Some understanding of diagnostic tests for pulmonary embolism

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CHAPTER 3

Use of a new monoclonal antibody-based enzyme immunoassay for soluble fibrin to exclude pulmonary embolism

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Summary

We prospectively evaluated the diagnostic performance of a new soluble fibrin assay in 303 consecutive patients with suspected pulmonary embolism and examined potentially useful cut-off levels at which this disease can be safely excluded. In addition, the diagnostic accuracy was calculated in the subgroups of in- and outpatients. The ROC curve of the assay in the total study cohort had an area under the curve of 0.69. The cut-off level associated with a sensitivity and negative predictive value of 100% was 20 ng/ml, but the specificity was only 4%. The cut-off level with a sensitivity of 90% was 30 ng/ml, which corresponded with a specificity and negative predictive value of 27% and 86%, respectively. The diagnostic performance was comparable in the subgroups of in- and outpatients. We conclude that the soluble fibrin assay has a low diagnostic accuracy and seems unsuitable as a screening test for the exclusion of pulmonary embolism.
Introduction

Soluble fibrin is produced when thrombin sequentially removes the fibrinopeptides A and B from the amino-terminal sections of the $\alpha\alpha$- and $\beta\beta$- chains of fibrinogen, yielding desAA- and desAABB fibrin monomers. These monomers display a tendency to polymerize because the new amino-terminal sections serve as the A- and B-polymerization sites that interact with the $\alpha$- and $\beta$- polymerization sites in the carboxyl-terminal D-domains of fibrinogen molecules (1). At a low concentration, fibrin monomers will be kept in solution by complexing with the relative excess of fibrinogen. These complexes are designated as “soluble fibrin” and elevated plasma levels reflect an increased thrombin activity (2, 3).

There is evidence that patients with pulmonary embolism have increased thrombin generation (4). Hence, it is conceivable that assays measuring soluble fibrin could potentially be useful in the diagnostic work-up of patients with clinically suspected pulmonary embolism. It has been shown that normal levels of soluble fibrin can be used for the exclusion of this disease (5, 6). Recently, a new quantitative enzyme immunoassay for the measurement of plasma levels of soluble fibrin has been developed (7). The assay was found to be specific (no interference with homologous antigens, such as fibrin(ogen) degradation products) and reproducible (intra-assay variation 4-8%, inter-assay variation 4-9%) (7). Therefore, this new assay is suitable for measuring soluble fibrin in patient plasma. However, it is presently unknown whether this assay has potential clinical utility for the exclusion of pulmonary embolism in a real clinical setting. This provided the impetus for the present study.

We sought to determine the diagnostic accuracy of this soluble fibrin assay in a large cohort of consecutive patients with clinically suspected pulmonary embolism and to examine potentially useful cut-off levels of soluble fibrin concentration at which pulmonary embolism can be excluded. In addition, we assessed the diagnostic accuracy of the assay in in- and outpatients separately.

Patients and Methods

Patients

The study was performed within the framework of a large multi-center study which evaluated various diagnostic methods for pulmonary embolism. From June 1997 through March 1998, consecutive in- and outpatients presenting in six Dutch teaching hospitals with clinically suspected pulmonary embolism were eligible. Patients were excluded if they were younger than 18 years of age, were pregnant, had already undergone objective diagnostic investigations for pulmonary embolism, had an indication for thrombolytic therapy, or if there was an expected inability to complete the diagnostic protocol within 48 hours. The study
protocol was approved by the Institutional Review Boards of all hospitals and written informed consent was obtained from all participating patients.

**Diagnostic Investigations**

All patients underwent a six-view perfusion lung scintigraphy, using 100 Mbq of $^{99m}$Technetium-labelled macroaggregates of albumin, within 24 hours of presentation. Ventilation lung scintigraphy was performed, using $^{81m}$Krypton gas, if at least one segmental or larger perfusion defect was seen. Ventilation-perfusion lung scans were classified according to previously described criteria (8) as being normal (no perfusion defects), high probability (at least one segmental or larger perfusion defect with locally normal ventilation) or non-diagnostic (ventilation-perfusion defects that did not qualify as high probability). Pulmonary angiography was indicated in all patients with a non-diagnostic lung scan. The maximum allowed time interval between the diagnostic investigations was 24 hours, although the diagnostic process was usually completed in one day.

