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BRIEF REPORT

Liquid Biopsy of Bile based on Targeted Mass Spectrometry for the Diagnosis of Malignant Biliary Strictures

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Bile holds biomarkers of malignant biliary strictures (MBS) but is unsuited for automated analyzers used in routine diagnostic laboratories. Selected reaction monitoring (SRM) is a flexible high-throughput analytical approach based on targeted mass spectrometry (MS) already implemented in clinical settings. We tested the hypothesis that SRM could be used to quantify cancer biomarkers in human bile. An SRM-based assay was developed to simultaneously quantify up to 37 peptides from 13 bile proteins in a developmental cohort of 15 patients (MBS, *n* = 8; benign biliary stricture or obstruction (BBS), *n* = 7). The most reliable biomarkers were then absolutely quantified by SRM in a verification cohort of 67 patients (MBS, *n* = 37; BBS, *n* = 30). The diagnostic performances of single and combined biomarkers were assessed. In the developmental cohort, SRM-based analysis revealed six protein biomarkers with significantly higher peptide ratios (endogenous vs. standard) in bile from MBS vs. BBS. In the verification cohort, five of these biomarkers proved good diagnostic ability (individual receiver operating characteristic-area under the receiver operating characteristic curve (ROC-AUC) up to 0.889, accuracies from 67.8% to 83.1%). Combining bile biomarkers and serum CA19-9 in 2 panels allowed differentiating MBS from BBS with up to 0.929 ROC-AUC and 89.8% accuracy. In this study, a newly developed SRM-based assay proved able to simultaneously quantify multiple biomarkers in bile samples. The combination of bile biomarkers with serum CA19-9 was highly accurate for the diagnosis of MBS. Liquid biopsy of bile based on targeted MS is eligible to support MBS diagnosis in clinical practice.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Bile contains biomarkers of malignant biliary strictures (MBS). Bile is not suited for automated analyzers used in routine diagnostic laboratories. Selected reaction monitoring (SRM) has already been implemented in clinical settings for the diagnosis of lung cancer and spontaneous preterm birth.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ We tested the hypothesis that SRM could be used to quantify bile biomarkers.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ The newly developed SRM assay allows the simultaneous measurement of multiple cancer biomarkers in human bile. Panels composed of bile biomarkers dosed by SRM and serum CA19-9 are highly accurate for the diagnosis of MBS.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Liquid biopsy of bile based on SRM is eligible to support MBS diagnosis in clinical practice.

Biliary strictures are mainly caused by pancreatic adenocarcinoma and cholangiocarcinoma and, less frequently, by benign diseases, including primary sclerosing cholangitis, IgG4-associated cholangitis, and chronic pancreatitis. The differential diagnosis between these etiologies is challenging because of nonspecific clinical symptoms and inaccurate diagnostic criteria.[1] As a consequence, 3–15% of surgical resections for suspected malignant biliary strictures (MBS) reveal benign disease.[2] In this differential diagnostic context, the shortfall of classical serum biomarkers and the lack of new valuable substitutes have stressed the need to complement conventional serum biochemistry with novel analytical approaches. Liquid biopsy of bile is emerging as a promising option for the molecular diagnosis of MBS as various bile biomarkers have been described, including proteins, metabolites, extracellular vesicles, and microRNAs.[3–8] Unfortunately,

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bile is not suitable for automated clinical chemistry analyzers used in routine diagnostic laboratories.[9] Nonautomated immunoassays (i.e., enzyme immunoassay/enzyme-linked immunosorbent assay kits, Luminex xMAP Technology) could be an alternative for measuring bile proteins and metabolites but antibodies are not available for all potential biomarkers and commercial assays often lack validation in bile. Such methodologies also present recurring costs and potential analytical bias due to cross-reactivity.[10] On the other hand, diagnostic tests based on extracellular vesicles and microRNAs are not yet implemented in clinical routine mainly due to the lack of standardized analytical workflows.[11,12]

Selected reaction monitoring (SRM), a targeted mass spectrometry (MS) approach, has the potential to overcome these limitations. This technology is suitable for use in a routine laboratory thanks to its flexibility, high-throughput capability, analytical specificity, and quantitative reliability.[13] In clinical practice, SRM has already been successfully implemented for the diagnosis of lung cancer and spontaneous preterm birth.[14]

In this work, we demonstrate the ability of SRM to simultaneously quantify multiple cancer biomarkers in human bile. We also evaluate the diagnostic performances of the most promising candidates and identify two panels of biomarkers able to discriminate between patients with an MBS or a benign biliary stricture or obstruction (BBS). These panels present a higher accuracy than serum CA19-9.

