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Plasma Proteome to Look for Diagnostic Biomarkers of Early Bacterial Sepsis after Liver Transplantation

A Preliminary Study

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ABSTRACT

Background: While outcome continuously improves after liver transplantation, sepsis remains the leading cause of early postoperative mortality. Diagnosis of infections remains particularly difficult in these patients. This study used plasma profiling coupling Proteinchip array with surface-enhanced laser desorption ionization time-of-fly mass spectrometry to search for biomarkers of postoperative sepsis in patients who underwent liver transplantation.

Methods: Diagnosis of sepsis at day 5 relied on widely accepted clinical signs and positive culture of microbiologic samples. Profiles of day 5 plasma were obtained from SELDI-TOF CM10 chip (BioRad, Marnes-la-Coquette, France) analysis. Mean peak intensity of proteins was compared between septic and nonseptic plasma by U test followed by analysis of the area under the receiver-operating characteristic for the significant peaks. Diagnostic performance of significant proteins was established in a derivation set and in a validation set.

Results: In the derivation set of 31 patients with and 30 without infection, 23 plasma protein peaks were differentially expressed between patients with and without sepsis. Combination of five peaks allowed sepsis diagnosis with a positive likelihood ratio of 12.5 and a C-statistics of 0.72, 95% CI 0.57–0.85. In the validation set of 31 patients with infection and 34 without infection, the five peaks were differentially expressed as well and allowed day 5 sepsis diagnosis with a positive likelihood ratio of 5.1 and C-statistics of 0.74 (0.58–0.85).

Conclusion: A combination of five plasma protein peaks may provide material for useful diagnostic biomarkers of postoperative sepsis in patients undergoing liver transplantation. However, these proteins remain to be identified.

What We Already Know about This Topic

- Bacterial sepsis is an important cause of mortality after liver transplantation
- Early diagnosis of sepsis in this setting based on clinical signs alone is difficult

What This Article Tells Us That Is New

- Using a proteomic approach in serum from patients 5 days after liver transplantation, a profile containing five proteins reliably identified sepsis
- The identity of these proteins is under investigation

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LIVER transplantation represents the sole acceptable treatment for most forms of acute and chronic hepatic failure. This surgery is now increasingly performed and is associated with continuous improvement of outcome, with a 10-yr survival rate of 70%.[1] However, postoperative mortality rate remains approximately 10% and accounts for more than half of the mortality observed in the first postoperative year (17%).[1] Bacterial infections are the leading cause of early mortality during the postoperative period and occur in almost half the patients who have undergone liver transplantation.[1,2] Early diagnosis of infection is one of the crucial steps in managing sepsis in transplanted patients. It allows prompt initiation of appropriate antimicrobial therapy, which turns to have a major impact on survival in septic patients.[3] However, diagnosis of bacterial infections remains difficult in the days after liver transplantation, particularly because of the ongoing immunosuppression regimen. Reliable biomarkers of sepsis could help for therapeutic decision making.[4] Procalcitonin and C-reactive protein (CRP) are commonly used as biomarkers of sepsis despite their modest diagnostic performance.[5,6] Moreover, in the specific setting of liver transplantation, previous reports have suggested that procalcitonin levels could be influenced by a noninfectious state such as rejection treatment or donor status.[7,8] Consequently, in the particular field of solid organ transplantation, no biomarker has definitely been proven to be reliable in helping physicians ascertain the presence of sepsis in the early postoperative period. Search for reliable biomarkers of infections is, therefore, still ongoing.[4]

Clinical proteomics is a nontargeted approach that allows the characterization of the whole or a part of the whole spectrum of proteins in any biologic sample.[9] The development of proteomic array technology, including serum profiling coupling Proteinchip array with Surface-enhanced laser desorption ionization time-of-fly mass spectrometry (SELDI-TOF Proteinchip technology, Bio-Rad Laboratories, Marnes-la-Coquette, France), provides a powerful tool for clinical research in developing biomarkers of cancer or a pathologic state such as cirrhosis.[10–12] We hypothesized that this approach could allow the identification of specific protein profiles that may present as good candidates for biomarkers of postoperative sepsis in the early postoperative context of liver transplantation. Therefore, the aim of the study was to look for sepsis biomarkers in liver recipients using proteomic analysis and to compare the diagnostic performance of the selected protein peaks with those of procalcitonin and CRP. For this purpose, we determined plasmatic protein profiles of liver recipients on postoperative day 5 and compared the profiles from patients with ongoing infection with the profiles obtained from patients without infection at day 5 to identify a diagnostic profile of infectious complications after liver transplantation.

