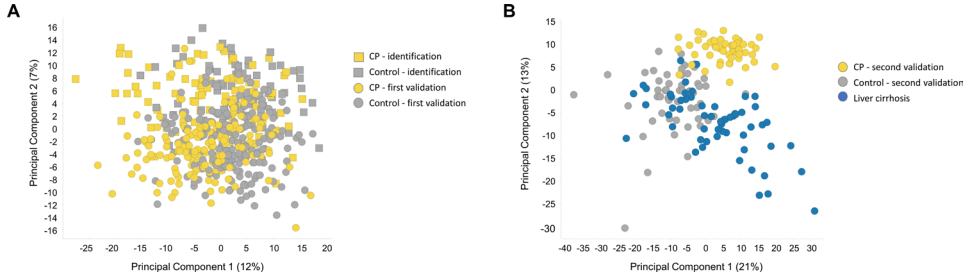

Figure 3 Multivariate analysis. (A) Principal component analysis (PCA) score plot of the identification study and the first validation study sets (EDTA plasma). The best separation of the control group (grey) and the CP group (amber) was observed plotting principal component (PC) 1 vs PC2. Together, these PCs account for 19% of the observed total variation within the dataset. (B) PCA score plot of the second validation study set (serum). A marked separation between the control group (grey), the CP group (amber), as well as the liver cirrhosis group (blue) was observed plotting PC1 vs PC2. While there was some overlap between the CP and the liver cirrhosis groups, the control group is markedly separated from the other two. Together, these PCs account for 34% of the observed total variation within the dataset. CP, chronic pancreatitis; EDTA, ethylenediaminetetraacetic acid.

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Table 2 List of metabolites selected for the biomarker signature

Metabolite name	Ontology class	Biological background
Beta-carotene	Vitamins, cofactors and related	Exogenous compound, lipid malabsorption
Cryptoxanthin	Miscellaneous (Subclass: diet related)	Exogenous compound, lipid malabsorption
Behenic acid (C22:0)	Complex lipids, fatty acids and related (Subclass: fatty acids)	Poorly absorbable, de novo synthesis, ceramide related
Indole-3-acetic acid	Amino acids and related	Altered bioavailability due to microbiome changes
Hippuric acid	Miscellaneous (Subclass: microbiome related)	
Mannose	Carbohydrates and related	Altered blood glucose levels due to impaired endocrine function inhibits mannose conversion to glucose.
Ceramide (d18:1, C24:1)	Complex lipids, fatty acids and related (Subclass: Sphingolipids)	Increased in response to cellular stress (ie, fibrosis and calcifications)
N-acetylcytidine	Nucleobases and related	Increased in chronic inflammation

table S2). The specificity was 0.86 and the sensitivity 0.71 (table 3); ROC curves see figure 4A.

Biomarker validation in independent validation cohorts

The biomarker signature was tested in a first validation study, comprising ethylenediaminetetraacetic acid (EDTA) plasma samples from 348 patients. Cut-off and coefficient values for the metabolites were transferred from the identification study, and the diagnostic performance was evaluated. CP (in comparison to control patients) was detected with an AUC of 0.85 (95% CI 0.81 to 0.89). This corresponded to a specificity of 0.66 and a sensitivity of 0.84 (table 3). The CIs of the AUCs in both studies were almost identical, indicating that the biomarker performance was robust enough to be successfully transferred to the first validation study. The ROC curve is shown in figure 4B.

Having obtained a steady performance in the first validation study, we tested whether the CP biomarker signature was valid in a different context and conducted a second validation study, including metabolomics data from 162 individuals. In this cohort, we used serum samples, and liver cirrhosis patients as additional controls.

Cut-off and metabolite coefficients were again transferred from the identification study to the second validation study without retraining, and the diagnostic performance was assessed. The AUC for CP versus control was 0.87 (95% CI 0.81 to 0.95). This CI showed considerable overlap with the AUC CI in the identification study, indicating a successful performance in the second validation. Specificity was 0.89 and sensitivity 0.78 (table 3). The ROC curve is shown in figure 4C. The biomarker signature was thus robust enough to work with serum samples.

When the algorithm was applied to liver cirrhosis samples, the results were more similar to the CP group than to the blood donor group.

Table 3 Performance characteristics for the biomarker signature

	Chronic pancreatitis versus control		
Dataset (matrix)	Identification study (plasma)	First validation study (plasma)	Second validation study (serum)
AUC (95% CI)	0.85 (0.79 to 0.91)	0.85 (0.81 to 0.89)	0.88 (0.81 to 0.95)
Sensitivity (LL – UL)	0.71 (0.60–0.80)	0.84 (0.77–0.89)	0.78 (0.64–0.87)
Specificity (LL – UL)	0.86 (0.77–0.92)	0.66 (0.60–0.73)	0.89 (0.78–0.95)
Cut-off	0.479	0.479	0.479

AUC, area under the curve; LL, lower limit; UL, upper limit.

Multivariate statistical analysis of the biomarker panel in the three study cohorts

Separate analysis of the three datasets revealed that the eight chosen metabolites were all significantly altered in CP patients versus controls in both plasma-based studies ($p<0.05$; FDR<0.2), and 6 of them also in the serum-based study. The variance analysis results (fold-changes) for the panel metabolites are shown in table 4. FDR-corrected p values (q values) are provided in online supplemental table S3. Of note, the fold-changes were in the same range across all studies. A striking feature was the very small p values for the panel metabolites in the first validation study. Due to the larger sample number in this study, the statistical significance was higher also for metabolites with small shifts in concentration levels between the groups. In addition, the PCAs show that the groups in the second validation study were less homogenous than in the first validation study, leading to higher p values in the MANOVA.

The calculation of prediction scores revealed the relative importance of the panel metabolites, with beta-carotene and cryptoxanthin having the highest impact (table 4). Boxplots indicate the inter-individual variability of the panel metabolite levels (figure 5A–H) in the different studies. They illustrate why the carotenoids were chosen as the most important metabolites by the algorithm, as they discriminated best between groups. Nevertheless, single metabolites were unable to discriminate between CP and controls. Only the computation of the biomarker panel yielded a sufficiently accurate diagnosis. The distribution of age and BMI over the biomarker signature score is shown in online supplemental figure S1.

Effect of pancreatic insufficiency

Because the identification of beta-carotene and cryptoxanthin suggested a pathophysiological mechanism of maldigestion/malabsorption, we analysed whether pancreatic exocrine insufficiency (PEI) and enzyme supplementation (pancreatic enzyme replacement therapy, PERT) had an effect on plasma carotenoid levels (online supplemental figure S2). No significant increase of carotenoid plasma levels in PEI with PERT was observed. However, a significant increase of the biomarker signature score values was seen when comparing CP patient from the identification and first validation cohorts with and without pancreatic insufficiency, indicating correlation of this metabolic biomarker signature with disease stage (online supplemental figure S3).

DISCUSSION

In our proof-of concept biomarker study following the TRIPOD guidelines[20] we show for the first time that a signature comprised of 8 metabolites of six different ontology classes can successfully differentiate between CP and controls with acceptable accuracy (AUC >0.8) in serum and EDTA-plasma samples.

There is no recommended blood-based biomarker for diagnosis of CP in medical guidelines.[2] Some proteins or miRNAs have been proposed to have a potential to take up this role, but validation studies in larger cohorts are still lacking.[28 29] Those biomarkers analytically validated for diagnosis using mass spectrometry are reviewed by Chou *et al*, but diagnostic accuracy is mostly unknown.[30] Studies in rodents employing spontaneous and pharmacologically induced models of CP suggested significant alterations to the pancreatic metabolome, including energy production, anabolism, lipid synthesis and ROS detoxification pathways.[31 32] Small and due to their heterogeneity inconclusive nuclear MR-spectroscopy-based human studies identified changes in citrate and adenosine levels in urine and

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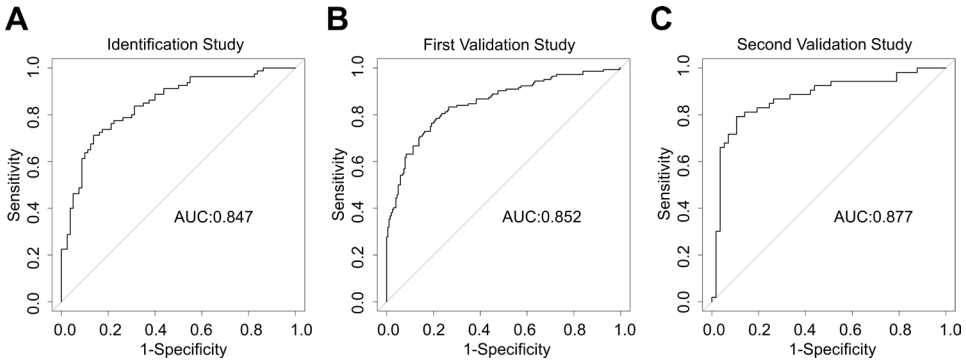

Figure 4 Performance of the biomarker signature for detection of chronic pancreatitis. Receiver operating characteristics and area under the curve (AUC) of the biomarker signature for differentiating chronic pancreatitis from control. (A) Identification study (EDTA plasma) (B) first validation study (EDTA plasma) (C) second validation study (serum). EDTA, ethylenediaminetetraacetic acid.

3-hydroxybutyrate, trimethylamine-N-oxide, acetate, acetone, isoleucine, acetylglycine, triglyceride and inosine levels in serum.[33 34] This prompted us to conduct a trial with a study design more adequate for biomarker development.

The main strength of the signature discovered in the presented study is its robustness. We used large and well-characterised patient cohorts with adequate controls for training and external validation. The comparability of results yielded from EDTA-plasma and serum samples underscores the validity of the assays used, despite the described high intraindividual and interindividual variability of the blood-metabolome.[21] The signature yielded in acceptable diagnostic accuracy in the three presented sub-studies with AUC varying between 0.85 and 0.87. Of note, we report considerable variation regarding sensitivity and specificity in the first validation study which is explained by a certain heterogeneity when recruiting at multiple sites. The relevance of this finding needs exploration in further studies.

On closer inspections, the identified metabolites fit pathophysiological concepts of CP. Levels of fat-soluble vitamins, antioxidants and trace elements have been reported to be lower in CP patients when compared with controls.[35–40] Whether the deficiency in beta-carotene and cryptoxanthin (exogenous compounds) is secondary to malabsorption remains unknown.[41 42] We could not discover an association between PEI or PERT and beta-carotene levels in the identification cohort. However, these data need to be interpreted with caution as previous studies have been inconsistent regarding the relationship of PEI and nutritional markers.[43 44] This is the first study to describe a decrease in beta-carotene levels as a diagnostic marker for CP.

