Quantification of Free Circulating DNA As a Diagnostic Marker in Lung Cancer

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Purpose: Analysis of circulating DNA in plasma can provide a useful marker for earlier lung cancer detection. This study was designed to assess the sensitivity and specificity of a quantitative molecular assay of circulating DNA to identify patients with lung cancer and monitor their disease.

Materials and Methods: The amount of plasma DNA was determined through the use of real-time quantitative polymerase chain reaction (PCR) amplification of the human telomerase reverse transcriptase gene (hTERT) in 100 non-small-cell lung cancer patients and 100 age-, sex-, and smoking-matched controls. Screening performance of the assay was calculated through the receiver operating characteristic (ROC) curve. Odds ratios were calculated using conditional logistic regression analysis.

Results: Median concentration of circulating plasma DNA in patients was almost eight times the value detected in controls (24.3 ± 3.1 ng/mL). The area under the ROC curve was 0.94 (95% CI, 0.907 to 0.973). Plasma DNA was a strong risk factor for lung cancer; concentrations in the upper tertile were associated with an 85-fold higher risk than were those in the lowest tertile.

Conclusion: This study shows that higher levels of free circulating DNA can be detected in patients with lung cancer compared with disease-free heavy smokers by a PCR assay, and suggests a new, noninvasive approach for early detection of lung cancer. Levels of plasma DNA could also identify higher-risk individuals for lung cancer screening and chemoprevention trials.

LUNG CANCER is the leading cause of cancer mortality throughout the world and is the cause of more than 1 million annual deaths. In Europe, of the more than 150,000 new patient cases diagnosed every year, only 10% can be cured and can benefit from long-term survival because of the absence of early detection plans, the frequency of metastases at diagnosis, and poor responsiveness to chemotherapy. However, survival of patients undergoing lung resection for small intrapulmonary cancers is greater than 80%. Despite major potential for prevention, complete eradication of smoking has proven difficult, and the risk of cancer remains high in former smokers. As a consequence, there is a need to develop new tests that may facilitate earlier diagnosis and more effective treatment. Low-dose spiral computed tomography (CT) scan of the chest has been effective in detecting small tumors, with a high proportion of resectable (96%) and stage I (80%) disease. Conversely, increased knowledge of molecular pathogenesis of lung cancer offers a basis for the use of molecular markers in biologic fluids for early detection as well as identification of higher-risk smokers.

Common genetic alterations in lung carcinogenesis include allelic loss and instability at loci on 3p (fragile histidine triad [FHIT]), 9p (p16 INK4A), and 17p (p53); aberrant promoter methylation of p16 INK4A, APC (adenomatous polyposis of the colon), and other tumor suppressor genes; and Kirsten rat sarcoma (KRAS)10 and p53 mutations. Detection of these changes in DNA derived from body fluids such as sputum, bronchial brush and lavage, and plasma or serum of lung cancer patients and chronic smokers has been proposed by several authors as a potential diagnostic tool. However, the sensitivity and specificity of detection assays in these biologic samples have been limited by the low frequency of alterations of each specific gene, relative low-sensitivity of used methodologies, and choice of appropriate markers.

Analysis of circulating DNA in plasma is a promising noninvasive diagnostic tool, requiring only a limited blood sample. The intent of this study was to set up a relatively simple blood test on the basis of a single marker, to be potentially applicable to large-scale trials for early lung cancer detection. In a previous report, using a DNA colorimetric assay, we have shown a higher plasma DNA concentration in 84 lung cancer patients than in 43 controls, regardless of tumor stage, suggesting that plasma DNA was an early event in lung carcinogenesis. In addition, changes in DNA level and in the presence of allelic imbalances at 3p loci correlated with the clinical status of patients during follow-up. These results were recently confirmed in a group of 54 patients with ovarian cancer and 31 controls, for whom plasma DNA concentrations and digital single-nucleotide polymorphism analy-
ysis of allelic imbalances were proposed as screening tools for ovarian cancer.26

To measure with greater accuracy the amount of free circulating DNA, a quantification approach based on real-time quantitative polymerase chain reaction (PCR) was developed. Using Epstein-Barr virus DNA as the target genome, real-time PCR has proven effective to monitor the progress of nasopharyngeal cancer and assess the effects of treatment.27 A single copy gene, the amplification of which is specific and robust, represents the ideal target for DNA-based quantitative real-time PCR assay. For this study, we selected an assay designed for the human telomerase reverse transcriptase (hTERT) genomic sequence that performed consistently in preliminary experiments. Amplification of hTERT was therefore used as a marker of the total amount of DNA present in plasma samples. We considered that hTERT expression and telomerase activity have been reported as prognostic factors in stage I non–small-cell lung cancer (NSCLC) patients.28 However, our working hypothesis was not based on the evaluation of hTERT expression at the transcriptional level as a tumor-associated marker, but was based instead on the use of a single copy gene such as hTERT as an indicator of the global amount of circulating DNA.