Materials and Methods

After Institutional Review Board approval (Comité d'évaluation de l'éthique des projets de recherche biomédicale du GHU Nord) and informed consent, this monocenter prospective study included consecutive patients who underwent liver transplantation in the Liver Transplantation Unit of Beaujon University Hospital between July 1, 2006, and October 31, 2008, (n = 253). Exclusion criteria were intraoperative infection at the time of the transplantation documented by positive microbiologic cultures of intraoperative samples, death in intensive care unit (ICU) before postoperative day 5 (D5), or inability to determine sepsis status at D5. Allocation of patient to derivation or validation set was based on chronological inclusion. Surgical technique of liver transplantation consisted of preservation of the inferior vena cava with or without piggy-back technique.[13] No venovenous bypass was used. Patients were discharged to the ICU after the operation was completed. Immunosuppression consisted of triple therapy for all patients. Prednisone was started intraoperatively (5 mg/kg intravenous bolus) and continued until day 7 (20 mg/day). Tacrolimus was started on day 1 and titrated to obtain residual levels between 8 and 10 UI/ml, and mycophenolate molfetil (1.5 g twice a day) was started on day 1. Antimicrobial prophylaxis consisted of cefoxitine 2 g intravenously given immediately before induction of anesthesia and 1 g every 2 h during the operative period only. If methicillin-resistant *Staphylococcus* carriage was detected preoperatively, intraoperative vancomycin was added. Antimicrobial prophylaxis was not continued after the end of the surgery. Ganciclovir was used for cytomegalovirus prophylaxis in case of cytomegalovirus seronegative recipient and cytomegalovirus-positive graft. Acyclovir was used for other status.

Diagnosis of Postoperative Infection

Criteria for infection combined at least two criteria among the criteria of systemic inflammatory response syndrome: temperature more than 38.3° or less than 36°C, leukocytosis more than 12,000 Giga/l or less than 4,000 Giga/l or more than 10% immature forms, heart rate more than 90/min, respiratory rate more than 20/min, blood glucose more than 7.7 mM, altered mental status, capillary recoloration time more than 2 s or lactactemia more than 2 mM, and microbiologically proven infections.[14] Ventilator-acquired pneumonia was considered when new pulmonary infiltrates were observed on chest x-ray and when at least three of the following criteria were fulfilled: (a) purulent respiratory secretions, (b) body temperature more than 38.3° or less than 36.5°C, (c) leukocyte count more than 10,000 Giga/l or less than 4,000 Giga/l, (d) deterioration of Pa_{CO_2}/F_{IO_2} ratio more than 20 in association with a positive distally protected sample yielding at least one pathogen.[15] Bacteriuria was considered present when culture of urine yielded at least 10^5 microorganisms per milliliter of urine with no more than two species.[16] Postoperative abdominal sepsis was retained in case of ascitis infection (positive ascitic fluid culture) and in case of intrabadominal infection defined by positive culture of percutaneous abdominal puncture or intraoperative samples of abdominal fluid. Diagnosis of central venous catheter-related sepsis was considered if local or general signs of sepsis

were associated with positive bacteriologic culture of the distal catheter tip more than 10^3 colony-forming units/ml.[17] Diagnosis of infection was retrospectively confirmed by an independent panel composed of two individuals with substantial expertise in infectious diseases. The panel used a three-level scale with 1 being a very high probability and 3 being a very low probability of infection according to the earlier definitions. All patients initially classified as "septic" have been confirmed with a very high or mean probability of infection by the panel. Of note, CRP or procalcitonin plasma levels were not available to the attending physician or the panel members at the time of diagnosis of infection. Patients considered as nonseptic had a strictly uneventful ICU stay with normal physiologic variables and absence of any organ dysfunction during the whole stay. Patients with documented infection between postoperative days 4 and 6 were defined as SEPSIS D5+. They were compared with noninfected patients at D5 (SEPSIS D5−).

Measurements of C-reactive Protein and Procalcitonin Plasma Levels

Plasma was sampled at day 5, frozen, and stored at −80°C until analysis was performed. Procalcitonin plasma level was determined with an electrochemiluminometric assay Elecsys® BRAHMS procalcitonin (BRAHMS, Hennigsdorf, Germany), performed on analyzer Elecsys® 2010 (Roche Diagnostics, Meylan, France). CRP level was measured by an immunoturbidimetric method using CRP Vario® assay (Sentinel Diagnostics, Milano, Italy) distributed by Abbott Diagnostics performed on analyzer Architect C8000®, (Abbott Laboratories, Abbott Park, IL).