Mannose and other carbohydrates were found to be significantly altered in a GC-MS based approach in studies identifying metabolic biomarkers for acute pancreatitis.[45] Although in their study, Xiao *et al* did not find a further increase after repeated episodes of acute pancreatitis, it underscores the role of the pancreas in sugar metabolism during health and disease.

Ceramides and other components related to sphingomyelin-metabolism have been associated with metabolome changes in caerulein-induced pancreatitis in mice[46] and were found to be a hallmark in a metabolome signature highly sensitive for the detection of pancreatic cancer in a previous study from our group.[19] The reduced levels of the poorly absorbable long-chain saturated behenic acid, found in our CP subjects could be directly related to ceramide pathways by ceramide synthetase 2.[47] To our knowledge, there is no published study linking behenic acid to pancreatitis.

Indol-3-acetic acid, which belongs to the group of uraemic toxins, is a tryptophan-derived byproduct of microbiota in the large intestine. It's bioavailability depends on the gut microbiome and has been linked to aryl hydrocarbon receptor expression,[48] which in turn regulates fibrosis of the pancreas.[49] The microbiome composition is sensitive to pancreatic enzyme secretion, even in non-clinically manifested CP.[50] Similarly, plasma levels of hippuric acid, another uraemic toxin, is sensitive to change of the gut microbiome related to diet, drugs and diseases.[51]

N-acetylcytidine is a post-transcriptional mRNA modification that can induce more efficient translation[52] and is implicated in inflammasome related interleukin (IL)1β production in patients with chronic inflammation.[53] Inflammasome activation, yet not a pancreas specific process, is involved in pancreatic healing and fibrosis.[54]

Taken together the signature is comprised of metabolites at least plausible to be involved in a variety of processes implicated with pathogenesis of CP. Failure of a single biomarker in an individual patient could, therefore, be compensated by other

Table 4 Statistical analysis results (linear model) of the signature metabolites (CP vs control)

Dataset (matrix)	Identification study (plasma)	First validation study (plasma)	Second validation study (serum)	Importance of rank in the algorithm
Metabolite	**Fold change (p value)**	**Fold change (p value)**	**Fold change (p value)**	
Beta-carotene	0.37 ($6.8x10^{-8}$)	0.38 ($2.5x10^{-21}$)	0.47 (0.00017)	1
Cryptoxanthin	0.40 ($8.8x10^{-8}$)	0.38 ($8.8x10^{-19}$)	0.48 (0.00043)	2
Mannose	1.47 ($5.9x10^{-8}$)	1.52 ($3.9x10^{-29}$)	2.80 ($7.3x10^{-14}$)	3
Behenic acid (C22:0)	0.72 (0.00024)	0.67 ($4.9x10^{-17}$)	1.14 (0.10586)	4
Ceramide (d18:1, C24:1)	1.27 (0.00016)	1.28 ($1.3x10^{-11}$)	1.96 ($6.7x10^{-13}$)	5
Indole-3-acetic acid	0.63 (0.00086)	0.62 ($4.2x10^{-9}$)	1.15 (0.33726)	6
Hippuric acid	0.68 (0.039)	0.49 ($2.2x10^{-8}$)	0.51 (0.00173)	7
N-acetylcytidine	1.22 (0.0096)	1.21 ($2.3x10^{-5}$)	2.36 ($3.3x10^{-13}$)	8

CP, chronic pancreatitis.

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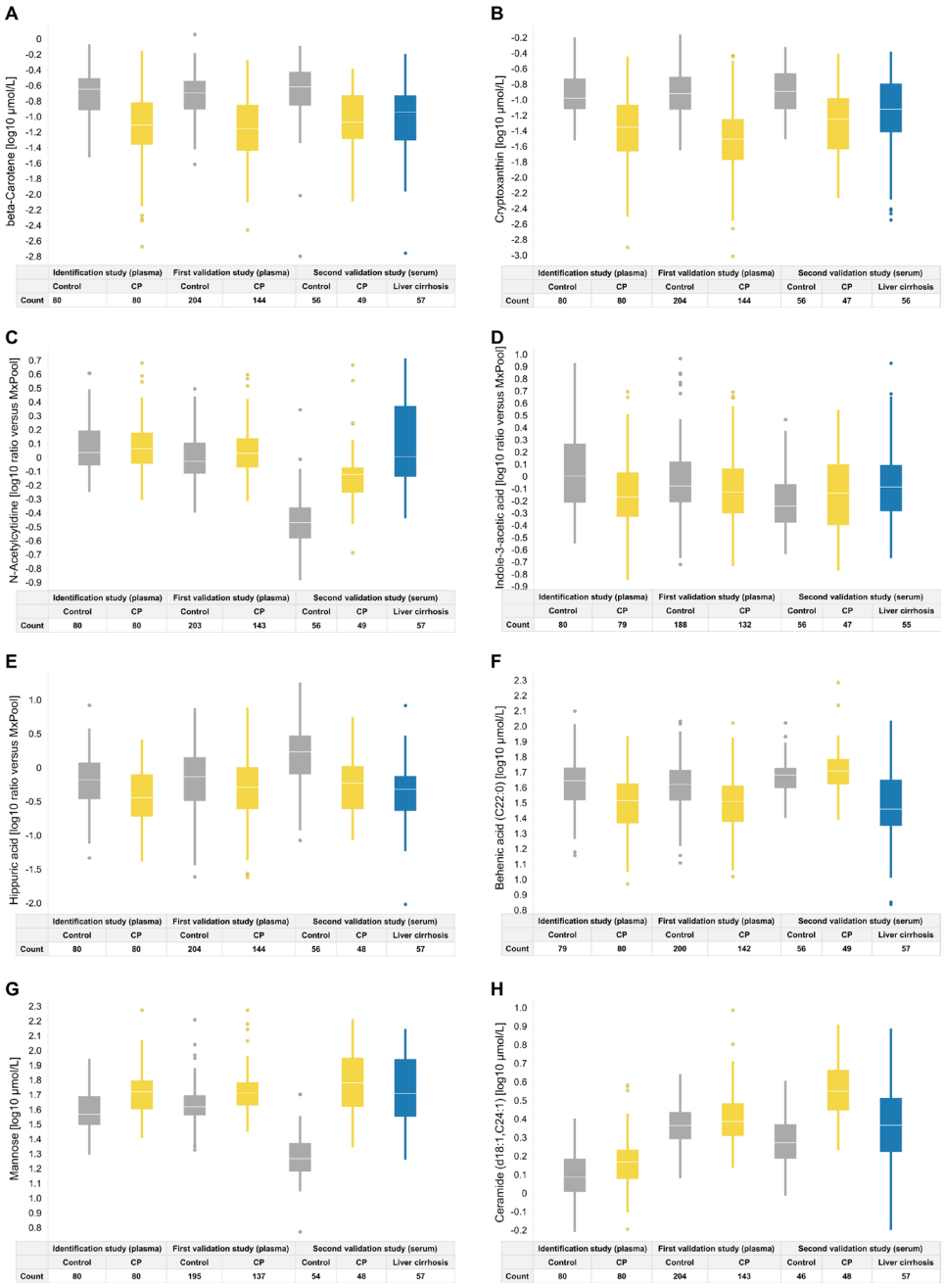

Figure 5 Boxplots of individual metabolite levels from the biomarker signature within the three studies, separated by group. CP, chronic pancreatitis.

components of the algorithm. This is further supported by the finding, that the biomarker score increases with presence of pancreatic insufficiency, a surrogate for disease stage.

A recent study by an US American consortium used a 62-plex luminex assay to identify potential biomarkers for diagnosis of CP. In blood samples of 41 controls, 20 recurrent acute pancreatitis (RAP) and 40 CP patients they found that GM-CSF, IFNb, Leptin, PDGFB and Resistin could distinguish between CP and control (AUC 0.86) and resistin, SCF, MIP-1a and IL-17F between RAP and CP (AUC 0.77).[55] Although the results are not comparable due to methodological differences it appears clear that only a combination of markers rather than a single protein allows for adequate discrimination. Independent validation of these data are pending.

A potential weakness of our study is the fact that CP patients and controls were unmatched for age, gender and BMI due to disease heterogeneity and consecutive recruitment. CP is diagnosed predominantly in middle-aged males at risk for malnutrition.[12 13] Non-pancreatic controls (eg, day-surgery patients or blood donors) comprise by default a different cohort. Consequently, gender, BMI and age were not included in the prediction model because we aimed to avoid the pitfall that a shift in age alone could be sufficient to change a positive to a negative classification or vice versa.

It cannot be ruled out that some of the differences in the metabolic profiles between the groups are due to these features. Nevertheless, the MANOVA statistics were corrected for age, gender and BMI, and still show a significant difference between the groups for all chosen metabolites. This supports our hypothesis that this metabolic signature is able to identify CP patients irrespective of gender, age and BMI.

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We found the metabolic signature developed to distinguish CP from controls to be less discriminative when applied to liver cirrhosis samples. The similarity was interpreted as being likely due to activation of fibrosis and alcohol abuse, a common feature of both disorders. Fortunately, having to discriminate between liver cirrhosis and CP is uncommon and diseases rarely overlap. In the second validation study however, an almost complete separation of the control group from CP was seen. In this cohort, the control group was comprised healthy blood donors instead of non-pancreatic patients, which explains the significant improvement.

A potential clinical use of this metabolic signature is the identification of CP patients early in the disease course (early CP), of patients with unexplained abdominal symptoms and a history of pancreatic disease, but (yet) no definitive morphological signs of CP (probable CP), or of patients with RAP at risk for developing CP.[16] These groups so far are only vaguely defined by international consensus diagnostic criteria. We, therefore, recruited patients with definitive CP for the sake of biomarker development. Whether the presented metabolic signature is sensitive enough under the above circumstances needs further testing in trials with long-term follow-up,[12] ideally in a design that includes disease staging via chronic pancreatitis prognosis score score.[44] Whether a metabolic biomarker can distinguish between CP and cirrhosis which share a common aetiology (alcohol), also needs to be addressed in future trials.

In conclusion, we have identified and validated an LC-MS/MS-based human blood-metabolome signature, which successfully discriminates between healthy individuals and patients with CP. Whether this biomarker has clinical value for diagnosing early stages of CP or can be used to monitor disease progression needs further prospective studies.