METHODS

Based on our previous quantitative investigations,[3,15] 11 proteins found to be overexpressed (neutrophil gelatinase-associated lipocalin, NGAL; galectin-3-binding protein, LG3BP; matrilysin, MMP7; mucin-5B, MUC5B; carcinoembryonic antigen-related cell adhesion molecule 6, CEAM6; olfactomedin-4, OLFM4; mucin-5AC, MUC5A; syntenin-2, SDCB2; osteopontin, OSTP; ras-related C3 botulinum toxin substrate 1, RAC1; Golgi membrane protein 1, GOLM1) and 2 proteins found to be isoexpressed (retinol-binding protein 4, RET4 and corticosteroid-binding globulin, CBG) in bile from patients with MBS vs. BBS were selected to serve as candidate biomarkers and control references, respectively.

The developmental and verification cohorts included, respectively, 15 patients (pancreatic adenocarcinoma (PAC), $n = 4$; cholangiocarcinoma, $n = 4$; chronic pancreatitis, $n = 4$; and biliary obstruction due to stones or other benign causes, $n = 3$) and 67 patients (PAC, $n = 20$; cholangiocarcinoma, $n = 10$; chronic pancreatitis, $n = 18$; biliary obstruction due to stones or other benign causes, $n = 19$) (**Table S1** and **Table S2**, respectively). The diagnosis of MBS was established by pathological examination of tissue samples and that of BBS was confirmed with a minimum follow-up of 1 year. To validate the repeatability and consistency of the results, 11 patients were included in both cohorts (**Table S1** and **Table S2**).

The measurement and data analysis methods are detailed in the **Supplementary Information**. Briefly, proteins from 5 µL of each crude bile sample were subjected to *in-gel* protein digestion using trypsin and the resulting peptides were mixed with heavy synthetic peptides, serving as internal standards for quantification. Crude and unquantified heavy synthetic peptides (PEPotec, Thermo Fisher Scientific, Waltham, MA) were used for relative quantification in the developmental phase, whereas high-purity AQUA synthetic peptides (Thermo Fisher Scientific) were used for absolute quantification in the verification phase. In total, 37 different peptides (1 to 4 peptides per protein) were quantified in the developmental phase (**Table S3**) and the most reliably detected peptide for each of the 5 selected biomarkers was further quantified in the verification phase (**Table S4**). To ensure maximum specificity,[16] 3 to 4 transitions (i.e., precursor and product ion pairs) per peptide were simultaneously quantified. After MS-based analysis, the ratios of endogenous (biliary) vs. standard (synthetic) peptides were extracted for each sample using Skyline software[17] and data were subjected to manual and statistical curation.