Proteomic Analysis

Plasma samples were collected at postoperative day 5, aliquotted, then immediately frozen and stored at −80°C for later assay. Stability of samples throughout various durations of storage has been confirmed previously. A total of 125 serum samples were analyzed. To determine the best conditions for identifying the most discriminating plasma protein profile, different experimental conditions were compared on two different Proteinchip arrays: weak cationic exchange (CM10) and immobilized metal ion affinity capture loaded with zinc. Finally, plasma samples were processed using CM10 Proteinchip array according to the manufacturer's protocols (Bio-Rad). All samples of each set were tested during the same experiment in duplicate and processed on a Biomek 2000 (Beckman Coulter, Tallerton, CA). Each plasma aliquot was thawed and diluted (1:10) in denaturing buffer (urea 7 M, thiourea 2 M, CHAPS 4% [3-{(3-cholamidopropyl) dimethylammonio}-1-propanesulfate], dithioerythritol 0.1%). The mixture was incubated with vigorous shaking at room temperature for 20 min. The array spots were preactivated with 10 mM HCl for 5 min at room temperature. This step was followed by two washes with H_2O and additional incubation with binding/washing buffer (0.1 M sodium acetate, [pH 5.0], triton 0.1%). After incubation, the diluted serum mixture was mixed in dilution (1:20) with binding buffer on spots. Samples were removed from the wells, and each well was washed twice with 200 μl of washing buffer for 5 min with agitation. This was repeated once with washing buffer without triton. Binding buffer was removed from the wells, and 200 μl HEPES (1 mM hydroxyethyl piperazine ethanesulfonic acid) was added to each well. Then, the Proteinchip was removed from the bioprocessor and dried at room temperature. One microliter of sinapinic acid (Bio-Rad) in 50% acetonitrile volume/volume (v/v) and 0.5% v/v trifluoroacetic acid was applied once to each spot and a second time after air-drying. The mass spectra of proteins were generated using an average of 530 lasers shots. For data acquisition of low-molecular weight proteins, the detection size range was between 1 and 10 kd at a laser intensity of 2400 nJ. For high-molecular weight proteins, the detection size range was between 10 and 200 kd, and the laser intensity was set at 2700 nJ. The mass-to-charge ratio (m/z) of each protein captured on the array surface was determined according to externally calibrated standards (all in one protein II, Bio-Rad). Interprotein chip array reproducibility was checked by spotting one sample of every two chips on the same array. Peaks are autodetected using Biomarker wizard set to a signal/noise ratio of more than 5 and present in 100% of spectra. The means of interprotein chip array coefficient of variations were 22.5 and 17.5% for the derivation set and the validation set, respectively. The different profiles were aligned and baseline was substrated with Proteinchip Software (Bio-Rad). Spectra were normalized by using the total ion current of all profiles; some of them were excluded when their normalization coefficient was not between 0.5 and 2.5.

Data Collection

Recipient preoperative variables (age, etiology of cirrhosis, and Model End-stage Liver Disease score) and graft criteria (duration of cold ischemia time, that is, time between aortic clamp in the donor until beginning of vascular anastomosis of the graft in the recipient) were recorded. Intraoperative blood products and duration of the surgical procedure were also recorded. Postoperative variables included Simplified Acute Physiologic Score (SAPS II) calculated on the worst values recorded during the 24 h after ICU admission,[18] vasopressors requirement, duration of postoperative mechanical ventilation and ICU stay, death rate in ICU, and biologic data on admission in ICU and at postoperative day 5. Procalcitonin and CRP dosages at day 5 were not available for the attending intensivist and did not influence decision making.

Statistical Analysis

Results are expressed as median (range) or n (percentage). A P value < 0.05 was considered significant. Clinical and biologic data obtained from SEPSIS D5+ and SEPSIS D5− groups of patients were compared using the following tests: categorical data were compared using a chi-square test (or a Fisher exact test when appropriate). Quantitative parameters were compared by the Mann-Whitney U test. Univariate

Table 1. Demographic and Intraoperative Data of Derivation and Validation Cohort

	Derivation Set		Validation Set	
	Sepsis D5+ (n = 31)	Sepsis D5− (n = 30)	Sepsis+ (n = 31)	Sepsis− (n = 34)
Age (yr)	52 (27–65)	57.5 (31–69)	54 (23–65)	56 (20–69)
Male/female	24/7	22/8	21/10	24/10
Cirrhosis (%)	26 (84)	26 (87)	26 (84)	28 (82)
HCC (%)	8 (26)	11 (37)	10 (45)	11 (46)
Acute liver failure (%)	3 (10)	1 (3.3)	5 (18)	3 (9)
MELD score	19 (7–40)	18 (6–32)	17 (6–40)	18 (8–33)
Kidney-liver transplantation	2 (6.5)	4 (13)	1 (3.2)	2 (6)
Total graft (%)	20 (64.5)	23 (77)	22 (71)	28 (85)
Cold ischemia duration (min)	490 (115–690)	438 (145–780)	**530 (270–900)**	**450 (130–720)**
Surgery duration (min)	**492 (375–810)**	**472 (300–720)**	457 (265–715)	427 (320–600)
Red packed cells (units)	**6 (0–17)**	**3 (0–26)**	**4 (0–12)**	**2.5 (0–11)**
Fresh frozen plasma (units)	8 (0–22)	3 (0–22)	4.5 (0–14)	3.5 (0–12)
Platelets (units)	1 (0–3)	0 (0–5)	1 (0–2)	0 (0–2)

Bold entries correspond to significantly different values between patients with sepsis (SEPSIS D5+) or without sepsis (sepsis D5−). D5 = postoperative day 5; HCC = hepatocellular carcinoma; MELD = model for end-stage liver disease.

logistic regression models were also used to analyze the data *via* a simple logistic regression of the diseased status.