Author affiliations
[1]Metanomics Health GmbH, Berlin, Berlin, Germany
[2]Department of Medicine II, Ludwig-Maximilians-Universitat Munchen, Munchen, Bayern, Germany
[3]TrinamiX GmbH, Ludwigshafen am Rhein, Rheinland-Pfalz, Germany
[4]Department of Surgery, Erlangen University Hospital, Erlangen, Bayern, Germany
[5]Clinic and Outpatient Clinic for Visceral-, Thorax- and Vascular Surgery, Dresden University Hospital, Dresden, Sachsen, Germany
[6]St. Josef Hospital, Department of Surgery, Ruhr University Bochum, Bochum, Nordrhein-Westfalen, Germany
[7]Askleipios Clinic Harburg, Department for General and Visceral Surgery, Asklepios Hospital Group, Hamburg, Hamburg, Germany
[8]Department of Surgery, Charité Universitätsmedizin Berlin Campus Charite Mitte, Berlin, Berlin, Germany
[9]Department of Medicine A, University Medicine Greifswald, Greifswald, Mecklenburg-Vorpommern, Germany

Correction notice This article has been corrected since it published Online First. The author, Tim Fahlbusch's, name has been corrected.

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Patient consent for publication Not required.

Ethics approval All studies were designed and conducted in adherence to the Declaration of Helsinki and approved by the local ethics review boards of all four participating centres.

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Data availability statement Data may be obtained from a third party and are not publicly available. Data are deidentified patient data, which are available from the corresponding author after appropriate application for reuse under research purpose only.

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ORCID iDs
Ujjwal M Mahajan http://orcid.org/0000-0002-7238-8775
Frank U Weiss http://orcid.org/0000-0003-3369-6369
Julia Mayerle http://orcid.org/0000-0002-3666-6459

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Supplement

Identification and validation of a multivariable prediction model based on blood plasma and serum metabolomics for the distinction of chronic pancreatitis subjects from non-pancreas disease control subjects

M. Gordian Adam#[1,2], Georg Beyer#[3], Nicole Christiansen[1,4], Beate Kamlage[1], Christian Pilarsky[5], Marius Distler[6], Tim Falbusch[7], Ansgar Chromik[8], Fritz Klein[9], Marcus Bahra[9], Waldemar Uhl[7], Robert Grützmann[5], F. Ulrich Weiss[10], Julia Mayerle#[3,10], and Markus M. Lerch#[10]*

[1] Metanomics Health GmbH, Tegeler Weg 33, 10589 Berlin, Germany
[2] Biocrates AG, Eduard-Bodem-Gasse 8, 6020 Innsbruck, Austria
[3] Department of Medicine II, University Hospital, Ludwig-Maximilians-Universität München, Marchioninistr. 15, 81377 Munich, Germany
[4] trinamiX GmbH, Industriestraße 31a, 67063 Ludwigshafen am Rhein, Germany
[5] Department of Surgery, University Hospital, Erlangen, Germany
[6] Clinic and Outpatient Clinic for Visceral, Thorax, and Vascular Surgery, Medical Faculty, TU Dresden, Dresden, Germany
[7] Ruhr-University Bochum, St. Josef Hospital, Department of Surgery, Bochum, Germany
[8] Asklepios Clinic Harburg, Department for General and Visceral Surgery, Hamburg, Germany
[9] Department of Surgery, Charité Campus Mitte and Charité Campus Virchow Klinikum, Charité-Universitätsmedizin Berlin, Berlin, Germany
[10] Department of Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruchstrasse, 17475 Greifswald, Germany
* Correspondence:

Prof. Dr. med. Markus M. Lerch
Department of Medicine A
University Medicine Greifswald
Ferdinand-Sauerbruchstrasse
17475 Greifswald
Germany
Phone: +49 (0) 3834 86 7230
Fax: +49 (0) 3834 86 7234
Markus.lerch@med.uni-greifswald.de

Suppl. Material and Methods

Study details

We conducted a type 3 study for multivariable prediction for individual prognosis according to the TRIPOD guidelines [1]. Patients with chronic pancreatitis, liver cirrhosis, healthy blood donors and preoperative patients with non-pancreatic or liver disease were consecutively recruited from university referral centers in Greifswald, Dresden, Berlin, and Bochum, all in Germany.

For the identification study, EDTA plasma samples were collected within a case-control study from 80 patients with CP and 80 non-pancreatic control patients, who underwent small, non-pancreas-related surgical procedures under general anesthesia (see below).

For the first validation study, 144 chronic pancreatitis patients and 204 non-pancreatic control patients were recruited at three different centers. Because it was acknowledged that CP patients are relatively young and mostly male, during patient recruitment, special care was taken to also recruit younger and mostly male patients for the control group with the aim to achieve a similar average patient age and a similar ratio of male to female subjects in both groups.

In the second validation study, a different sample type was utilized: serum samples taken from 49 chronic pancreatitis patients, 56 controls, and 57 liver cirrhosis patients were analyzed. These samples were collected in a fourth independent center. Furthermore, the control group consisted of healthy blood donors instead of patients waiting to undergo a small surgery.

The key study dates for the three studies were as follows: accrual for the identification study was started on 2009-01-13, end of accrual was on 2013-08-01. Accrual for the first validation study was started on 2013-09-09 and ended on 2015-09-28. Accrual for the second validation study started on 2002-10-23 and ended on 2010-06-10.

The general inclusion criteria for all groups included written informed consent prior to any study procedures, age ≥ 18 to 85 years and eight hours fasting prior to blood draw.

The general exclusion criteria for all groups included type I diabetes, pregnancy or lactation phase, known viral infections like hepatitis B, hepatitis C, HIV, major surgery within the last 4 weeks before sample collection, acute anemia (Hb<9 g/dl or <5,58 mmol/l), malignant tumors within the last 5 years.

Chronic pancreatitis patients were included if one or more of the following criteria were met and no other diagnosis was more likely [2, 3, 4]: recurrent bouts of pancreatic pain with documented rise in amylase or lipase activity for a duration of more than one year plus radiological evidence supporting the diagnosis, pancreatic calcifications, histological proof

of chronic pancreatitis, unequivocal changes in pancreatic duct morphology, severely abnormal pancreatic function tests with maldigestion. Calcifications were identified on CT-scan, diabetes was diagnosed as suggested by the WHO definition and exocrine insufficiency was determined by either fecal elastase measurement or concurrent pancreatic enzyme supplementation. Pancreatitis patients were excluded if they had undergone pancreatitis surgery within 6 months before sample collection, bile duct stent placement or surgery, endoscopically assisted pancreatic aspiration <5 days before sample collection or had known liver cirrhosis.

Liver cirrhosis patients were included if preexisting liver cirrhosis had been diagnosed based on imaging and clinical chemistry. Liver cirrhosis patients were excluded if concomitant chronic pancreatitis was present.

Control patients were included if they were undergoing minor non-pancreatic surgery under general anesthesia. Control patients were excluded if they had chronic pancreatitis or liver cirrhosis or if a hernia was due to solid organ transplantation.

For the blood donors, only the standard blood donor inclusion criteria applied, i.e. the donors had to be in good general health, body weight at least 110 pounds. Participants with diabetes type II were excluded from the blood donor group because of the requirement of a fasting period of at least 8 hours which was not considered feasible for diabetics.

From the patients in the non-pancreatic control group in the identification study, 20 patients underwent vascular surgery, 18 patients received a hernia repair, 3 were resected for goiter and 39 received various other small surgical procedures under general anesthesia. From the patients in the non-pancreatic control group in the first validation study, 164 underwent hernia repair surgery and 40 were resected for thyroid goiter. None were operated in metabolically deranged state. Thus, the data in the first validation study differed from the identification study as it was a multicentric study, and in the composition of the non-pancreatic controls. The second validation study differed in the matrix used for analysis (serum instead of plasma), the center where the samples were obtained, the control group (healthy blood donors instead of non-pancreatic controls), and the inclusion of liver cirrhosis patients as an additional control group. Furthermore, 22.5% of the non-pancreatic controls in the identification study were diabetes type II patients, while 13.5% of the patients suffered from diabetes type II in the first validation study, and diabetes patients were excluded as control in the second validation study. As opposed to the identification study, the genesis of pancreatitis, calcifications, exocrine insufficiency, and enzyme supplementation were only partially available in the validation studies.

Sample Storage

Before freezing, the EDTA plasma samples and serum samples were aliquoted to avoid freeze-thaw cycles during the measurement period. Samples were stored at the respective center at -80°C until transport to the measurement location, which occurred on dry ice. Samples were stored at the measurement location at -80°C until measurement.

Our own work has shown that there were no significant differences in the metabolome of plasma samples due to storage when stored at -80°C for up to 7 years [5]. Even though the sample collection for one of the studies employed here started in 2003 already, all samples were measured within 7 years after sample collection. The longest sample storage time before measurement was 1637 days (about 4.5 years). Thus, a marked influence of freeze-thaw cycles or sample age on the results of this study can be excluded.

Metabolite profiling details

Briefly, proteins were removed from the samples by precipitation, using three volumes of acetonitrile. Polar and nonpolar fractions were separated by adding water and a mixture of ethanol and dichloromethane (2:1, v/v). For GC-MS analysis, the nonpolar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The polar and nonpolar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride to convert oxo-groups to O-methyl-oximes, and subsequently with N-methyl-N-(trimethylsilyl)trifluoroacetamide prior to analysis. For LC-MS/MS analysis, both fractions were dried and reconstituted in appropriate solvent mixtures. High-performance liquid chromatography was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied as described in patent WO2003073464 [6] which allows targeted and high-sensitivity multiple reaction monitoring (MRM) profiling in parallel to a full screen analysis. In brief, mass spectrometric detection was performed with repetitive cycles of MRM transitions for pre-selected metabolites followed by a full scan from a mass-to-charge ratio of 100 to 1000. The instrument was operated in positive ionization mode for metabolites in the nonpolar fraction, and in negative ionization mode for metabolites in the polar fraction. Metabolite identification was done by comparing sample data to authentic standards where applicable, as outlined previously [7].

MxP® Lipids covered profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). Total lipids were extracted from the sample by liquid/liquid extraction using chloroform/methanol. The lipid extracts were subsequently fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to [8, 9]. The fractions were analyzed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with detection of specific MRM transitions for preselected sphingolipids.

Metabolite profiling generated semi-quantitative data of metabolite concentrations calculated by determining metabolite levels in each study sample relative to metabolite concentrations in reference pool samples that were created from aliquots of all study samples. The normalization to reference pool samples compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences were analyzed by different devices. To allow comparison of data sets between the different studies, the semi-quantitative data were further normalized to the median of MxPool™ samples representing a pool of commercial human EDTA plasma containing more than 2,000 different metabolites of known concentrations. A one-point calibration was used to calculate quantitative absolute concentrations for those metabolites present in the MxPool. Both types of pooled reference samples were run in parallel through the entire process.

