Total Discrimination of Peritoneal Malignant Ascites from Cirrhosis- and Hepatocarcinoma-Associated Ascites by Assays of Ascitic Cholesterol and Lactate Dehydrogenase

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No laboratory test completely distinguishes malignant ascites (MA) from ascites associated with cirrhosis and (or) hepatocellular carcinoma (A/C-HC). Ascitic cytology is highly specific but has a diagnostic sensitivity of only 40–60%. We determined 11 ascitic analytes and cytology in 58 patients with cirrhosis, 15 with hepatocellular carcinoma, and 21 with MA (10 ovarian cancers, 4 mesotheliomas, 6 gastrointestinal neoplasias, 1 leukemia). Ascitic total protein, cholesterol, pseudouridine, and lactate dehydrogenase (LD), and the ascitic:serum ratios of total protein and of LD showed the most significant differences between the two groups of patients. Stepwise multiple linear discriminant analysis (applied the Wilks' lambda criterion) of several variables, corroborated by the "jackknife" reallocation procedure, showed that the ascitic cholesterol and ascitic LD association correctly identified 100% of MA and A/C-HC; cytology had a diagnostic specificity of 100%, but identified only 48% of MA. This association may represent a primary tool for the discrimination of ascites of unknown origin, particularly in the presence of negative cytology findings.

Indexing Terms: liver disease/cancer/cytology/multivariate analysis/receiver-operating characteristic curve

In patients with ascites of unknown origin, distinguishing between malignant ascites (MA) and ascites from cirrhosis and (or) hepatocellular carcinoma (A/C-HC) is a major problem (1–3). Regardless of etiology, ascites associated with hepatocellular carcinoma (HC), which in Italy very frequently derives from cirrhosis, is often referred to as "nonmalignant" (2, 3) because HC is practically never associated with the implant of negative cells in the peritoneum, which is one of the characteristics used to define MA (2, 3).

The formation of MA is frequently caused by peritoneal metastatic tumors, e.g., ovarian, endometrial, and cervical neoplasias in females, and gastrointestinal cancer in males (4, 5). In some cases, MA can also be due to breast cancer, abdominal lymphoma, or a primary peritoneal tumor (2–4). About 10% of MA cases are caused by cancer of uncertain origin (4, 5).

Although cytology is considered the "gold standard" in terms of diagnostic specificity, its diagnostic sensitivity in detecting MA is only 40–60% (3, 6). To increase diagnostic sensitivity, cytologic evaluation has been coupled with the analysis in both serum and ascitic fluid for total protein (7), various enzymes (8), fibronectin (7, 9–11), tumor antigens (12, 13), and lipids (14–16). Diagnostic performance has been enhanced with ascitic analytes plus cytology (16), but complete discrimination between MA and A/C-HC has never been reported.

In an attempt to identify a reliable test to discriminate between MA and A/C-HC, we evaluated ascitic cytology and various biochemical markers in serum and ascitic fluid of patients with ascites arising from cirrhosis, HC, ovarian cancer, gastrointestinal neoplasias, and mesothelioma. We found that a test association of ascitic cholesterol and lactate dehydrogenase (LD) discriminates between MA and A/C-HC with an efficiency of 100%.

Materials and Methods

Patients

We collected ascitic fluid by paracentesis from 94 patients: 58 with liver cirrhosis, 15 with HC, and 21 with peritoneal neoplasia (10 ovarian cancers, 6 gastrointestinal neoplasias, 4 mesotheliomas, and 1 leukemia). The procedures followed in our study were in accordance with the ethical standards of the Ethics Committee of our medical school. Within 2–3 h of sampling, the fluids were processed for cell count and cytology, microbiological assays, and analyses for LD and its isoenzymes, triglycerides, total protein, γ-glutamyltransferase, cholesterol, and pseudouridine. Blood was also collected from the patients and processed for the biochemical analytes as indicated in Results. We obtained a complete clinical history in all cases. Diagnosis was histologically assessed from laparoscopic or surgical biopsies in all patients with MA. Cirrhosis was confirmed by means of laparoscopy or biopsy, except in a low percentage (~20%) of cases in which invasive procedures were contraindicated because of changes in markers of coagulation. HC diagnosis was made by ultrasound-guided fine-needle biopsy. Examination once or twice a year of patients with A/C-HC showed no clinical, instrumental, or laboratory signs of peritoneal metastasis.
Methods

Cytologic examination was performed on pellets of ascitic fluid previously centrifuged (10 min, 3000g) and stained with Giemsa. We used cellulose acetate electrophoresis, followed by colorimetric visualization and densitometric scanning (17), to estimate ascitic and serum LD isoenzymes (materials from Helena Laboratories, Beaumont, TX). We assayed ascitic and serum LD, γ-glutamyltransferase, cholesterol, triglycerides, and total protein with a Hitachi 705 analyzer, using reagents from Boehringer Biochemica (Mannheim, Germany). Ascitic pseudouridine was analyzed with a previously described HPLC procedure (18, 19).