The lung scans were interpreted by a panel of experienced nuclear medicine physicians and final conclusions were reached by consensus. The angiograms were interpreted independently by two radiologists and in case of disagreement, the interpretation of a third was decisive. Patients were classified as having pulmonary embolism on the basis of a high probability lung scan or an abnormal pulmonary angiogram and the disease was considered absent in case of a normal perfusion scan or a normal angiogram.

**Laboratory Investigations**

Upon study inclusion, and prior to or within 24 hours of the start of heparin therapy, 4.5 ml venous blood was drawn into a vacutainer tube (Becton Dickinson, New Jersey; USA) containing 1 ml of 0.105 M sodium citrate solution. Plasma was immediately centrifuged at 2500 g for 15 minutes at 4°C. The plasma was then aliquoted into plastic tubes, snapfrozen and stored at -80°C. Samples were thawed only once for batch-wise analysis at the end of patient inclusion.

The plasma levels of soluble fibrin were measured, using a described enzyme immunoassay procedure (7), by laboratory technicians who were unaware of the final diagnosis regarding the presence or absence of pulmonary embolism. Soluble fibrin levels are expressed as fibrinogen equivalent units (FEU) per ml. The assay is based on a monoclonal capture antibody directed against a neo-epitope ($\gamma312-324$) in the gamma chain of fibrinogen. This neo-epitope is not accessible to anti-$\gamma312-324$ in native fibrinogen, but becomes exposed upon fibrin polymerization. The horse-radish peroxidase labeled monoclonal antibody (G8), with its epitope in the carboxyl-terminal section of intact $\alpha$-chains of soluble fibrin, serves as the tagging antibody. Since the latter epitope is degraded early in the course of plasmin-mediated proteolysis of fibrin and fibrinogen, the assay is specific for nonplasmin-degraded soluble fibrin.
Statistical Analysis
The Mann-Whitney-U test was used to calculate the significance of difference between the median plasma levels of soluble fibrin in patients with and without pulmonary embolism. The diagnostic accuracy of the assay was calculated in the total study cohort and in in- and outpatients separately using Receiver Operated Characteristics (ROC) analysis and expressed as the area under the ROC curve (AUC). The AUCs and their 95% confidence intervals (CI) were calculated with a non-parametric method (9). An ideal test providing a sensitivity and specificity of 100% generates an AUC of 1, while a fully non-informative test generates an AUC of 0.5.

We examined the cut-off levels which had a sensitivity of 90% or above in the total study cohort. The sensitivity, specificity, negative predictive value and the exclusion efficiency, defined as the proportion of patients with a “normal” soluble fibrin result, were calculated for these cut-off levels using standard methods. The corresponding 95% confidence intervals were calculated according to the binomial distribution.

Two-tailed p-values of less than 0.05 were regarded as statistically significant. Analyses were performed with statistical software (SPSS; version 9.0).

Results
A total of 807 consecutive patients with clinically suspected pulmonary embolism were screened during the study period. Of these patients, 130 were excluded on the basis of one of the predefined exclusion criteria. Hence, a total of 677 patients were eligible for inclusion in the study of whom 440 (65%) gave informed consent. A final diagnosis regarding the presence or absence of pulmonary embolism was not reached in 70 of the 440 included patients due to withdrawal of informed consent, clear evidence of an alternative diagnosis that explained the presenting symptoms, or technical failure. In 67 other patients, blood samples were not collected due to logistic reasons. The study cohort therefore consisted of 303 patients of whom the clinical characteristics are shown in Table 3.1.

Of the 303 study patients, 94 (31%) were classified as having pulmonary embolism. Seventy-nine of these 94 patients had a high probability lung scan and 15 patients had an abnormal angiogram. The diagnosis of pulmonary embolism was refuted in the remaining patients on the basis of a normal perfusion scan in 129 patients and a normal angiogram in 80 patients.