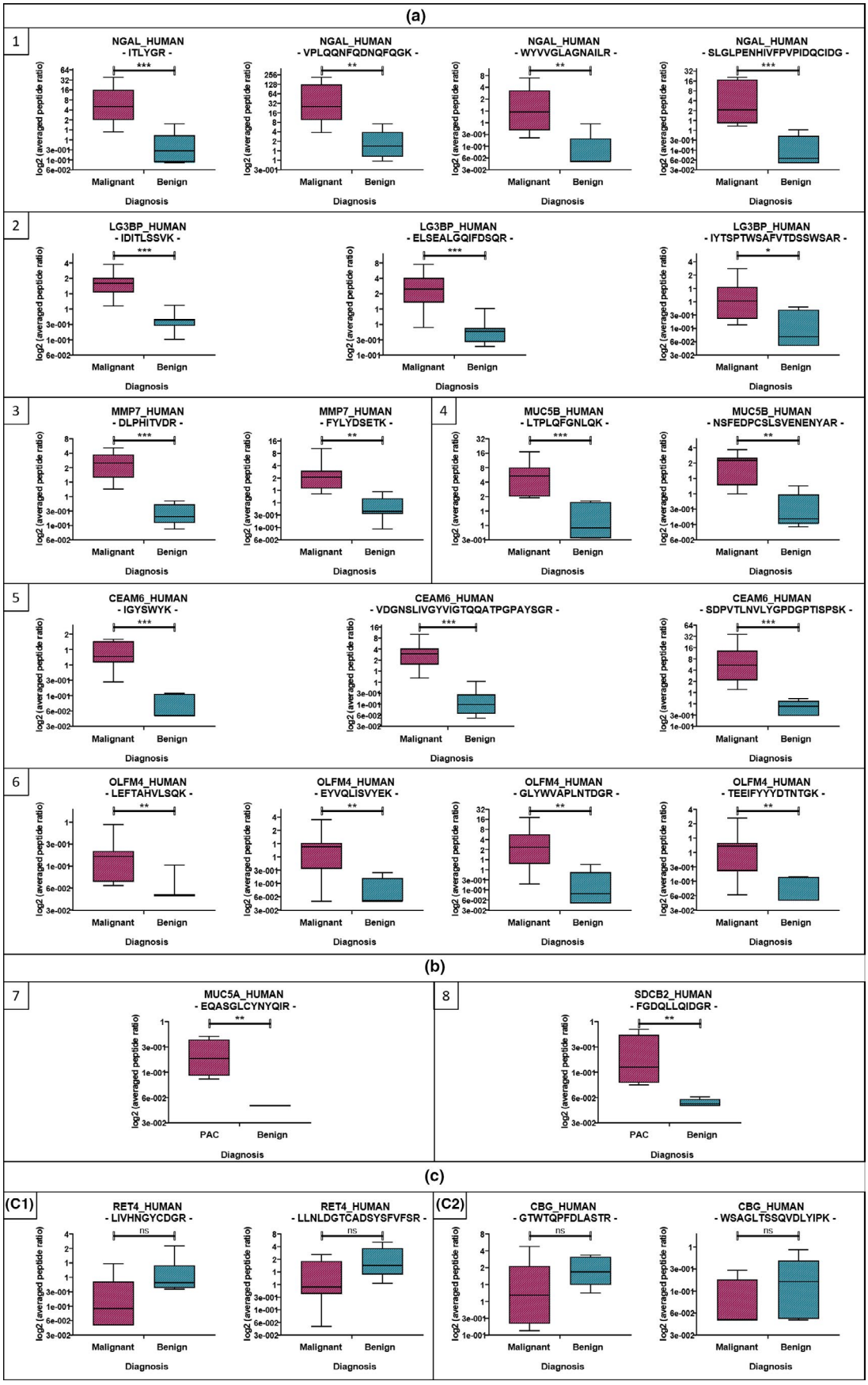
Statistical comparisons between patient groups and correlation analyses were performed using Prism 8.0.2 (GraphPad Software, La Jolla, CA). Diagnostic performances of single biomarkers and panels were evaluated by using the pROC package[18] for TIBCO Spotfire S+ 8.2 (TIBCO Software, Palo Alto, CA). The best performing panel was identified by using the PanelomiX platform for combination of biomarkers.[19]

The study was approved by the Ethics Committees of the Geneva University Hospitals (Geneva, Switzerland) and of the Erasme University Hospital (Brussels, Belgium). Written informed consent was obtained from all the patients before enrollment in the study.