Statistics

Univariate statistical analysis was performed with the nonparametric Mann–Whitney U-test to compare the mean values of the various analytes of the two groups. Diagnostic sensitivity and specificity, and positive and negative predictivity, were calculated according to Galen and Gambino (20); cutoff values were determined with the receiver-operating characteristic (ROC) curve procedure (21).

Multivariate analysis was based on stepwise multiple linear discriminant analysis with the Wilks’ lambda criterion (ratio of within-groups sum of squares to total sum of squares) (22). Before the discriminant analysis, the fitting of the variable distribution to a gaussian distribution was checked by the Kolmogorov–Smirnov test and by skewness and kurtosis evaluation. In all cases, the Kolmogorov–Smirnov test did not show any significant deviation from gaussian; skewness, if significant, was successfully corrected by using natural logarithms or square roots (23).

We examined collinearity between any pair of variables by calculating the Pearson correlation matrix. Where the correlation coefficient for a pair of variables exceeded 0.70, one of the pair would be excluded from the discriminant analysis. The reallocation procedure was as follows: For each subject, the discriminant score was estimated by substituting the observed numerical value of the variables, selected by the discriminant analysis, into the discriminant function. We calculated the posterior probability for the appropriate diagnostic group—MA or A/C-HC—with Bayes’ rule, using the estimated discriminant score after adjusting the posterior probability with reference to the prior probability. The prior probability was calculated as the proportion of cases in the analysis that fell into each group. Each individual was then classified in the group with the higher bayesian probability. The diagnostic sensitivity and specificity of the discriminant function obtained were determined by the two-way table compiled by this reallocation procedure. Because this simple procedure may overestimate the accuracy of correct discrimination of another independent sample of patients, we tested the reliability of discriminant analysis by the reallocation jackknife algorithm, which removes much of the bias of the simple reallocation method (24).

Results

Cytologic evaluation of ascites was negative in all cases of cirrhosis and HC. The difference between cirrhosis and HC patients was not significant (Mann–Whitney U-test) for any serum and (or) ascitic biochemical analyte except ascitic pseudouridine (P <0.001). These data confirm that ascites associated with HC or with cirrhosis may be grouped together for this type of study (10). Therefore, in accordance with accepted usage (2, 10), we divided the patients into two groups: A/C-HC patients and MA patients.

We compared the mean values of several serum analytes (total protein, cholesterol, triglycerides, γ-glutamyltransferase, and LD and its isoenzymes) in the two groups with the Mann–Whitney U-test. The only difference was a significantly lower concentration of serum cholesterol in the A/C-HC group (2.92 ± 2.02 mmol/L vs 4.42 ± 3.13 mmol/L; P <0.001).

Microbiological analysis of ascites gave negative results for all patients. The analytes indicated in the previous paragraph, plus pseudouridine, were determined in ascitic fluid samples from the two groups of patients. The ascitic:serum ratios of total protein and LD were also calculated. The most significant differences (P <0.0005 at least) between the mean values of the analytes in the two groups of patients were found for total protein, cholesterol, LD, and pseudouridine, and for the ascitic:serum ratios for total protein and for LD (Table 1). Triglycerides, γ-glutamyltransferase, the LD isoenzymes, and cell count (mean 850 × 10⁶/L for A/C-HC vs 950 × 10⁶/L for MA) were less significant or not significant.

<table>
<thead>
<tr>
<th>Marker</th>
<th>MA</th>
<th>A/C-HC</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein, g/L</td>
<td>n: 17</td>
<td>Mean: 57.12</td>
<td>SE: 3.8</td>
</tr>
<tr>
<td>Total protein, A:S</td>
<td>n: 9</td>
<td>Mean: 0.75</td>
<td>SE: 0.10</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>n: 21</td>
<td>Mean: 3.14</td>
<td>SE: 0.24</td>
</tr>
<tr>
<td>Pseudouridine, µmol/L</td>
<td>n: 10</td>
<td>Mean: 7.48</td>
<td>SE: 0.72</td>
</tr>
<tr>
<td>LD, U/L</td>
<td>n: 21</td>
<td>Mean: 823</td>
<td>SE: 107</td>
</tr>
<tr>
<td>LD, A:S</td>
<td>n: 9</td>
<td>Mean: 3.85</td>
<td>SE: 0.96</td>
</tr>
</tbody>
</table>

* Mann–Whitney U-test.