The median soluble fibrin level in patients with pulmonary embolism (49 ng FEU/ml, interquartile range 39-67) was significantly higher than in patients without the disease (37 ng FEU/ml, interquartile range 29-52) (p<0.001), albeit the respective interquartile ranges showed a large overlap. The ROC curve of the
Table 3.1 Clinical characteristics of the 303 study patients with clinically suspected pulmonary embolism

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>121</td>
<td>(40%)</td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>50</td>
<td>(18)</td>
</tr>
<tr>
<td>Outpatients, n (%)</td>
<td>243</td>
<td>(80%)</td>
</tr>
<tr>
<td>Median duration of symptoms, days (interquartile range)</td>
<td>3</td>
<td>(1-11)</td>
</tr>
<tr>
<td>Previous VTE, n (%)</td>
<td>51</td>
<td>(17%)</td>
</tr>
<tr>
<td>Family history of VTE, n (%)</td>
<td>63</td>
<td>(21%)</td>
</tr>
<tr>
<td>Risk-period for VTE, n (%)</td>
<td>103</td>
<td>(34%)</td>
</tr>
<tr>
<td>Active malignancy, n (%)</td>
<td>27</td>
<td>(9%)</td>
</tr>
</tbody>
</table>

1 venous thromboembolism
2 period of immobilization, surgery or trauma in period of 3 months before presentation

Soluble fibrin assay in the total study cohort had an AUC of 0.69 (95% CI 0.63-0.74) (Fig. 3.1). The accuracy indices and the exclusion efficiency of the soluble fibrin assay at several examined cut-off levels are shown in Table 3.2. The cut-off level associated with a sensitivity (and negative predictive value) of 100% was 20 ng FEU/ml and corresponded with a specificity of 4%. Pulmonary embolism could be accurately excluded on basis of soluble fibrin levels of ≤ 20 ng FEU/ml in only 9 (3%) of the 303 symptomatic patients. The cut-off level giving a sensitivity of 90% was 30 ng FEU/ml and corresponded with a specificity of 27%. Of all study patients, 66 (22%) patients had soluble fibrin levels below this cut-off level of whom 9 were classified as having pulmonary embolism.

Table 3.2 Accuracy indices and exclusion efficiency at several cut-off levels of soluble fibrin in the total study cohort

<table>
<thead>
<tr>
<th>Cut-off level (ng FEU/ml)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>NPV (95% CI)</th>
<th>Exclusion efficiency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 20</td>
<td>100% (96-100)</td>
<td>4% (2-8)</td>
<td>100% (66-100)</td>
<td>3% (1-5)</td>
</tr>
<tr>
<td>≤ 22</td>
<td>99% (95-100)</td>
<td>8% (6-8)</td>
<td>94% (70-100)</td>
<td>6% (3-8)</td>
</tr>
<tr>
<td>≤ 24</td>
<td>96% (90-99)</td>
<td>11% (8-12)</td>
<td>85% (65-95)</td>
<td>9% (5-12)</td>
</tr>
<tr>
<td>≤ 26</td>
<td>93% (86-97)</td>
<td>15% (12-17)</td>
<td>82% (66-92)</td>
<td>13% (9-16)</td>
</tr>
<tr>
<td>≤ 28</td>
<td>92% (85-96)</td>
<td>19% (16-21)</td>
<td>83% (70-92)</td>
<td>16% (11-20)</td>
</tr>
<tr>
<td>≤ 30</td>
<td>90% (83-95)</td>
<td>27% (24-29)</td>
<td>86% (76-93)</td>
<td>22% (17-26)</td>
</tr>
</tbody>
</table>

NPV = negative predictive value
The assay generated a slightly larger AUC in the subgroup of outpatients (0.70, 95% CI 0.64-0.76) than in the subgroup of inpatients (0.60, 95% CI 0.46-0.72), but the difference did not reach statistical significance (p=0.19).

A small proportion of the study patients (5%) were already receiving intravenous heparin therapy at the time the blood samples were obtained. Reanalysis of the data after exclusion of these patients did not change our results significantly (data not shown).