RESULTS

In the developmental phase, reliable quantification was obtained for 31 of the 37 measured peptides (**Table S3**). Overall, the relative quantification of the 11 proteins previously found to be overexpressed in MBS showed significantly higher peptides ratios (endogenous vs. standard) in MBS vs. BBS samples (median 1.861 (interquartile range 3.463) vs. 0.174 (interquartile range 0.338), respectively; Mann–Whitney P value < 0.001; **Table S3**). Among them, six potential biomarkers for gastrointestinal cancers (i.e., NGAL, LG3BP, MMP7, MUC5B, CEAM6, and OLFM4) had all of their measured peptide ratios significantly higher in MBS vs. BBS conditions (**Figure 1a**, **Table S3**), whereas MUC5A and SDCB2 proved slightly overexpressed only in

Figure 1 Box-and-whisker (minimum to maximum) plots of the log2-transformed averaged ratio of each peptide quantified by selected reaction monitoring in bile samples included in the developmental cohort composed of malignant (pancreatic cancer (PAC), $n = 4$; cholangiocarcinoma, $n = 4$), and benign (chronic pancreatitis, $n = 4$; biliary obstruction due to stones or other benign causes, $n = 3$) biliary strictures. (**a**) Cancer proteins significantly overexpressed in malignant vs. benign samples: 1, neutrophil gelatinase-associated lipocalin, NGAL; 2, galectin-3-binding protein, LG3BP; 3, matrilysin, MMP7; 4, mucin-5B, MUC5B; 5, carcinoembryonic antigen-related cell adhesion molecule 6, CEAM6; and 6, olfactomedin-4, OLFM4. (**b**) Cancer proteins significantly overexpressed in PAC vs. benign samples: 7, mucin-5AC, MUC5A; and 8, syntenin-2, SDCB2. (**c**) Two control references: C1, retinol-binding protein 4, RET4; and C2, corticosteroid-binding globulin, CBG. ns, not significant; *P value < 0.05; **P < 0.01; ***P < 0.001.


(a)
1
NGAL_HUMAN - ITLYGR -
NGAL_HUMAN - VPLQQNFQDNQFQGK -
NGAL_HUMAN - WYVVGLAGNAILR -
NGAL_HUMAN - SLGLPENHIVFPVPIDQCIDG -
2
LG3BP_HUMAN - IDITLSSVK -
LG3BP_HUMAN - ELSEALGQIFDSQR -
LG3BP_HUMAN - IYTSPTWSAFVTDSSWSAR -
3
MMP7_HUMAN - DLPHITVDR -
MMP7_HUMAN - FYLYDSETK -
4
MUC5B_HUMAN - LTPLQFGNLQK -
MUC5B_HUMAN - NSFEDPCSLSVENENYAR -
5
CEAM6_HUMAN - IGYSWYK -
CEAM6_HUMAN - VDGNSLIVGYVIGTQQATPGPAYSGR -
CEAM6_HUMAN - SDPVTLNVLYGPDGPTISPSK -
6
OLFM4_HUMAN - LEFTAHVLSQK -
OLFM4_HUMAN - EYVQLISVYEK -
OLFM4_HUMAN - GLYWVAPLNTDGR -
OLFM4_HUMAN - TEEIFYYYDTNTGK -
(b)
7
MUC5A_HUMAN - EQASGLCYNYQIR -
8
SDCB2_HUMAN - FGDQLLQIDGR -
(c)
(C1)
RET4_HUMAN - LIVHNGYCDGR -
RET4_HUMAN - LLNLDGTCADSYSFVFSR -
(C2)
CBG_HUMAN - GTWTQPFDLASTR -
CBG_HUMAN - WSAGLTSSQVDLYIPK -
log2 (averaged peptide ratio)
Malignant
Benign
PAC
Diagnosis
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ns

PAC vs. BBS (**Figure 1b**, **Table S3**). The three remaining proteins (i.e., OSTP, RAC1, and GOLM1) and the two control references (i.e., RET4 and CBG) showed an overall unvarying expression in all samples (**Figure 1c**, **Table S3**)