A:S, ascitic:serum ratio.
Within the MA group, the Mann–Whitney U-test indicated that no serum or ascitic analyte was significantly different between the positive (10 of 21 = 48%) and the negative cytology subgroups. This confirms that, in MA patients, positive cytology does not distinguish between subgroups that show different biochemical characteristics.

Figure 1 shows the scattergrams of some analytes used to discriminate between MA and A/C-HC. Panels A, B, and C, respectively, refer to ascitic LD, ascitic cholesterol, and ascitic total protein. The two A/C-HC subgroups, namely HC and cirrhosis, overlap widely in all three instances. Ascitic pseudouridine (Fig. 1D), however, differs significantly among the three groups of patients (HC, cirrhosis, and MA).

Figure 2 shows the ROC plots for each of the variables listed in Table 1. Interestingly, neither in our study nor in other reports (2, 3, 7–9, 11, 12, 14–16) did diagnostic sensitivity and specificity together reach a diagnostic efficiency of 100%. Cytologic results were negative in all cases of A/C-HC and positive in 10 of 21 (48%) cases of MA; therefore, also in this instance the overall diagnostic efficiency did not reach 100%. Consequently, we decided to use a multivariate approach (see Materials and Methods), the stepwise linear discriminant analysis based on Wilks’ lambda criterion (22), to improve the diagnostic performances of the biochemical analytes that were most efficient in the discrimination between A/C-HC and MA, i.e., those in which the mean differences between the two groups were statistically significant with \( P < 0.0001 \) at least (see Table 1).

We constructed a first discriminant mathematical function with which to differentiate between MA and A/C-HC, using a subset of 67 cases (9 MA and 58 A/C-HC) for which we had the values of all the variables (ascitic total protein, cholesterol, and LD; and ascitic: serum ratios for both total protein and LD) in which the mean values differed significantly (at least \( P < 0.0001 \)) between the two groups. After calculating the correlation matrix, we eliminated the ascitic:serum ratio of total protein from subsequent analyses because the Pearson correlation coefficient with ascitic total protein exceeded 0.7 (see Statistics). Thus, among the four remaining variables, the Wilks’ discriminant analysis selected two variables for the discriminant function: ascitic cholesterol and ascitic LD, expressed in ln scale. The corresponding equation is:

\[
\text{discriminant score} = \ln \text{ascitic cholesterol} (\text{mmol/L})
\times 0.539 + \ln \text{ascitic LD (U/L)} \times 1.638 - 7.677 \quad (1)
\]

For the set of 67 patients, the above discriminant score produced an overall diagnostic efficiency (i.e., correct classification of all patients belonging to the two groups) of 100% when the Bayes’ classification rule was applied. To validate the procedure, we applied the jackknife re-allocation algorithm to the results for all 67 patients (see Statistics); the algorithm verified that all cases were correctly classified.

To further validate and extend the discriminant function, we classified on the basis of Eq. 1 the other 27
cases, 12 MA and 15 A/C-HC, for which the values of some analytes were missing, but for all of which ascitic cholesterol and ascitic LD were available. Again, with the bayesian rule, all cases were correctly classified (diagnostic efficiency = 100%).

Transforming the ascitic cholesterol and ascitic LD values to their natural logarithms, we calculated (including all 94 cases) a final discriminant function:

\[
\text{discriminant score} = \ln \text{ascitic cholesterol (mmol/L)}
\times 0.799 + \ln \text{ascitic LD (U/L)} \times 1.459 - 7.119 \quad (2)
\]

This function correctly classified all 94 cases, applying Bayes’ rule with a prior probability of 1:3.5, for MA and A/C-HC. The cutoff value of the discriminant score (corresponding to a bayesian probability of 0.50) was 1.39 (see Fig. 3). Furthermore, also for Eq. 2, we repeated the jackknife reallocation algorithm on the total set of 94 patients and confirmed the overall diagnostic efficiency of 100%.

Figure 4 is a ln/ln plot of ascitic cholesterol vs ascitic LD concentration for all 94 cases. In this format, the capability of the two variables to discriminate completely between the two sets of patients is readily appreciable.

Discussion

We report an association of two ascitic analytes that completely discriminates between MA and A/C-HC. No one of the analytes used so far to distinguish MA from A/C-HC has a 100% diagnostic efficiency (7–16).

Our data confirm the high diagnostic specificity (100%) and the low diagnostic sensitivity of cytology in distinguishing MA from A/C-HC (3, 7, 8, 12, 14, 16). This lack of sensitivity may be due to the low number of neoplastic cells present in some ascitic samples, or to the low number of cells collected in the sample (1, 4–6). False-negative results can also be caused by the difficulty in distinguishing between neoplastic and atypical inflammatory cells (1, 4, 10).