**Discussion**

This prospective study in a large series of consecutive patients with clinically suspected pulmonary embolism shows by means of ROC analysis that the soluble fibrin assay has a disappointing low diagnostic accuracy (AUC 0.69, 95% CI 0.63-0.74). At a cut-off level of 20 ng FEU/ml, the assay had a sensitivity of 100%, but the specificity was poor at only 4%. The low specificity corresponding with this cut-off level would limit the clinical utility of the assay, since almost all patients would then have an "abnormal" soluble fibrin result and thus still require further diagnostic investigations. By adapting the cut-off level to 30 ng FEU/ml, the sensitivity decreased to 90%, while the specificity increased to 27%. Although the exclusion efficiency, i.e. the proportion of patients with a normal soluble fibrin test result, increased to 22% by applying this cut-off level, pulmonary embolism was still present in a large proportion (14%) of these patients. The diagnostic accuracy of the assay was not different in the subgroups of in- and outpatients.
These results imply that the assay is unsuitable as a screening test for the exclusion of pulmonary embolism.

What is a potential explanation for the low diagnostic accuracy of the soluble fibrin assay? We hypothesize that the specific measurement of nonplasmin-degraded soluble fibrin may have contributed to the low diagnostic accuracy of the assay. The actual soluble fibrin production may be underestimated by the assay in patients with pulmonary embolism, since these patients usually have an increased fibrinolysis (10). This may negatively affect the sensitivity of the assay for the diagnosis of pulmonary embolism. In addition, the measured plasma levels of nonplasmin-degraded soluble fibrin in patients with pulmonary embolism may tend to overlap with the levels in patients without this disease, thereby decreasing the specificity of the assay. This hypothesis finds support in the present study. Although the median plasma level of soluble fibrin in the study patients with pulmonary embolism was higher than in the study patients without the disease, the respective levels were low and the corresponding interquartile ranges showed a large overlap. Hence, it appears that nonplasmin-degraded soluble fibrin is a less useful biochemical plasma marker to discriminate between patients with and without pulmonary embolism.

The present study is the first that has prospectively evaluated the diagnostic accuracy of this particular soluble fibrin assay in a large cohort of patients with clinically suspected pulmonary embolism. Our findings are partly discrepant with those of a retrospective study of Bos et al. (7) in which the same assay was used. The soluble fibrin levels found in the 81 apparently healthy volunteers in their study (median 40 ng FEU/ml, 10-90 percentiles 26-59) are in agreement with the respective levels for patients without pulmonary embolism in our study. However, the soluble fibrin levels in a small group of 29 patients with pulmonary embolism in their study (median 527 ng FEU/ml, 10-90 percentiles 84-1234) were much higher than in our study patients with this disease. The reasons for this considerable difference are not clear. We introduced no difference in the enzyme immunoassay procedure or the collection and preparation of blood samples. A potential explanation may be the relatively small number of patients with pulmonary embolism in the study of Bos et al. as compared with our study. The very wide range of soluble fibrin levels in their patients with pulmonary embolism appears to be a reflection of this small sample size. Another plausible explanation could be the retrospective design of their study which may have caused selection bias and thereby a difference in the spectrum of patients studied. Ginsberg et al. (5) evaluated a slightly different soluble fibrin assay, employing the same capture antibody (anti-γ312-324) but with another pan-specific tagging antibody (DD-4D2/182), in 195 unselected patients with clinically suspected pulmonary embolism. At a sensitivity of 100% (95% CI 88.8-100), the specificity for pulmonary embolism was also low at 12.8% (95% CI 7.7-17.9). Reber et al. (6) also used another assay, the Enzymun-Test FM®, to quantify the plasma levels of
fibrin monomers in 426 patients with clinically suspected pulmonary embolism. At a sensitivity of 100% (95% CI 97.1-100), the specificity for pulmonary embolism was 32.8% (95% CI 25.7-38.1). Differences in sample handling and the employed antibody may explain the more favourable diagnostic accuracy of that assay. The Enzymun-Test FM® is performed after sample pretreatment with thiocyanate solution, which exposes the epitopes for the employed monoclonal antibody (MoAb 2B5) in nearly all soluble fibrin complexes (11). It has further been suggested that these epitopes probably remain intact and thus detectable in plasmin-degraded crosslinked fibrin fragments (6). This might also explain the very high soluble fibrin levels measured with the Enzymun-Test FM®.

We conclude that the evaluated soluble fibrin assay has a low diagnostic accuracy in our study cohort and seems unsuitable as a screening test for the exclusion of pulmonary embolism.

References