For the verification phase, a second SRM assay was designed to simultaneously quantify the five potential biomarkers found to be most consistently overexpressed in MBS vs. BBS (Mann–Whitney P value ≤ 0.001 for a majority of measured peptides). Absolute quantification was carried out using, for each protein, the peptide most reliably detected in the developmental cohort. The dosages confirmed the ability of LG3BP, MMP7, MUC5B, NGAL, and CEAM6 to distinguish patients with MBS from patients with BBS (Mann–Whitney P values ≤ 0.001; **Table S4**). Interestingly, none of these proteins correlated with serum CA19-9, direct bilirubin, or total bilirubin (Pearson's correlation coefficient, $r \leq |0.7|$, data not shown). By further analyzing the 11 reference samples included in both cohorts, a very strong correlation ($r \geq |0.9|$) was observed between all the measurements performed using the two different SRM assays, with the only exception of MUC5B ($r = 0.649$), which was consequently excluded from the subsequent assessment of biomarker combination (**Figure S1**).

Among individual bile protein biomarkers, CEAM6 showed the highest area under the receiver operating characteristic curve (ROC-AUC, 0.889), whereas NGAL and LG3BP showed, respectively, the highest sensitivity (92.6%, threshold 0.2335 ng/µL) and specificity (96.9%, threshold 1.132 ng/µL) for diagnosing MBS. When considered in panel (maximum number of biomarkers = 3), the measurement of bile LG3BP, CEAM6, and serum CA19-9 showed an ROC-AUC of 0.914 with 88.1% accuracy, 88.9% sensitivity, and 87.5% specificity (respective thresholds: 1.132 ng/µL, 0.8565 ng/µL, and 37 kU/L). In an alternative panel (maximum number of biomarkers = 4), the combination of bile MMP7, LG3BP, CEAM6, and serum CA19-9 allowed to reach the highest ROC-AUC, accuracy, and specificity (0.929, 89.8%, and 96.9%, respectively), but a lower sensitivity (81.5%), in discriminating between patients with MBS and patients with BBS (respective thresholds: 0.034 ng/µL, 1.271 ng/µL, 1.762 ng/µL, and 37 kU/L). Both the panels outperformed the results obtained with serum CA19-9 alone (0.822 ROC-AUC, 76.3% accuracy, 77.8% sensitivity, and 75.0% specificity) at the clinical threshold of 37 kU/L (**Table 1**).

DISCUSSION

We developed an SRM assay able to reliably quantify 31 peptides at the same time in human bile. This strategy allowed to confirm the ability of various cancer-related bile proteins to distinguish MBS from BBS. The diagnostic value of two identified panels of biomarkers proved to be superior to that of serum CA19-9 alone.

Our study is the first to show that SRM has the potential to differentiate MBS from BBS in clinical practice. Besides its versatility in processing complex biological fluids, critical advantages of SRM include its ability to simultaneously measure various proteins on a single sample; its virtual capacity to quantify any human protein; its independence from the availability of commercial antibodies; and its affordability, even when a small number of samples is processed. A final crucial strength of the proposed method is its flexibility. The identified biomarker panels are indeed not strictly linked to the protocol, which could be adapted to include any new bile biomarker able to improve MBS diagnosis. So far, other potential

Table 1 Diagnostic performances of single and combined biomarkers in paired cohorts of patients

Marker	Threshold[a]	ROC-AUC	95% CI	SE	SP	PPV	NPV	ACC
Serum CA19-9	37 kU/L	0.822	0.694–0.951	77.8%	75.0%	72.4%	80.0%	76.3%
Bile NGAL	0.2335 ng/µL	0.741	0.614–0.867	**92.6%**	46.9%	59.5%	**88.2%**	67.8%
Bile MMP7	0.034 ng/µL	0.755	0.626–0.884	88.9%	62.5%	66.7%	87.0%	74.6%
Bile MUC5B	7.747 ng/µL	0.752	0.617–0.888	63.0%	90.6%	85.0%	74.4%	78.0%
Bile LG3BP	1.132 ng/µL	0.841	0.729–0.954	66.7%	**96.9%**	**94.7%**	77.5%	**83.1%**
Bile CEAM6	3.077 ng/µL	**0.889**	0.807–0.970	74.1%	90.6%	87.0%	80.6%	**83.1%**
Panel 1: Positive when 2 markers > threshold								
Bile LG3BP	1.132 ng/µL	0.914	0.839–0.989	88.9%	87.5%	85.7%	**90.3%**	88.1%
Bile CEAM6	0.8565 ng/µL							
Serum CA19-9	37 kU/L							
Panel 2: Positive when 3 markers > threshold								
Bile MMP7	0.034 ng/µL	**0.929**	0.866–0.991	81.5%	**96.9%**	**95.7%**	86.1%	**89.8%**
Bile LG3BP	1.271 ng/µL							
Bile CEAM6	1.762 ng/µL							
Serum CA19-9	37 kU/L							