To characterize the plasma protein peaks of interest for the diagnosis of sepsis at D5, plasma profiles of patients (SEPSIS D5+) were compared with plasma profiles from patients (SEPSIS D5−). Note that because of lack of existing data on the expected quantifiable difference between septic and nonseptic patients, it was not possible to precisely calculate the number of patients to be included.[19] Consequently, the sample size was chosen based on previous study using the same technology.[20] Mean peak intensity of each protein was calculated and compared with a nonparametric test (Mann-Whitney U test). Peaks were considered as significantly differently expressed for a P value < 0.05. Significant peaks were entered in a stepwise logistic regression model to identify independent predictive peaks of sepsis. The performance of selected peaks for the diagnosis of sepsis was assessed by determination of the area under the curve (AUC) of the receiver-operating characteristic (ROC) curves. An AUC value of 0.5 means that distributions are similar in both populations. Conversely, an AUC value of 1.0 means that the two population distributions do not overlap at all. The sensitivity and specificity of a test cannot be used to estimate the probability of disease in individual patients. They can, however, be combined into a single measure called the likelihood ratio (LHR), which is clinically more useful as they provide a summary of how many times more likely patients with a disease are to have a particular result than patients without the disease.[21] Consequently, for every possible cutoff point, positive LHRs were computed, and the point with the best positive LHR was chosen as the best cutoff point. Proteins or combination of proteins that were significantly differentially expressed in the derivation set were then studied in the validation set. The same cutoff was applied, and LHRs were computed. A similar analysis was performed with procalcitonin and CRP levels. Statistical analysis was performed using the Statistical Analysis System (SAS Institute Inc., Cary, NC) version 9.1.3.

Results

During the study period, 230 liver transplantations have been performed on 220 patients; 98 patients have been excluded because of death before day 5 (n = 11), lack of blood samples (n = 34), or inability to determine presence or absence of sepsis at day 5 (n = 53).

Derivation Set

Demographic Data. Sixty-one patients (31 with sepsis at D5 and 30 without) have been included in the derivation set. Clinical preoperative and intraoperative data are displayed in table 1. No preoperative characteristic was different between septic and nonseptic patients. Patients who developed sepsis at postoperative D5 had significantly longer duration of surgery and received significantly more red-packed cells than patients who did not develop infection at D5. The increase in transfusion requirement by one unit of red-packed cells was associated with a nonsignificant increased risk of sepsis at D5 (odds ratio = 1.06; 95% CI 0.99–1.12, $P = 0.0521$). Data on ICU admission and postoperative clinical course are shown in table 2. Patients who developed sepsis at D5 had a significantly greater SAPS II on admission and longer duration of postoperative mechanical ventilation and ICU stay. One point increase of SAPS II was associated with an increased risk of developing sepsis at D5 (odds ratio = 1.13; 95% CI 1.01–1.27, $P = 0.037$). Mortality rate did not reach statistical difference between patients with and without sepsis. No significant difference was noted in biologic data on admission into the ICU between patients with and without sepsis.

Clinical presentation and type of infection observed at D5 are presented in table 3. Half the patients suffered from septic shock. Bacterial pneumonia accounted for almost half of the documented infections and more than one-third of the patients experienced blood stream infections. Biologic data of patients with or without sepsis at D5 are presented in table 4. No usual biologic variables, particularly leukocyte count, were signifi-

Table 2. Biologic Data on Admission in Intensive Care Unit after Liver Transplantation and Postoperative Course

	Derivation Set		Validation Set	
	Sepsis D5+ (n = 31)	Sepsis D5− (n = 30)	Sepsis D5+ (n = 31)	Sepsis D5− (n = 34)
Prothrombin time D0 (%)	36 (18–55)	40 (19–68)	36 (16–54)	38 (20–50)
Platelets count D0 (Giga/l)	**78 (21–216)**	**110 (33–249)**	87 (13–201)	98 (29–305)
Bilirubin D0 (μM)	87 (11–240)	68 (4–289)	80 (12–318)	63 (4–289)
Lactate D0 (mM)	2.8 (1–9)	1.8 (0–9)	2 (0–15)	1.6 (1–6)
SAPS II	**38 (18–58)**	**35 (15–53)**	**43 (25–88)**	**34 (22–69)**
Norepinephrin infusion (mg/h)	1.2 (0–15)	1 (0–4)	1.5 (0–8)	0.8 (0–7)
Mechanical ventilation (d)	**5 (1–63)**	**1 (0–4)**	**1 (0–4)**	**1 (0–4)**
Reoperation, n (%)	9 (29)	5 (17)	10 (32)	3 (9)
Length ICU stay (d)	**14 (4–65)**	**5 (2–12)**	**13 (2–48)**	**6 (2–31)**
ICU Death, n (%)	4 (13)	0	5 (16)	3 (9)

Bold entries correspond to significantly different values between patients with sepsis (sepsis D5+) or without sepsis (sepsis D5−). D0 = day of admission in intensive care unit; D5 = postoperative day 5; ICU = intensive care unit; SAPSII = Simplified Acute Physiological Score.

cantly different between septic and nonseptic patients. At D5, plasma CRP and procalcitonin levels were significantly greater in septic patients than in nonseptic patients. The performance of CRP for the diagnosis of infection at D5 corresponded to an AUC = 0.73, 95% CI 0.57–0.87. The best CRP positive LHR was 10.4, 95% CI 1.4–16.8 for a threshold of 47.9 mg/l, with a sensitivity of 36.0%, 95% CI 18.0–57.5 and a specificity of 96.6%, 95% CI 82.2–99.9. The performance of procalcitonin plasma levels for the diagnosis of infection at D5 corresponded to an AUC = 0.73, 95% CI 0.59–0.87. The best procalcitonin positive LHR was 9.3, 95% CI 1.2–69.2 for a threshold of 1.45 μg/l with a sensitivity of 32.0%, 95% CI 14.9–53.5 and a specificity of 96.6%, 95% CI 82.2–99.9.