Biomarker selection

The metabolites for the biomarker panel were nominated based on biomedical expertise. In a first step, features that markedly differentiate CP patients from controls that could have an influence on the metabolome were considered. CP patients frequently suffer from lipid malabsorption and gut microbiome changes due to reduced bile acid secretion, reduced endocrine pancreatic function, pancreatic tissue fibrosis, and pancreatic inflammation. In a second step, metabolite groups that were expected to be different between CP patients and controls based on these physiological differences were collected: nutritional lipids that would be affected from malabsorption, microbiome-derived metabolites that could be affected by gut microbiome changes, carbohydrate metabolites that that would be affected by the reduced endocrine function, metabolites that would be altered in response to fibrosis, and metabolites that would be altered in response to inflammatory processes. In a third step, single representative metabolites from these groups were chosen for the signature panel based on methodical experience (the metabolites needed to allow for robust measurements above the limit of detection), available literature, and experience from previous experiments with CP patients and controls.

Prediction model

One prediction model was employed for all three study cohorts, i.e. the beta coefficients obtained from the first cohort were then applied to the individuals from the other 2 cohorts. Our prediction model, consisting of the biomarker signature, the corresponding algorithm, and the established cut-off, predicts whether a patient suffers from chronic pancreatitis. The biomarker enables a clinical diagnosis, supporting the standard diagnostic means for

diagnosis of chronic pancreatitis (see above). The biomarker is not designed to be applied for screening of the general population.

To avoid any bias when analyzing the concentrations of the metabolites present in the biomarker signature, the diagnosis was blinded to the scientists measuring the samples using mass spectrometry. The concentration values in the plasma samples of the 8 metabolites present in the biomarker signature are the only predictors used in the prediction model. Furthermore, the calculation of the biomarker score by the algorithm and selection of the cut-off was done fully automated, without human interference. After the initial calculation based on the identification study results, there were no subsequent interventions like patient exclusions, cut-off optimization, or re-training of the algorithm. Vice versa, the clinical diagnosis was established in the participating clinical centers according to the criteria mentioned above before the plasma samples were taken and analyzed in this study. Thus, the outcome obtained with the prediction model did not have any effect on the clinical diagnosis.

Statistical analysis details

Power analysis was performed to estimate an adequate sample size using representative metabolite profiling standard deviations that were determined by metanomics GmbH in earlier studies. Primary goal of the study was to determine a 20% metabolic difference on a 5% significance level with approximately 72-99% power on the basis of the patient samples. Metabolic difference was defined as absolute or relative difference in concentrations of individual metabolites. Power estimates were based on t-test statistics.

Missing data were handled differently depending on the analysis. For the Naive Bayes algorithm and the principal component analysis, missing values were imputed with the NIPALS (Non-linear Iterative PArtial Least Squares) algorithm [10]. In the second validation study, BMI values were not available for all participants. For the inclusion of the BMI as a confounding factor in the MANOVA, the missing BMI values were imputed using K-means clustering [11] for this purpose.

All R packages used, sorted by analysis step and including the utilized functions, are listed in supplemental **Table S1**.

Suppl. Results

Metabolomic analyses details

The metabolomics data underwent a strict quality control after which 505 known and 115 unknown metabolites remained for statistical analysis in the datasets based on plasma samples. Most of these metabolites could also be detected in the study conducted with serum samples. In this dataset, 498 known and 118 unknown metabolites remained for statistical analysis that met the quality control criteria.

Concentration data were missing for beta-carotin from 2 samples in the second validation study, for cryptoxanthin in 7 samples from the second validation study, for N-acetylcytidine in 2 samples from the first and 1 sample from the second validation study, for behenic acid in 1 sample from the identification study, 6 samples from the first validation study, and 1 sample in the second validation study, for mannose in 16 samples from the first validation study and 5 samples from the second validation study, for indole-3-acetic acid for 1 sample in the identification study, for 28 samples in the first validation study, and for 7 samples in the second validation study, for hippuric acid for 1 sample in the second validation study, and for ceramide (d18:1,C24:1) for 1 sample in the first validation study and 17 samples in the second validation study. Normalized to the number of samples in each study, this means that a maximum of 1% of the values for a given metabolite were missing in the identification study, maximally 8% in the first validation study and maximally 10% in the second validation study. There were no cases where the outcome (diagnosis) was unknown or missing.

In total, 60 metabolites were not significantly different ($p > 0.05$ or $q > 0.2$) between CP and control groups in any of the three studies. 516 metabolites were significantly different in some of the studies and 39 metabolites were significantly ($p < 0.05$ and $q < 0.2$) different in all of the three studies. 6 of the 8 metabolites from the signature metabolite panel had a p value below the significance threshold ($p < 0.05$) in all three studies (see **Table 4**). The other 2 of the 8 metabolites from the panel (behenic acid and indole-3-acetic acid) were significantly different between the groups in the identification and the first validation study, but not in the second validation study that had serum as a sample matrix.

The panel metabolites were not the best 8 discriminators between CP patients and controls. Beta-carotene and cryptoxanthin were among the top 3 discriminators in the plasma-based studies (identification and first validation study), with lycopene being the best discriminator in the plasma studies. Looking at all three studies together, beta-carotene, cryptoxanthin, and mannose were among the top 5 discriminators, with 3-hydroxybutyrate being the best discriminator.

The distribution of age and BMI over the biomarker signature score is shown in supplemental **Figure S1**. The age gap between CP patients and non-pancreatic controls is markedly higher in the identification study than in the validation. In the first validation study, the age of the patients follows an even Gaussian distribution for both CP patients and non-

pancreatic controls. In both studies, the score is markedly higher for CP patients compared to non-pancreatic controls irrespective of the age. As the BMI was calculated with a decimal, there are a lot of potential sublevels, which results in more data being needed for Gaussian curves. Nevertheless, the BMI of non-pancreatic controls in the validation study also follows a Gaussian distribution, while the BMI of CP patient is clearly skewed due to the increased frequency of patients with low BMI. This is an inherent feature of the disease concomitant with the malnutrition caused by CP. These trends can also be observed in the identification study. Despite the uneven BMI distribution, the graphs show that the biomarker score is markedly higher for CP patients compared to non-pancreatic controls irrespective of the BMI.

The full prediction model can be used universally. The weighting of the metabolites as shown in supplemental **Table S2** can be used as coefficients to be multiplied with the respective concentrations of the eight metabolites in the biomarker signature (in µmol / L) to calculate the biomarker signature score. Whether the score is above or below the cut-off value of 0.479 determines whether the patient is evaluated as positive or negative for the diagnosis “chronic pancreatitis”.

Effect of exocrine insufficiency and enzyme supplementation on carotenoid levels

Because the identification of beta-carotene and cryptoxanthin suggested a pathophysiological mechanism of malabsorption, it was analyzed whether pancreatic exocrine insufficiency and enzyme supplementation had an effect on plasma carotenoid levels. This analysis was limited to the identification study because the full information was available for this cohort only. Almost all patients with exocrine insufficiency also received enzyme supplementation so that a separate comparison of the effect of exocrine insufficiency alone was not possible. As obvious from **Figure S2**, there was no significant increase of carotenoid levels in plasma of chronic pancreatitis patients supplemented with enzymes to treat exocrine insufficiency.

Biomarker signature score increases with disease severity

In clinical daily routine, patients that will are tested for chronic pancreatitis are not always as healthy as the control groups used in this study. We therefore wanted to investigate whether the biomarker signature score was more accurate in patients with advanced disease than in less severe cases. Because other clinical data regarding disease severity was elusive, we used the information whether the patients suffered from pancreatic endocrine or exocrine insufficiency, which is a good surrogate marker for severity and time

since disease onset. 199 chronic pancreatitis patients from the identification and the first validation study for whom this information was available together were categorized in three groups: those without insufficiencies, those with either endo- or exocrine insufficiency, and those with both endo- and exocrine insufficiencies. The distribution of biomarker signature score values in the three groups is shown in **Figure S3**. The average biomarker signature score was 0.68 in patients without insufficiencies, 0.78 in patients with either endo-or exocrine insufficiency, and 0.90 in patients with both endo- and exocrine insufficiency. An ANOVA was employed to test whether the differences in the biomarker signature score were significant. While the group with one pancreatic insufficiency did not have a significantly different score compared to the other groups, the scores of the groups without pancreatic insufficiencies and with both endo- and exocrine insufficiencies were significantly different ($p = 0.0018$). This indicates that the biomarker signature score is higher in patients with more severe pancreatic disease.

Using the established cut-off of 0.479, we concluded for each patient in the three groups whether the diagnosis based on the signature score was correct or a false negative. A chi-squared test was employed to investigate whether the diagnosis and the severity were co-dependent. The result of $p = 0.056$ shows a clear trend towards a higher fraction of correct diagnoses in more severe cases, although there was no significant dependency between the group affiliation and the diagnosis.

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Suppl. Figures and Tables:

Figure S1. Distribution of age (A,C) and BMI (B,D) over biomarker signature score in the identification study (A,B) and the first validation study (C,D). Demographics are shown on the x axes, the biomarker signature score on the y axes. Stacked columns for age and side-by-side columns for BMI are colored according to outcome (diagnosis). Horizontal lines / functions represent Gaussian approximation of the data.

Figure S2. Boxplots of effect of enzyme supplementation on plasma carotenoid levels. Shown are data for chronic pancreatitis patients of the identification study (plasma). There was no significant increase of carotenoid levels in plasma of patients supplemented with enzymes to treat exocrine insufficiency.

Figure S3. Boxplots of biomarker signature scores in chronic pancreatitis patients from the identification study and the first validation study, which were categorized depending on whether they suffer from endo- and/or exocrine insufficiencies as a measure of disease stage. The average biomarker signature score increased with disease severity and this increase was significant comparing patients without insufficiency and patients with both endo- and exocrine insufficiency.