ACC, accuracy; CA19-9, carbohydrate antigen 19-9; CEAM6, carcinoembryonic antigen-related cell adhesion molecule 6; CI, confidence interval; LG3BP, galectin-3-binding protein; MMP7, matrilysin; MUC5B, mucin-5B; NGAL, neutrophil gelatinase-associated lipocalin; NPV, negative predictive value; PPV, positive predictive value; ROC-AUC, area under the receiver operating characteristic curve; SE, sensitivity; SP, specificity.
Bile LG3BP in panel 2 is considered positive when its value is greater than, or equal to, its threshold. In all other cases, markers are considered positive when their values are greater than their threshold.
Blue and bold, best performance among single biomarkers; **red and bold**, best performance among all biomarkers; red, better performance of combined vs. single biomarkers.
[a]For bile markers, the number of digits does not reflect the relative precision of the mass spectrometry measurement, and the margin of errors of a future clinical assay should be calculated through dedicated studies.

bile protein biomarkers have already been highlighted, which yield promising results in preliminary studies and could be tested using the technique described here. These include: CD276 antigen (81.2% sensitivity and 81.6% specificity in a cohort of 323 patients),[20] dysbindin (81.9% sensitivity and 84.7% specificity in a cohort of 550 patients),[21] as well as insulin-like growth factor (91.4% sensitivity and 89.5% specificity) and vascular endothelial growth factor A (90.3% sensitivity and 84.9% specificity), both in a cohort of 109 patients.[22] Further large-scale screenings would be needed to select additional proteins and suitable peptides able to increase the clinical value of the newly developed assay.

With respect to the duration of the analysis, sample preparation for SRM analysis has already been optimized for other sample types, such as cerebrospinal fluid, allowing for automation and biomarker dosage in hours.[23,24]

Limitations of this study include the use of synthetic peptides for absolute quantification. The use of full-length labeled proteins would have allowed accounting for any potential variation introduced by sample prefractionation, proteolysis efficiency, or poor sequence coverage, thus ensuring a maximum reliability of the results, at the expense of higher costs.[25] However, the strong correlation between the dosages obtained by using separate prefractionation/digestion procedures and different quantitative assays supports the reliability of our preliminary investigation. Before transfer to clinical practice, calibration curves would also need to be prepared for each biomarker, allowing to demonstrate the linear response and determine the limits of quantification. In both cohorts, a few peptides were not dosed in all samples because of detection limits and signal interferences. Such problems should be solved using the latest MS instruments. Finally, our results should be confirmed in larger cohorts of patients, including other biliary diseases that may mimic MBS, such as primary sclerosing cholangitis and autoimmune pancreatitis.

In conclusion, we succeeded in developing a method for cancer biomarker dosage in bile, which owns the potential to be translated into clinical settings. The high accuracy of some bile biomarkers for the diagnosis of MBS has been confirmed, and two panels of biomarkers outperforming the diagnostic accuracy of serum CA19-9 have been proposed.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

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Conflicts of Interest. The authors declared no competing interests for this work.

Author Contributions. A.F. and Y.C. wrote the manuscript. A.F., Y.C., and J.-M.D. designed the research. A.A., Y.C., and I.A.-R. performed the research. A.F., Y.C., and A.A. analyzed the data. J.-M.D., M.D., and J.-L.F. contributed new reagents/analytical tools.

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