Proteomic Analysis. In the derivation set (61 plasma samples), a mean of 317 protein peaks for each plasma were generated with the Chip-array CM10. Comparison of the protein profiles obtained in the septic group (n = 31) showed a total of 29 differentially expressed protein peaks in comparison with those of the nonseptic group (n = 30; $P <$ 0.05). Fourteen were upregulated in the septic group, whereas 15 were down-regulated. The most significant protein peaks were CM-10 3079 and CM-4152 ($P = 0.0001$) in the derivation set. Expression of these two peaks allowed sepsis diagnosis with a positive LHR 11.6, 95% CI 1.6–83 and an AUC of the ROC curve of 0.86, 95% CI 0.76–0.95.

Table 3. Clinical Presentation and Type of Infections at Postoperative Day 5

	Sepsis D5+ Derivation (n=31)	Sepsis D5+ Validation (n = 31)
Sepsis (%)	13 (42)	14 (45)
Severe sepsis (%)	5 (16)	3 (9)
Septic shock (%)	11 (35)	13 (42)
Pneumonia (%)	14 (45)	17 (55)
Urinary tract infection (%)	7 (23)	5 (16)
Abdominal infections (%)	4 (13)	6 (18)
Bacteriemia (%)	12 (39)	11 (35)
Miscellaneous (%)	7 (23)	2 (6)

Abdominal infections include bacterial infection of ascitis and peritonitis or intrabadominal abcess. Miscellaneous includes central venous catheter infection, cellulites, and angiocholitis.

Validation Set

Demographic Data. Sixty-five additional patients (31 with and 34 without sepsis at D5) have been included in the validation set. Clinical preoperative and intraoperative data are displayed in table 1. No preoperative characteristic was different between septic and nonseptic patients. Intraoperatively, patients having developed sepsis at D5 had received significantly more red-packed cells than patients who did not develop infection at D5. Duration of surgery was not different between septic and nonseptic patients. The increase of transfusion requirement by one unit of red-packed cells was associated with an increased risk of developing sepsis at D5 (odds ratio = 1.07; 95% CI 1.01–1.12, $P = 0.014$). ICU admission data and postoperative clinical course are shown in table 2. Similar to the derivation set, patients who developed sepsis at D5 in the ICU had a significantly greater SAPS II on admission and longer durations of postoperative mechanical ventilation and ICU stay. One point increase of SAPS II was associated with an increased risk of D5 sepsis estimated by an odds ratio = 1.24; 95% CI 1.05–1.46, $P = 0.0133$. Mortality rate did not reach statistical difference between patients with and without sepsis. Similar to the derivation set, no biologic recorded data on admission to the ICU were significantly different between patients with or without sepsis.

Clinical presentation of infection was not different in the two cohorts of patients ($P = 0.74$, Fisher exact test; table 3). Only in the validation set, septic patients displayed a significantly lower prothrombin time, platelets count, and a significantly greater serum bilirubin and creatinin levels at D5 (table 4). At D5, plasma CRP and procalcitonin levels were signifi-

Table 4. Biologic Data on Postoperative Day 5 in the Derivation and Validation Cohorts

	Derivation Set		Validation Set	
	Sepsis D5+ (n = 31)	Sepsis D5− (n = 30)	Sepsis D5+ (n = 31)	Sepsis D5− (n = 34)
Prothrombin time D5 (%)	76 (33–115)	90 (51–130)	**76 (48–115)**	**88 (34–130)**
Platelets count D5 (Giga/l)	61 (8–152)	69 (24–197)	**46 (8–109)**	**73 (19–398)**
Bilirubin D5 (mM)	35 (10–324)	31 (8–93)	**67 (10–302)**	**26 (8–189)**
ASAT D5 (UI/l)	63 (18–338)	73 (23–273)	96 (18–390)	86 (19–700)
Creatinin D5 (μM)	81 (47–362)	78 (34–131)	**76 (40–296)**	**72 (42–207)**
White cells count D5 (Giga/l)	6,8 (1–15)	8 (2–45)	7.1 (1–17)	7.1 (1–18)
CRP D5 (mg/l)	**38.5 (4–137)**	**15.5 (9–62)**	**40 (9–192)**	**21 (5–80)**
PCT D5 (μg/l)	**0.9 (0–51)**	**0.5 (9–62)**	**1.7 (0–50)**	**0.6 (0–7)**

Bold entries correspond to significantly different values between patients with sepsis (sepsis D5+) or without sepsis (sepsis D5−).
ASAT = aspartate aminotransferase; CRP = C-reactive protein; D5 = postoperative day 5; PCT = procalcitonin.

cantly greater in septic patients than in nonseptic patients. The performance of CRP for the diagnosis of infection at D5 in the validation set corresponded to an AUC = 0.69, 95% CI 0.53–0.83. The performance of procalcitonin plasma levels for the diagnosis of infection at D5 in the validation set corresponded to an AUC = 0.68, 95% CI 0.53–0.82.