352 **Table S1**: R packages and functions used

Analysis	Packages	Functions
PCA	Stats (included in R core pkge)	prcomp()
Biomarker training and test	E1071 ROCR ROCR pROC	naivebayes() performance() prediction() roc()
ANOVA/MANOVA	slme stats (included in R core pkge)	lme() p.adjust()
Visualizations	ggplot2	

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356 **Table S2**: Weightings of all metabolites in the biomarker signature

Metabolite	**Weight**	**Mean**	**SD ±**
Beta-carotene	1.1749422	-0.9281	0.4641
Cryptoxanthin	1.2061438	-1.1714	0.4558
Mannose	0.7865473	1.6548	0.1678
Behenic acid (C22:0)	0.6728898	1.5537	0.2095
Ceramide (d18:1/ C24:1)	0.6066595	0.1294	0.1427
Indole-3-acetic acid	0.5846360	-0.0213	0.3273
Hippuric acid	0.5009786	-0.3020	0.4408
N-Acetylcytidine	0.1068720	0.0807	0.1897

357 SD: standard deviation

358 **Table S3**. Statistical analysis results (linear model) of the signature metabolites (CP vs.
359 control) with FDR corrected p-values (q values).

Dataset (matrix)	Identification study (plasma)	First validation study (plasma)	Second validation study (serum)
Metabolite	**Fold change (q-value)**	**Fold change (q-value)**	**Fold change (q-value)**
Beta-carotene	0.37 (9.8×10^{-6})	0.38 (1.8×10^{-19})	0.47 (0.00039)
Cryptoxanthin	0.40 (9.9×10^{-6})	0.38 (4.5×10^{-18})	0.48 (0.00090)
Mannose	1.47 (9.9×10^{-6})	1.52 (6.7×10^{-27})	2.80 (8.4×10^{-13})
Behenic acid (C22:0)	0.72 (0.0080)	0.67 (1.0×10^{-15})	1.14 (0.14)
Ceramide (d18:1, C24:1)	1.27 (0.0059)	1.28 (1.0×10^{-10})	1.96 (5.9×10^{-12})
Indole-3-acetic acid	0.63 (0.013)	0.62 (2.4×10^{-8})	1.15 (0.40)
Hippuric acid	0.68 (0.15)	0.49 (1.2×10^{-7})	0.51 (0.0034)
N-Acetylcytidine	1.22 (0.058)	1.21 (8.1×10^{-5})	2.36 (3.2×10^{-12})

360 CP: Chronic pancreatitis

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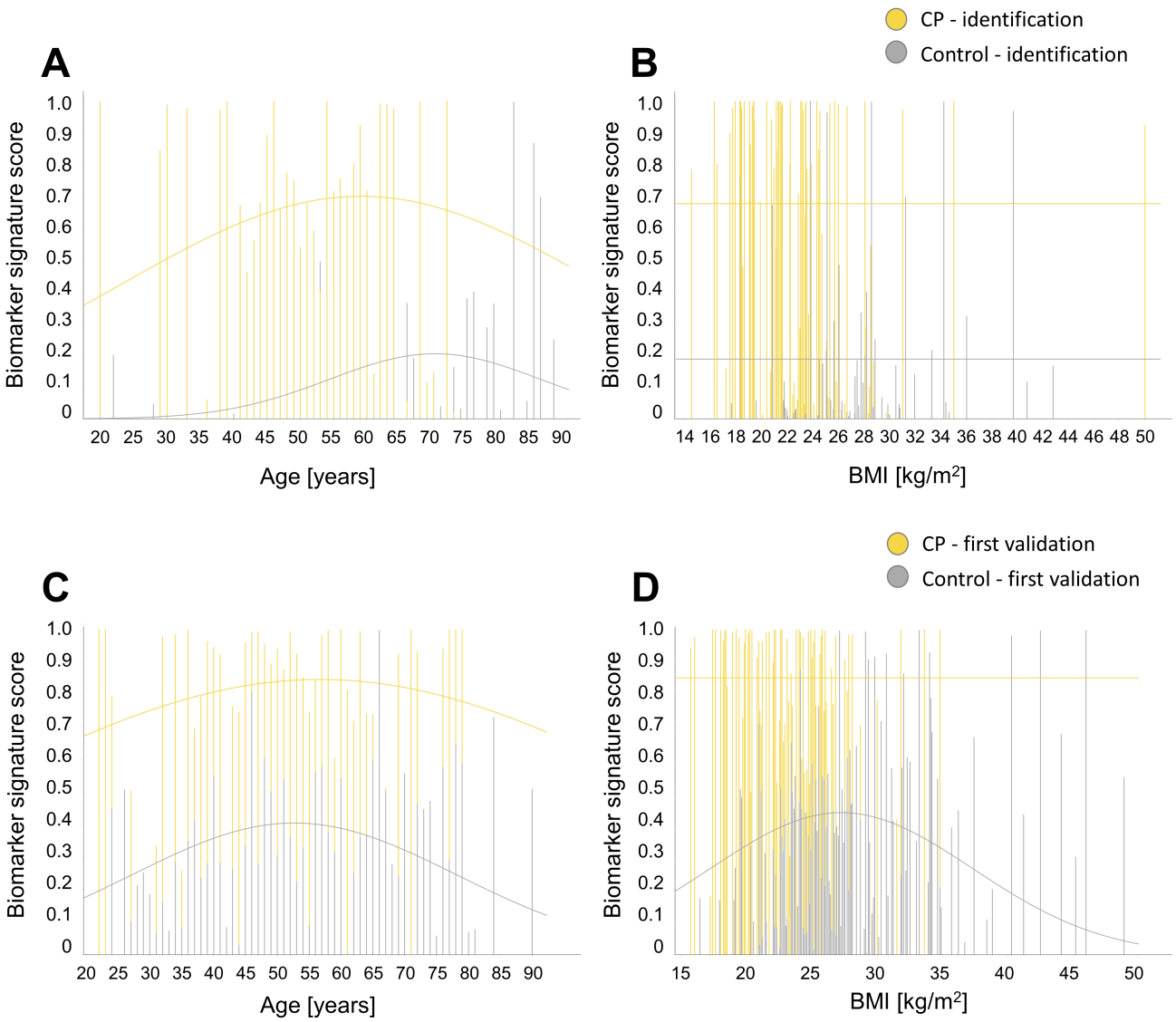
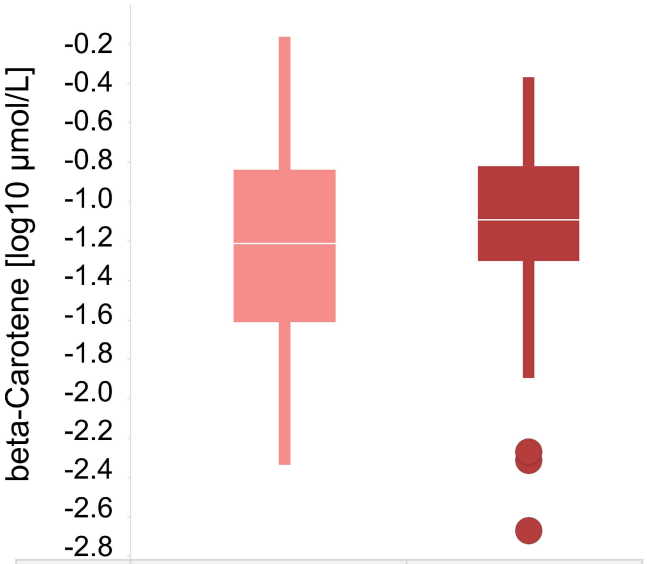
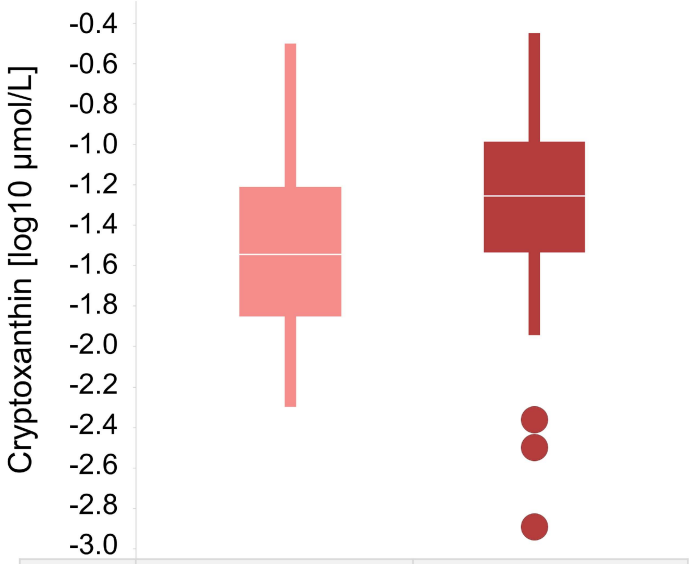
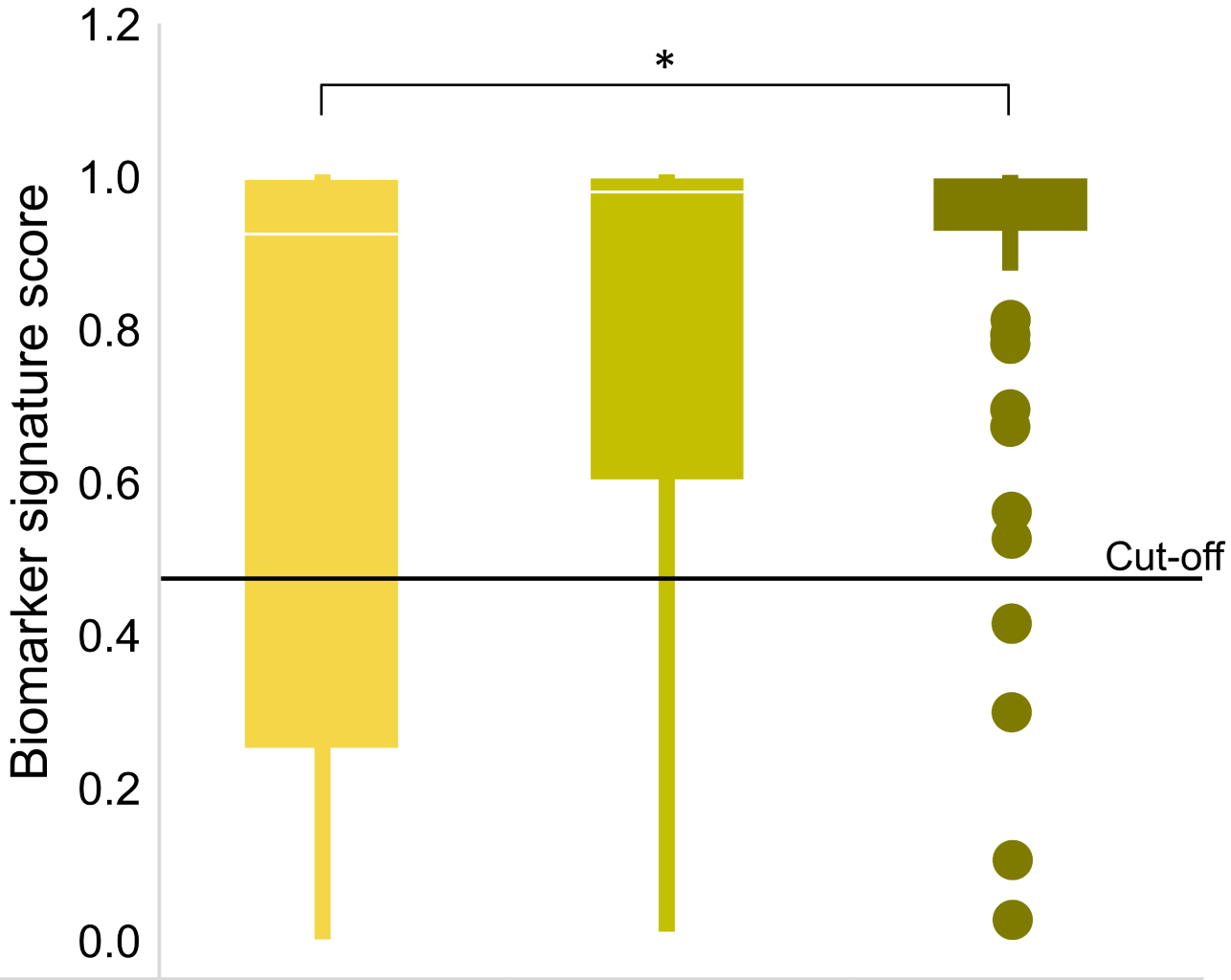