Patients affected by cirrhosis and HC show lower concentrations of ascitic cholesterol than do patients affected by MA (14–16). Our data confirm lower ascitic cholesterol concentrations in A/C-HC than in MA (mean 0.71 vs 3.13 mmol/L) but, contrary to the report of Collerodo Mels et al. (10), ascitic cholesterol did not differentiate between positive and negative cytology in MA patients. Increased cholesterol in ascitic fluid of MA patients has been attributed to increased vascular permeability (25), increased cholesterol synthesis (26), and release from neoplastic cells (26, 27). Contrary to a previous report (15), our MA patients had higher concentrations of serum and ascitic cholesterol than did A/C-HC patients. This suggests that the increased ascitic cholesterol observed in MA could be derived directly from serum. Similarly, our results contrast with the hypothesis of a blockage of lymphatic drainage (15), because ascitic triglyceride concentrations did not differentiate between MA and A/C-HC patients. This finding is in agreement with Collerodo Mels et al. (10), but differs from the results of Jungst et al. (14).

The diagnostic specificity previously reported (2, 7, 9, 11, 14, 16) for ascitic total protein in MA vs A/C-HC varies widely (between 68% and 97%); in particular, as many as 25% false-positive results have been reported in chronic liver diseases (14). These different specificities could be due in part to the cutoff values used. In our experience, even when we used a cutoff higher (35 g/L) than those used by others (2, 7, 9, 11, 14, 16), the positive predictivity at the prevalence used in our study was only 73%. Moreover, serum total protein values were roughly normal in both MA and A/C-HC patients. Therefore, the ascitic:serum ratio of total protein did not
improve discrimination between MA and A/C-HC. These findings suggest that the higher amounts of ascitic total protein observed in MA with respect to A/C-HC (2, 7, 9, 11, 14, 16) were due to increased vascular membrane permeability, which, in turn, could be due to the production in blood of soluble factors from tumors. This mechanism has been demonstrated in rat experimental ascites (6). The large number of false-positive results obtained for A/C-HC with ascitic total protein could be caused by factors produced by inflammatory cells (28). A diagnostic sensitivity of 100% and a diagnostic specificity of 96% have been reported for the serum/ascites albumin gradient (11); however, this signal may be primarily due to portal hypertension (29, 30).

Pseudouridine is a modified nucleoside derived mainly from tRNA catabolism (31, 32). It cannot be reutilized by the cell and so is eliminated via urine. When RNA turnover is increased, as during neoplastic growth and human immunodeficiency virus infection, serum and urine concentrations of pseudouridine increase (33). In the present study, the first report of ascitic pseudouridine concentrations in patients with MA and A/C-HC, mean pseudouridine concentrations in A/C-HC were significantly lower than in MA (P < 0.0005). However, data for the different groups of patients overlap widely; thus, ascitic pseudouridine is less useful for the discrimination of neoplastic ascites of unknown origin.

Serum LD activity is enhanced in various neoplasias. The efficiency of both ascitic LD and the ascitic-serum LD ratio in the differential diagnosis of ascites of unknown origin is reportedly satisfactory (8, 16, 29). In our study, the serum LD concentration was similar in MA and in A/C-HC patients, whereas ascitic LD was lower in A/C-HC than in MA (104 vs 823 U/L). Therefore, in contrast to earlier reports (16, 29), the use of the ascitic-serum LD ratio did not improve the discrimination between MA and A/C-HC. Furthermore, the dissociation of the increase in serum and ascitic LD suggests that the increased ascitic LD values in MA originate mainly from neoplastic cells implanted in the peritoneal membrane. A wide range of diagnostic sensitivities and specificities have been reported for ascitic LD in discriminating MA from A/C-HC (8, 16, 29). This variation is probably due in part to the different cutoff values selected in the various studies and in part to the methods used for LD enzyme assay (pyruvate or lactate as substrates).

Taken together, these findings indicate that the single analytes estimated in this paper, be they in serum or in ascitic fluid, are not sufficient to discriminate with complete efficiency between MA and A/C-HC. With the multivariate stepwise discriminant approach, we found that the association of noncorrelated variables that best discriminated between MA and A/C-HC was ascitic cholesterol and ascitic LD. Each of these analytes singly had a high diagnostic performance, but in association their diagnostic performance improved because, as described above, their increase is due to different biochemical mechanisms. We verified by various statistical procedures the excellent discriminatory efficiency of this test association and consistently found that all MA and A/C-HC patients were correctly classified (100% diagnostic efficiency).

In conclusion, being based on only two noninvasive laboratory assays, the clinical biochemistry approach described here is very promising for the evaluation of ascites of unknown origin, particularly when the cyologic result is negative, because the two analytes are increased in MA of both positive and negative cytologic findings. However, because this study was conducted in only one cohort of patients, our results should be verified by other clinical institutions.

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References