Proteomic Analysis. In the validation set (64 samples), a mean of 188 protein peaks for each plasma were generated with the Chip-array CM10. Comparison of the protein profiles obtained in the septic group (n = 31) with those of the nonseptic group (n = 33) showed a total of 56 differentially expressed protein peaks (31 down-regulated and 25 upregulated) ($P < 0.05$). The most significant protein peak in the derivation set (CM-10 3079) was not retrieved in the validation set. Expression of the following five protein peaks CM-10 4152, 4627, 5744, 5812, and 5912 were significantly different in septic patients in comparison with nonseptic patients in both sets of patients (table 5; fig. 1). In the derivation set, the performance for sepsis diagnosis at D5 of the combination of expression of these five peaks revealed an AUC of the ROC curve of 0.72, 95% CI 0.57–0.85. The best positive LHR of 12.5 was associated with a sensitivity of 46.4% and specificity of 96.3%. In the validation set, the performance for sepsis diagnosis of the combination of expression of these five peaks was assessed with an AUC of the ROC curve of 0.75, 95% CI 0.58–0.85. Applying the same cutoffs than those defined in the derivation set, the positive LHR was 5.1 with a sensitivity of 30% and a specificity of 94.2%.

In an additional analysis, we compared plasma protein profiles according to the severity of the clinical presentation of sepsis, that is, septic shock or not. In the derivation cohort of septic patients, we found 18 differentially expressed protein peaks between septic shock and nonseptic shock patients (8 down-regulated and 10 upregulated). One of these peaks, CM-10 4152, was significantly less expressed in septic shock patients and was part of the combination of the five protein peaks retained for sepsis diagnosis. In the validation cohort of septic patients, seven protein peaks were differentially expressed between septic shock and nonseptic shock patients (two down-regulated and five upregulated). CM-10 4152 was the only common differentially expressed protein peak between derivation and validation groups.

Discussion

This study focused on plasma proteomics in two prospective cohorts of patients admitted in the ICU after liver transplantation to identify noninvasive biomarkers of postoperative sepsis. We showed that expression of five protein peaks (CM10 4152.7, CM10 4627.2, CM10 5744.7, CM10 5812.9, and CM10 5912.3) was significantly different in patients who developed a proven sepsis at postoperative day 5 in comparison with nonseptic patients. This combination revealed a diagnostic performance of sepsis that may offer an interesting alternative to more traditional sepsis biomarkers,

Table 5. Levels of Expression of the Five Significantly Differentially Expressed Protein Peaks Used for Sepsis Diagnosis in the Derivation and Validation Sets

	Derivation Set		Validation Set	
	Sepsis D5+	Sepsis D5−	Sepsis D5+	Sepsis D5−
CM-10 4152	106 (23–958)	78 (24–381)	177 (34–1064)	113 (0–818)
CM-10 4627	135 (69–464)	120 (71–335)	270 (106–468)	179 (4–1064)
CM-10 5744	27 (7–214)	15 (4–126)	60 (5–342)	22 (1–311)
CM-10 5812	37 (4–234)	21 (1–186)	76 (11–434)	33 (3–340)
CM-10 5912	39 (8–297)	24 (7–367)	28 (6–177)	21 (1–206)

Results are expressed as median (range). $P < 0.05$ between plasma from patients with infection (sepsis D5+) and patients without infection at D5 (sepsis D5−).
CM-10 = weak cationic exchange Proteinchip; D5 = postoperative day 5.

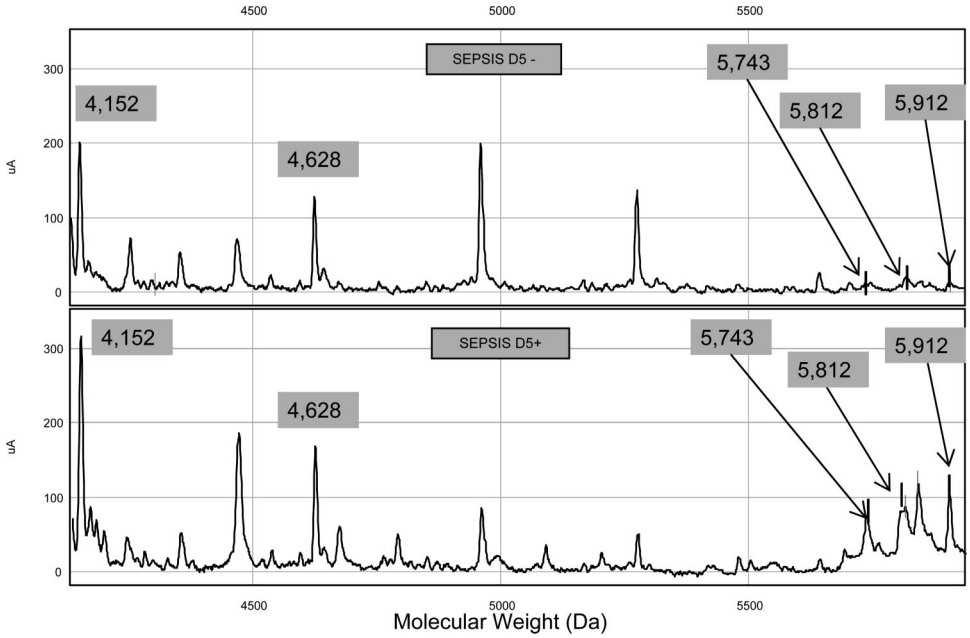

Fig. 1. Example of plasma protein profiles of patient without sepsis at day 5 (SEPSIS D5−) and patient with SEPSIS at day 5 (SEPSIS D5+), showing the differential expression of the five diagnostic protein peaks: CM-10 4152, 4627, 5744, 5812, and 5912. Other peaks were not significantly differentially expressed throughout the analysis of all samples.

such as procalcitonin or CRP. To our knowledge, assessing proteomics analysis for search for sepsis biomarkers in liver transplant patients has not been performed before.