A
B
C
D
CP - identification
Control - identification
CP - first validation
Control - first validation
Biomarker signature score
Age [years]
BMI [kg/m²]



	Enzyme supplementation	No enzyme supplementation
Count	34	46



	Enzyme supplementation	No enzyme supplementation
Count	34	46



	No insufficiencies	Endo- or exocrine insufficiency	Endo- and exocrine insufficiency
Count	**57**	**85**	**57**

Supplement

Identification and validation of a multivariable prediction model based on blood plasma and serum metabolomics for the distinction of chronic pancreatitis subjects from non-pancreas disease control subjects

M. Gordian Adam#[1,2], Georg Beyer#[3], Nicole Christiansen[1,4], Beate Kamlage[1], Christian Pilarsky[5], Marius Distler[6], Tim Falbusch[7], Ansgar Chromik[8], Fritz Klein[9], Marcus Bahra[9], Waldemar Uhl[7], Robert Grützmann[5], F. Ulrich Weiss[10], Julia Mayerle#[3,10], and Markus M. Lerch#[10]*

[1] Metanomics Health GmbH, Tegeler Weg 33, 10589 Berlin, Germany
[2] Biocrates AG, Eduard-Bodem-Gasse 8, 6020 Innsbruck, Austria
[3] Department of Medicine II, University Hospital, Ludwig-Maximilians-Universität München, Marchioninistr. 15, 81377 Munich, Germany
[4] trinamiX GmbH, Industriestraße 31a, 67063 Ludwigshafen am Rhein, Germany
[5] Department of Surgery, University Hospital, Erlangen, Germany
[6] Clinic and Outpatient Clinic for Visceral, Thorax, and Vascular Surgery, Medical Faculty, TU Dresden, Dresden, Germany
[7] Ruhr-University Bochum, St. Josef Hospital, Department of Surgery, Bochum, Germany
[8] Asklepios Clinic Harburg, Department for General and Visceral Surgery, Hamburg, Germany
[9] Department of Surgery, Charité Campus Mitte and Charité Campus Virchow Klinikum, Charité-Universitätsmedizin Berlin, Berlin, Germany
[10] Department of Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruchstrasse, 17475 Greifswald, Germany
* Correspondence:

Prof. Dr. med. Markus M. Lerch
Department of Medicine A
University Medicine Greifswald
Ferdinand-Sauerbruchstrasse
17475 Greifswald
Germany
Phone: +49 (0) 3834 86 7230
Fax: +49 (0) 3834 86 7234
Markus.lerch@med.uni-greifswald.de

Suppl. Material and Methods

Study details

We conducted a type 3 study for multivariable prediction for individual prognosis according to the TRIPOD guidelines [1]. Patients with chronic pancreatitis, liver cirrhosis, healthy blood donors and preoperative patients with non-pancreatic or liver disease were consecutively recruited from university referral centers in Greifswald, Dresden, Berlin, and Bochum, all in Germany.

For the identification study, EDTA plasma samples were collected within a case-control study from 80 patients with CP and 80 non-pancreatic control patients, who underwent small, non-pancreas-related surgical procedures under general anesthesia (see below).

For the first validation study, 144 chronic pancreatitis patients and 204 non-pancreatic control patients were recruited at three different centers. Because it was acknowledged that CP patients are relatively young and mostly male, during patient recruitment, special care was taken to also recruit younger and mostly male patients for the control group with the aim to achieve a similar average patient age and a similar ratio of male to female subjects in both groups.

In the second validation study, a different sample type was utilized: serum samples taken from 49 chronic pancreatitis patients, 56 controls, and 57 liver cirrhosis patients were analyzed. These samples were collected in a fourth independent center. Furthermore, the control group consisted of healthy blood donors instead of patients waiting to undergo a small surgery.

The key study dates for the three studies were as follows: accrual for the identification study was started on 2009-01-13, end of accrual was on 2013-08-01. Accrual for the first validation study was started on 2013-09-09 and ended on 2015-09-28. Accrual for the second validation study started on 2002-10-23 and ended on 2010-06-10.

The general inclusion criteria for all groups included written informed consent prior to any study procedures, age ≥ 18 to 85 years and eight hours fasting prior to blood draw.

The general exclusion criteria for all groups included type I diabetes, pregnancy or lactation phase, known viral infections like hepatitis B, hepatitis C, HIV, major surgery within the last 4 weeks before sample collection, acute anemia (Hb<9 g/dl or <5,58 mmol/l), malignant tumors within the last 5 years.

Chronic pancreatitis patients were included if one or more of the following criteria were met and no other diagnosis was more likely [2, 3, 4]: recurrent bouts of pancreatic pain with documented rise in amylase or lipase activity for a duration of more than one year plus radiological evidence supporting the diagnosis, pancreatic calcifications, histological proof

of chronic pancreatitis, unequivocal changes in pancreatic duct morphology, severely abnormal pancreatic function tests with maldigestion. Calcifications were identified on CT-scan, diabetes was diagnosed as suggested by the WHO definition and exocrine insufficiency was determined by either fecal elastase measurement or concurrent pancreatic enzyme supplementation. Pancreatitis patients were excluded if they had undergone pancreatitis surgery within 6 months before sample collection, bile duct stent placement or surgery, endoscopically assisted pancreatic aspiration <5 days before sample collection or had known liver cirrhosis.

Liver cirrhosis patients were included if preexisting liver cirrhosis had been diagnosed based on imaging and clinical chemistry. Liver cirrhosis patients were excluded if concomitant chronic pancreatitis was present.

Control patients were included if they were undergoing minor non-pancreatic surgery under general anesthesia. Control patients were excluded if they had chronic pancreatitis or liver cirrhosis or if a hernia was due to solid organ transplantation.

For the blood donors, only the standard blood donor inclusion criteria applied, i.e. the donors had to be in good general health, body weight at least 110 pounds. Participants with diabetes type II were excluded from the blood donor group because of the requirement of a fasting period of at least 8 hours which was not considered feasible for diabetics.

From the patients in the non-pancreatic control group in the identification study, 20 patients underwent vascular surgery, 18 patients received a hernia repair, 3 were resected for goiter and 39 received various other small surgical procedures under general anesthesia. From the patients in the non-pancreatic control group in the first validation study, 164 underwent hernia repair surgery and 40 were resected for thyroid goiter. None were operated in metabolically deranged state. Thus, the data in the first validation study differed from the identification study as it was a multicentric study, and in the composition of the non-pancreatic controls. The second validation study differed in the matrix used for analysis (serum instead of plasma), the center where the samples were obtained, the control group (healthy blood donors instead of non-pancreatic controls), and the inclusion of liver cirrhosis patients as an additional control group. Furthermore, 22.5% of the non-pancreatic controls in the identification study were diabetes type II patients, while 13.5% of the patients suffered from diabetes type II in the first validation study, and diabetes patients were excluded as control in the second validation study. As opposed to the identification study, the genesis of pancreatitis, calcifications, exocrine insufficiency, and enzyme supplementation were only partially available in the validation studies.

Sample Storage

Before freezing, the EDTA plasma samples and serum samples were aliquoted to avoid freeze-thaw cycles during the measurement period. Samples were stored at the respective center at -80°C until transport to the measurement location, which occurred on dry ice. Samples were stored at the measurement location at -80°C until measurement.

Our own work has shown that there were no significant differences in the metabolome of plasma samples due to storage when stored at -80°C for up to 7 years [5]. Even though the sample collection for one of the studies employed here started in 2003 already, all samples were measured within 7 years after sample collection. The longest sample storage time before measurement was 1637 days (about 4.5 years). Thus, a marked influence of freeze-thaw cycles or sample age on the results of this study can be excluded.

Metabolite profiling details

Briefly, proteins were removed from the samples by precipitation, using three volumes of acetonitrile. Polar and nonpolar fractions were separated by adding water and a mixture of ethanol and dichloromethane (2:1, v/v). For GC-MS analysis, the nonpolar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The polar and nonpolar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride to convert oxo-groups to O-methyl-oximes, and subsequently with N-methyl-N-(trimethylsilyl)trifluoroacetamide prior to analysis. For LC-MS/MS analysis, both fractions were dried and reconstituted in appropriate solvent mixtures. High-performance liquid chromatography was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied as described in patent WO2003073464 [6] which allows targeted and high-sensitivity multiple reaction monitoring (MRM) profiling in parallel to a full screen analysis. In brief, mass spectrometric detection was performed with repetitive cycles of MRM transitions for pre-selected metabolites followed by a full scan from a mass-to-charge ratio of 100 to 1000. The instrument was operated in positive ionization mode for metabolites in the nonpolar fraction, and in negative ionization mode for metabolites in the polar fraction. Metabolite identification was done by comparing sample data to authentic standards where applicable, as outlined previously [7].