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of a normal or a pathologic process or a response to a pharmacologic intervention.[22,23] It is also a biologic molecule associated with a disease (diagnostic biomarker) or a high probability of developing a disease (prognostic biomarker).[24] More than one hundred molecules have been proposed as useful biologic markers of sepsis highlighting the conviction that identifying such tools would represent an important advance in sepsis research.[4] Sepsis is indeed the leading cause of death in the ICU and the very first cause of early mortality in liver recipients.[1] In the current study, septic patients showed a higher mortality rate. Moreover, we found that sepsis was associated with greater morbidity assessed through longer duration of mechanical ventilation and length of stay in ICU. A useful biomarker of sepsis would help to promptly recognize the disease. This represents a crucial issue because this step associated with rapid and effective antimicrobial therapy are the cornerstones of the management of critically ill septic patients.[3,25] A combination expression of five protein peaks showed a diagnostic performance for sepsis in the derivation set, which was confirmed in the validation set evaluated by an AUC between 0.72 and 0.74. Because procalcitonin and CRP are currently thought to be acceptable biomarkers for infection, we performed concomitant comparative measurements of the diagnostic performance of CRP and procalcitonin plasma levels.[4,26] According to the values of AUC of the ROC curves, diagnostic performance of sepsis using the combination of protein peaks was similar to procalcitonin. LHR summarizes information about a diagnostic test by combining sensitivity and specificity. Moreover, Fagan's nomogram is a graphical tool that allows LHR to be used in conjunction with a patient's pretest probability of disease to estimate the posttest probability of disease.[21] Positive LHR values greater than 10 highlight very good discriminatory value of a biomarker.[27] According to the choice of the best threshold, the best positive LHRs were 12.5, 10.4, and 9.5 for the combination of proteins peaks, CRP, and procalcitonin levels, respectively, in the derivation set, thus showing an excellent discriminatory value for the protein combination. Note that the usual positive LHR of CRP or procalcitonin for sepsis diagnosis in ICU patients are consistently lower.[28,29] Finally, it should be emphasized that none of the current sepsis biomarkers reaches perfect diagnostic performance. Consequently, they should always be interpreted according to the clinical conditions.

Expressional proteomics provides quantitative measures and comparisons of proteins between samples.[30] In contrast to studies targeting specific proteins, this technique provides an unselected profile of major proteins present in a plasma sample and an unbiased approach.[4,20] SELDI-TOF Proteinchip mass spectrometry is an easy-to-use proteomic technique with automation and high throughput that has been widely used for the discovery of biomarkers for cancer, viral, or bacterial infection using various biologic samples.[10,31–33] However, methodologic considerations need to be addressed. Because of the experimental design of the technique, combining sample preparation, choice of array, and intrinsic sensibility, the protein profile obtained is restricted, which does not allow an exhaustive analysis of the entire plasma protein spectra.[24] Consequently, the reported analysis is only

a part of the potentially analyzable plasma proteome and could influence our results at least partially.[34] Moreover, the reproducibility of the SELDI-TOF Proteinchip mass spectrometry analysis has been questioned.[35–37] Various parameters may affect reproducibility, such as technical condition of samples collection or conservation, clinical evaluation of the studied phenotype, and treatment.[38,39] In the current study, determination of the phenotype, that is, sepsis or not, was based on strict defined criteria and was confirmed by an independent panel. Doubtful situations have been excluded. We chose microbiologic confirmation of infection because this diagnostic criterion is the best linked with clinically relevant decisions, which is to initiate or not antibiotic therapy. This methodology ensures a very homogeneous population with well-defined criteria for infection. Technically, collection and conservation of the plasma samples were standardized. Samples were processed through a robot for higher technical reproducibility. Finally, to confirm our results and to reduce verification bias, we checked the results obtained in the derivation set in a validation cohort composed of additional patients.[27] All these considerations allow us to be confident in the reliability of our results. From a statistical point of view, it has to be underlined that a P value < 0.05 has been considered for statistical significance particularly in the proteomic analysis. This choice was based on the explanatory design of the study, which was conducted for the screening of potential biomarkers.[40]

Clinical response to infection varies greatly between individuals.[41,42] This clinical response is probably associated with specific cellular processes, which could be responsible for respective modifications of plasma proteome. Consequently, the combination of proteins peaks that we found in septic patients could be at least in part due to differences in the clinical expression of the response to infection. It is noteworthy that one of the "septic" protein (CM10 4152) was also differentially expressed in patients with septic shock *versus* those with nonseptic shock. This result underlines the potential interaction of response to disease and the disease itself in the observed proteomic profile. To date, none of the five proteins peaks have been identified. However, human proteomics studies have already identified various proteins that are differentially expressed in patients with systemic inflammatory response syndrome or sepsis.[30] Complement factor B, haptoglobin, clusterin, 1-B-glycoprotein, complement C4, CRP precursor, plasminogen precursor, and transthyretin precursor have been observed in the plasma of systemic inflammatory response syndrome patients.[30] CM10 4152 was one of the protein peaks of the diagnostic combination and was also differentially expressed in patients with septic shock *versus* those with nonseptic shock. In a human study of plasma proteome after low-dose endotoxin challenge in healthy volunteers, Kasthuri *et al.*[40] found profound changes of plasma proteome with many proteins showing quantitative oxidation, appearance, or disappearance of baseline components. Interestingly, the authors identified a new component at 4154 mass units as the activation peptide of the C1 esterase inhibitor. Resemblance in mass units allowed us to believe that our 4152 protein peak could reasonably be the same peptide. Appearance of this peptide could be related to blood proteolytic degradation occurring in septic patients.[43] Finally, whether the protein combination could have a specific role in sepsis beyond its diagnostic value will be determined after the process of identification.

Among the routine clinical and biologic data, intraoperative transfusion of red-packed cells and SAPS II on ICU admission were associated with occurrence of postoperative sepsis at D5. Among routine clinical and biologic recorded data, very few have been found to be associated with postoperative day 5 sepsis. Among perioperative data, the volume of red-packed cell transfusion was consistently associated with an increased risk of postoperative sepsis. This has been largely suggested already.[44,45] However, the links between transfusion requirement, postoperative infections, and mortality are not fully sorted out.[44,46] SAPS II on admission has also been found to be associated with ulterior occurrence of postoperative sepsis. Measured after surgery, this score is a marker of the intensity of physiologic derangement caused by the surgery. These organ dysfunctions have already been identified as potential risk factors for subsequent sepsis particularly after liver transplantation.[46] At the time of occurrence of sepsis, that is, day 5, none of the usual recorded biologic data were consistently different between patients with or without sepsis. It is noteworthy that leukocyte counts were not different. This underlines that sepsis diagnosis based on routine data can be difficult to determine in liver recipients.

Our study has shown that the combination of protein peaks may have potential as a biomarker for infection in liver recipients with a good diagnostic performance and an excellent discriminatory value. However, it has to be emphasized that for the purpose of our exploratory study, we used highly selected patients. Thus, to confirm the interest of the combination of protein peaks, studies will have to be performed on nonselected ICU patients with pretest probability, that is, prevalence of the disease.[27,32] Because the aim of the study was to look for diagnostic biomarkers of sepsis, the prognostic value of the combination of peaks has not been examined. Besides, because of limited samples of identical bacteria species, the influence of species of bacteria responsible for infection has not been studied. However, one study on bacterial endocarditis has previously suggested that species of bacteria did not influence the serum proteomic signature of infection.[47] After identification of the five protein peaks, diagnostic test using more routine techniques, such as ELISA technique, could be implemented. Once completed, these proteins might have the potential to be developed as routine easy-to-use biomarkers for diagnosing early postoperative sepsis in patients undergoing liver transplantation.

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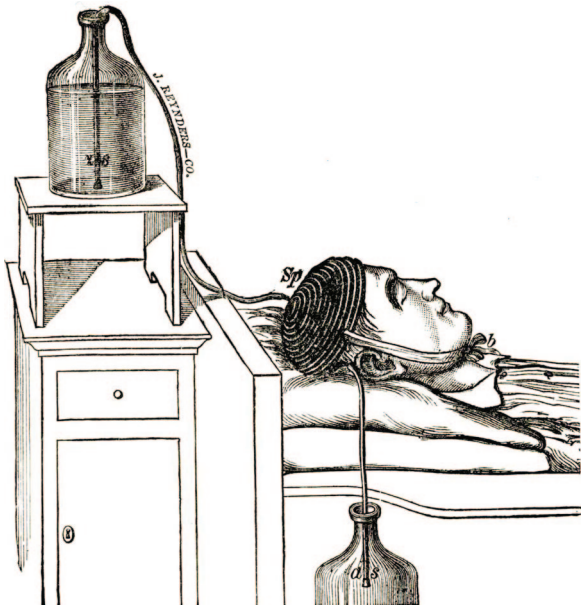
ANESTHESIOLOGY REFLECTIONS

Corning's "Ice-Water Cap" for Congestive Headaches



Many anesthesiologists recognize James Leonard Corning, M.D. (1855–1923, New York neurologist), for having accidentally conducted the first known cocaine spinal anesthetic in 1885 (after inadvertent dural puncture in a dog). Three years later, Corning published the first of eventually three editions of *A Treatise on Headache and Neuralgia*. According to his text, congestive or hyperemic headache produces "severe tensive pain" and "a sensation of fullness, as though the cranium were too small for its contents." As illustrated in his book (*above,* courtesy of the Wood Library-Museum), congestive headache can be treated by "a series of india-rubber spirals, which are wound around the head in the form of a cap. By passing a stream of cold water through these spirals it is possible to maintain the water in them at an exceedingly low temperature." Corning observed that such congestive headaches are "especially prone to occur as the consequence of excessive sexualism and the protracted abuse of alcoholic stimulants." (This image appears in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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