MxP® Lipids covered profiling of sphingolipids (ceramides, sphingomyelins, and sphingobases). Total lipids were extracted from the sample by liquid/liquid extraction using chloroform/methanol. The lipid extracts were subsequently fractionated by normal phase liquid chromatography (NPLC) into different lipid groups according to [8, 9]. The fractions were analyzed by LC-MS/MS using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with detection of specific MRM transitions for preselected sphingolipids.

Metabolite profiling generated semi-quantitative data of metabolite concentrations calculated by determining metabolite levels in each study sample relative to metabolite concentrations in reference pool samples that were created from aliquots of all study samples. The normalization to reference pool samples compensates for inter- and intra-instrumental variation, i.e. variability that occurs when different analytical sequences were analyzed by different devices. To allow comparison of data sets between the different studies, the semi-quantitative data were further normalized to the median of MxPool™ samples representing a pool of commercial human EDTA plasma containing more than 2,000 different metabolites of known concentrations. A one-point calibration was used to calculate quantitative absolute concentrations for those metabolites present in the MxPool. Both types of pooled reference samples were run in parallel through the entire process.

Biomarker selection

The metabolites for the biomarker panel were nominated based on biomedical expertise. In a first step, features that markedly differentiate CP patients from controls that could have an influence on the metabolome were considered. CP patients frequently suffer from lipid malabsorption and gut microbiome changes due to reduced bile acid secretion, reduced endocrine pancreatic function, pancreatic tissue fibrosis, and pancreatic inflammation. In a second step, metabolite groups that were expected to be different between CP patients and controls based on these physiological differences were collected: nutritional lipids that would be affected from malabsorption, microbiome-derived metabolites that could be affected by gut microbiome changes, carbohydrate metabolites that that would be affected by the reduced endocrine function, metabolites that would be altered in response to fibrosis, and metabolites that would be altered in response to inflammatory processes. In a third step, single representative metabolites from these groups were chosen for the signature panel based on methodical experience (the metabolites needed to allow for robust measurements above the limit of detection), available literature, and experience from previous experiments with CP patients and controls.

Prediction model

One prediction model was employed for all three study cohorts, i.e. the beta coefficients obtained from the first cohort were then applied to the individuals from the other 2 cohorts. Our prediction model, consisting of the biomarker signature, the corresponding algorithm, and the established cut-off, predicts whether a patient suffers from chronic pancreatitis. The biomarker enables a clinical diagnosis, supporting the standard diagnostic means for

diagnosis of chronic pancreatitis (see above). The biomarker is not designed to be applied for screening of the general population.

To avoid any bias when analyzing the concentrations of the metabolites present in the biomarker signature, the diagnosis was blinded to the scientists measuring the samples using mass spectrometry. The concentration values in the plasma samples of the 8 metabolites present in the biomarker signature are the only predictors used in the prediction model. Furthermore, the calculation of the biomarker score by the algorithm and selection of the cut-off was done fully automated, without human interference. After the initial calculation based on the identification study results, there were no subsequent interventions like patient exclusions, cut-off optimization, or re-training of the algorithm. Vice versa, the clinical diagnosis was established in the participating clinical centers according to the criteria mentioned above before the plasma samples were taken and analyzed in this study. Thus, the outcome obtained with the prediction model did not have any effect on the clinical diagnosis.

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Suppl. Results

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The panel metabolites were not the best 8 discriminators between CP patients and controls. Beta-carotene and cryptoxanthin were among the top 3 discriminators in the plasma-based studies (identification and first validation study), with lycopene being the best discriminator in the plasma studies. Looking at all three studies together, beta-carotene, cryptoxanthin, and mannose were among the top 5 discriminators, with 3-hydroxybutyrate being the best discriminator.

The distribution of age and BMI over the biomarker signature score is shown in supplemental **Figure S1**. The age gap between CP patients and non-pancreatic controls is markedly higher in the identification study than in the validation. In the first validation study, the age of the patients follows an even Gaussian distribution for both CP patients and non-

pancreatic controls. In both studies, the score is markedly higher for CP patients compared to non-pancreatic controls irrespective of the age. As the BMI was calculated with a decimal, there are a lot of potential sublevels, which results in more data being needed for Gaussian curves. Nevertheless, the BMI of non-pancreatic controls in the validation study also follows a Gaussian distribution, while the BMI of CP patient is clearly skewed due to the increased frequency of patients with low BMI. This is an inherent feature of the disease concomitant with the malnutrition caused by CP. These trends can also be observed in the identification study. Despite the uneven BMI distribution, the graphs show that the biomarker score is markedly higher for CP patients compared to non-pancreatic controls irrespective of the BMI.

The full prediction model can be used universally. The weighting of the metabolites as shown in supplemental **Table S2** can be used as coefficients to be multiplied with the respective concentrations of the eight metabolites in the biomarker signature (in µmol / L) to calculate the biomarker signature score. Whether the score is above or below the cut-off value of 0.479 determines whether the patient is evaluated as positive or negative for the diagnosis “chronic pancreatitis”.

Effect of exocrine insufficiency and enzyme supplementation on carotenoid levels

Because the identification of beta-carotene and cryptoxanthin suggested a pathophysiological mechanism of malabsorption, it was analyzed whether pancreatic exocrine insufficiency and enzyme supplementation had an effect on plasma carotenoid levels. This analysis was limited to the identification study because the full information was available for this cohort only. Almost all patients with exocrine insufficiency also received enzyme supplementation so that a separate comparison of the effect of exocrine insufficiency alone was not possible. As obvious from **Figure S2**, there was no significant increase of carotenoid levels in plasma of chronic pancreatitis patients supplemented with enzymes to treat exocrine insufficiency.

Biomarker signature score increases with disease severity

In clinical daily routine, patients that will are tested for chronic pancreatitis are not always as healthy as the control groups used in this study. We therefore wanted to investigate whether the biomarker signature score was more accurate in patients with advanced disease than in less severe cases. Because other clinical data regarding disease severity was elusive, we used the information whether the patients suffered from pancreatic endocrine or exocrine insufficiency, which is a good surrogate marker for severity and time

since disease onset. 199 chronic pancreatitis patients from the identification and the first validation study for whom this information was available together were categorized in three groups: those without insufficiencies, those with either endo- or exocrine insufficiency, and those with both endo- and exocrine insufficiencies. The distribution of biomarker signature score values in the three groups is shown in **Figure S3**. The average biomarker signature score was 0.68 in patients without insufficiencies, 0.78 in patients with either endo-or exocrine insufficiency, and 0.90 in patients with both endo- and exocrine insufficiency. An ANOVA was employed to test whether the differences in the biomarker signature score were significant. While the group with one pancreatic insufficiency did not have a significantly different score compared to the other groups, the scores of the groups without pancreatic insufficiencies and with both endo- and exocrine insufficiencies were significantly different ($p = 0.0018$). This indicates that the biomarker signature score is higher in patients with more severe pancreatic disease.

Using the established cut-off of 0.479, we concluded for each patient in the three groups whether the diagnosis based on the signature score was correct or a false negative. A chi-squared test was employed to investigate whether the diagnosis and the severity were co-dependent. The result of $p = 0.056$ shows a clear trend towards a higher fraction of correct diagnoses in more severe cases, although there was no significant dependency between the group affiliation and the diagnosis.

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Suppl. Figures and Tables:

Figure S1. Distribution of age (A,C) and BMI (B,D) over biomarker signature score in the identification study (A,B) and the first validation study (C,D). Demographics are shown on the x axes, the biomarker signature score on the y axes. Stacked columns for age and side-by-side columns for BMI are colored according to outcome (diagnosis). Horizontal lines / functions represent Gaussian approximation of the data.

Figure S2. Boxplots of effect of enzyme supplementation on plasma carotenoid levels. Shown are data for chronic pancreatitis patients of the identification study (plasma). There was no significant increase of carotenoid levels in plasma of patients supplemented with enzymes to treat exocrine insufficiency.

Figure S3. Boxplots of biomarker signature scores in chronic pancreatitis patients from the identification study and the first validation study, which were categorized depending on whether they suffer from endo- and/or exocrine insufficiencies as a measure of disease stage. The average biomarker signature score increased with disease severity and this increase was significant comparing patients without insufficiency and patients with both endo- and exocrine insufficiency.

352 **Table S1**: R packages and functions used

Analysis	Packages	Functions
PCA	Stats (included in R core pkge)	prcomp()
Biomarker training and test	E1071 ROCR ROCR pROC	naivebayes() performance() prediction() roc()
ANOVA/MANOVA	slme stats (included in R core pkge)	lme() p.adjust()
Visualizations	ggplot2	

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356 **Table S2**: Weightings of all metabolites in the biomarker signature

Metabolite	**Weight**	**Mean**	**SD ±**
Beta-carotene	1.1749422	-0.9281	0.4641
Cryptoxanthin	1.2061438	-1.1714	0.4558
Mannose	0.7865473	1.6548	0.1678
Behenic acid (C22:0)	0.6728898	1.5537	0.2095
Ceramide (d18:1/ C24:1)	0.6066595	0.1294	0.1427
Indole-3-acetic acid	0.5846360	-0.0213	0.3273
Hippuric acid	0.5009786	-0.3020	0.4408
N-Acetylcytidine	0.1068720	0.0807	0.1897

357 SD: standard deviation

358 **Table S3**. Statistical analysis results (linear model) of the signature metabolites (CP vs.
359 control) with FDR corrected p-values (q values).

Dataset (matrix)	Identification study (plasma)	First validation study (plasma)	Second validation study (serum)
Metabolite	**Fold change (q-value)**	**Fold change (q-value)**	**Fold change (q-value)**
Beta-carotene	0.37 (9.8×10^{-6})	0.38 (1.8×10^{-19})	0.47 (0.00039)
Cryptoxanthin	0.40 (9.9×10^{-6})	0.38 (4.5×10^{-18})	0.48 (0.00090)
Mannose	1.47 (9.9×10^{-6})	1.52 (6.7×10^{-27})	2.80 (8.4×10^{-13})
Behenic acid (C22:0)	0.72 (0.0080)	0.67 (1.0×10^{-15})	1.14 (0.14)
Ceramide (d18:1, C24:1)	1.27 (0.0059)	1.28 (1.0×10^{-10})	1.96 (5.9×10^{-12})
Indole-3-acetic acid	0.63 (0.013)	0.62 (2.4×10^{-8})	1.15 (0.40)
Hippuric acid	0.68 (0.15)	0.49 (1.2×10^{-7})	0.51 (0.0034)
N-Acetylcytidine	1.22 (0.058)	1.21 (8.1×10^{-5})	2.36 (3.2×10^{-12})

360 CP: Chronic pancreatitis

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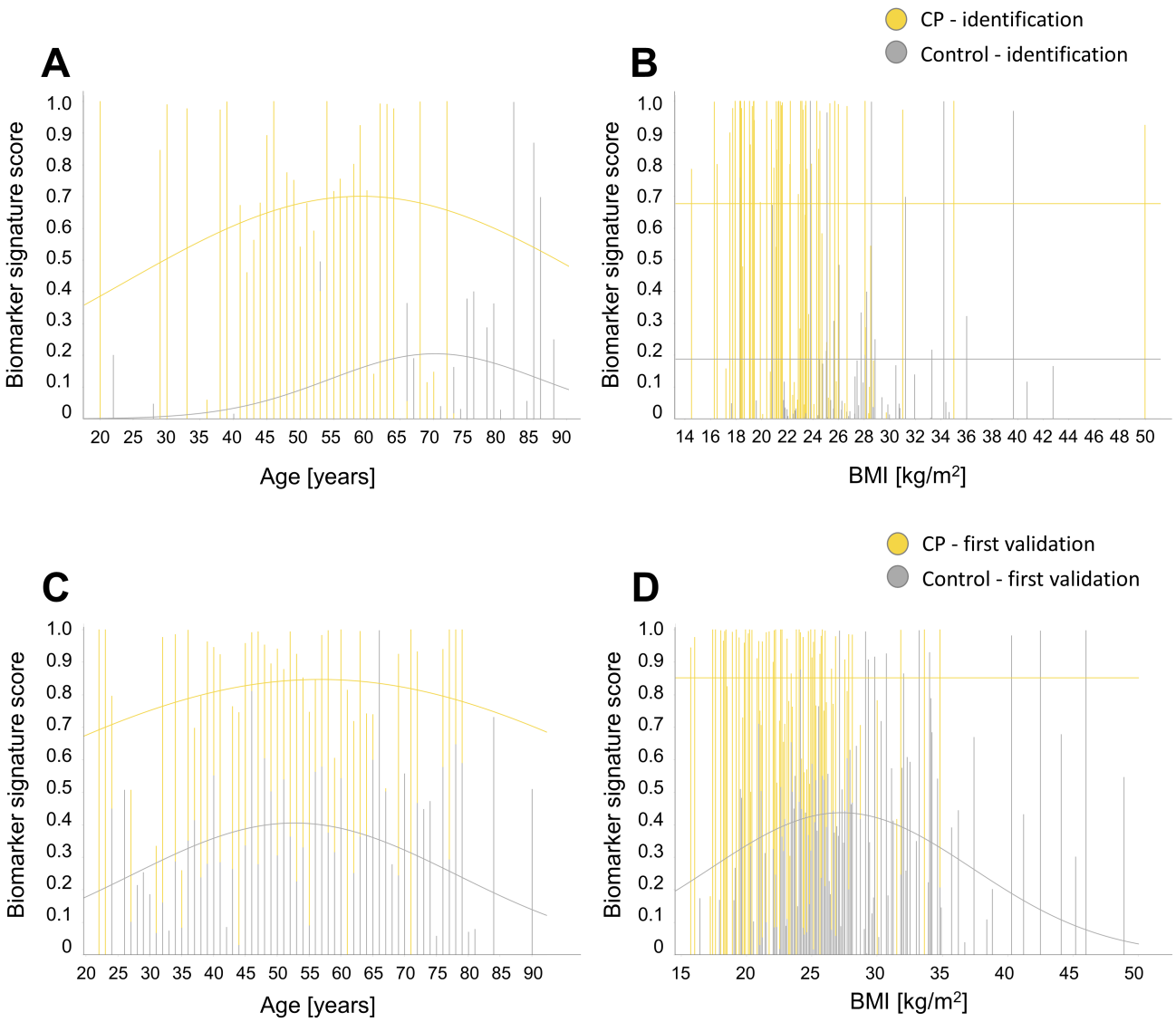
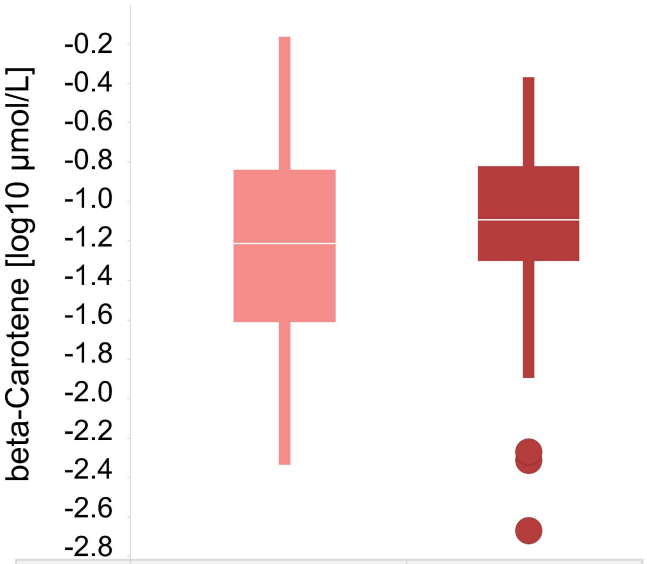
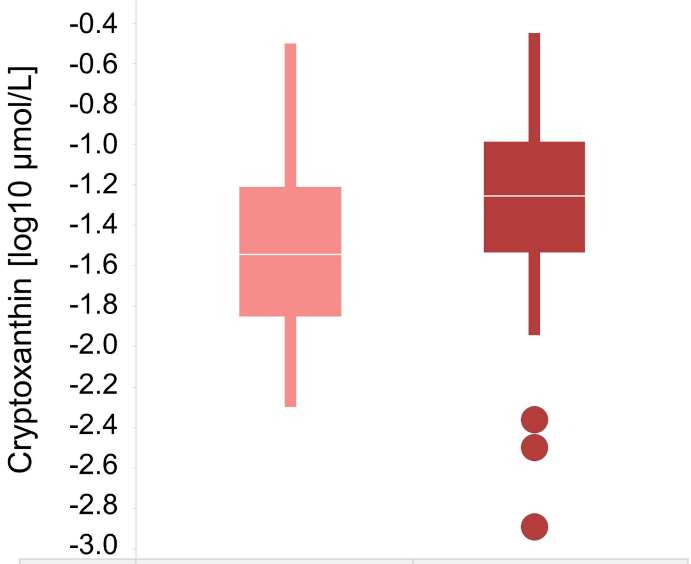
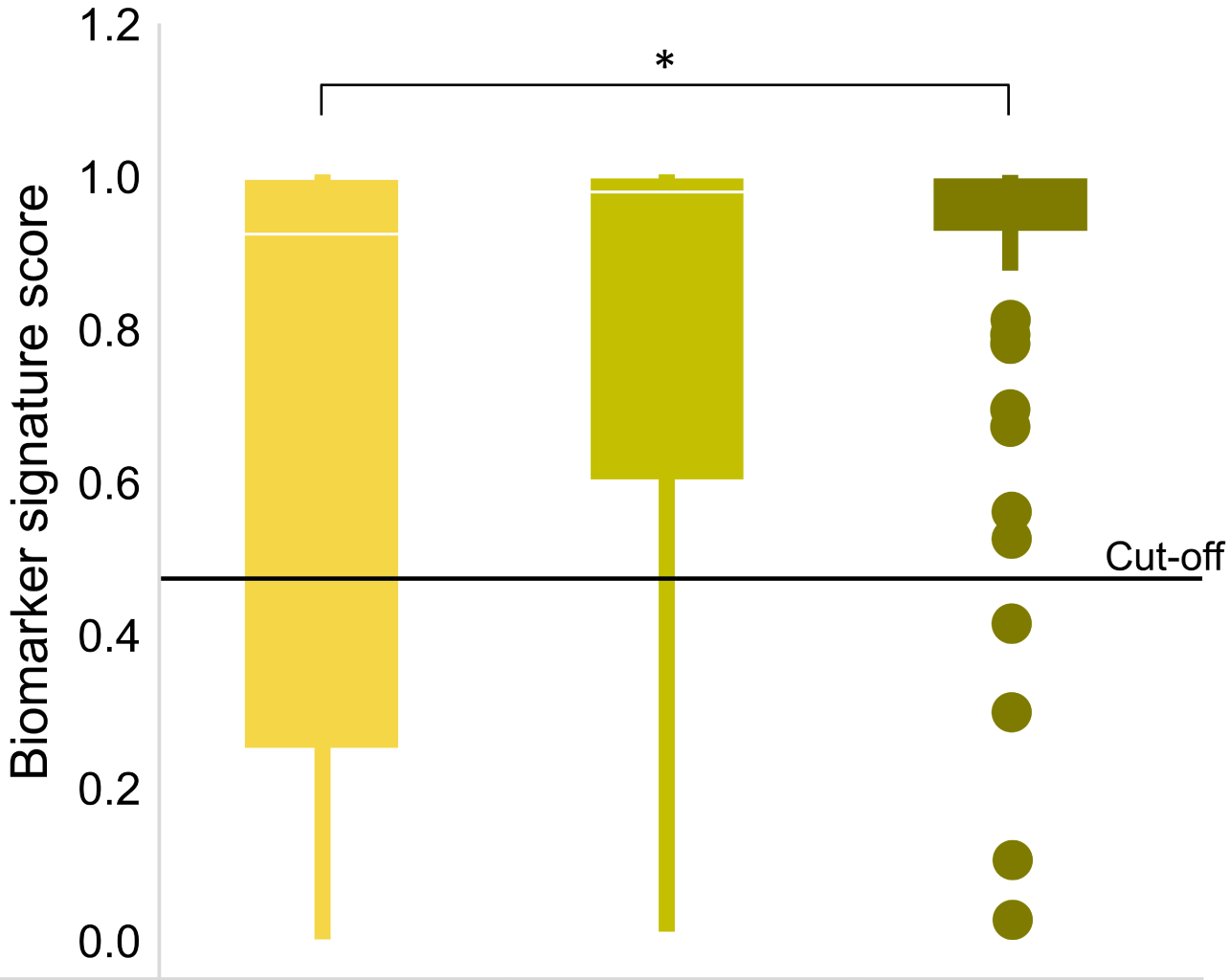

A
B
C
D
CP - identification
Control - identification
CP - first validation
Control - first validation
Biomarker signature score
Age [years]
BMI [kg/m²]



	Enzyme supplementation	No enzyme supplementation
Count	34	46



	Enzyme supplementation	No enzyme supplementation
Count	34	46



	No insufficiencies	Endo- or exocrine insufficiency	Endo- and exocrine insufficiency
Count	**57**	**85**	**57**