The sensitivity and the specificity of the test were validated in a large case-control study of 200 individuals and in a group of age-matched individuals who had never smoked.

**MATERIALS AND METHODS**

**Patients and Control Series**

We evaluated 100 consecutive patients with NSCLC, 81 men and 19 women, who were not previously treated with chemoradiotherapy and were included in the European Institute of Oncology tissue bank from 2000 to 2001. All patients had primary cancers and were receiving first treatment; no patients with disease relapse or follow-up were included.

One control was selected for each patient, matched by sex, age, and smoking habits. Mean age ± standard deviation was 65.1 ± 8.9 years in patients and 64.1 ± 8.2 years in controls; average smoking duration was 40.5 ± 10.9 years in patients and 41.7 ± 9.5 years in controls. The population included seven case-control pairs of never smokers. The 93 years or older who were current or former smokers with a minimum pack/yr smoking habits. Mean age ± standard deviation was 65.1 ± 8.9 years in patients and 64.1 ± 8.2 years in controls; average smoking duration was 40.5 ± 10.9 years in patients and 41.7 ± 9.5 years in controls. The population included seven case-control pairs of never smokers. The 93

**Sample Collection and DNA Isolation**

A 7.5-mL sample of peripheral blood was collected in tubes containing EDTA, from patients before surgery and from controls at the time of spiral CT examination, and stored at −140°C. Plasma separation and DNA extraction were performed as previously reported.28 The DNA purified from 1 mL of plasma was eluted in a final volume of 50 mL of water. Testing of plasma DNA was performed by technicians with no knowledge of the patient or control status.

**DNA Quantification in Plasma**

To quantify the circulating DNA in plasma, we used a real-time quantitation PCR approach based on the 5' nucleotide method. This methodology is based on continuous monitoring of a progressive fluorescent PCR by an optical system.29,30 The PCR system uses two amplification primers and an additional amplicon-specific and fluorogenic hybridization probe, the target sequence of which is located within the amplicon. The probe is labeled with two fluorescent dyes. One serves as a reporter on the 5' end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is quenched by a second fluorescent dye at the 3' end (Tamra; Applied Biosystems). If amplification occurs, the 5' to 3' exonuclease activity of the AmpliTaq (Applied Biosystems) DNA polymerase cleaves the reporter from the probe during the extension phase, thus releasing it from the quencher.31 The resulting increase in fluorescent emission of the reporter dye is monitored during the PCR process.

Primers and probes were designed to specifically amplify the ubiquitous gene of interest, the hTERT single copy gene mapped on 5p15.33 The amplicon size of the hTERT gene was 98 bp (position 13059 to 13156, GenBank accession number AF128893). The sequences of the primers and of the probe were the following: primer forward, 5'-GGC ACA CGT GGC TTT TCG-3'; primer reverse, 5'-GTT GAA CCT CGT AAG TTT ATG CAA-3'; probe, VIC-5'-TCA GGA CGT CGA GTG GAC ACG GTG-3' TAMRA.

Fluorogenic PCR was carried out in a reaction volume of 50 mL on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Fluorogenic probe and primers were custom synthesized by Applied Biosystems. Each PCR reaction mixture consisted of 25 mL of TaqMan Universal Master Mix (Applied Biosystems), 0.67 mL of probe (15 mmol/L), 0.45 mL of primer forward (10 mmol/L), 0.45 mL of primer reverse (10 mmol/L), and 18.43 mL of sterile water. DNA solution (5 mL) was used in each real-time PCR reaction. Thermal cycling was initiated with a denaturation step at 50°C for 2 minutes and then 95°C for 10 minutes. The thermal profile for the PCR was 95°C for 15 seconds and 60°C for 1 minute. Data obtained during 50 cycles of amplification were analyzed.

Amplifications were carried out in 96-well plates in a GeneAmp 5700 Sequence Detection System. Each plate consisted of patient samples in triplicates and multiple water blanks as negative control. For construction of the calibration curve on each plate, we used a standard TaqMan Control Human Genomic DNA (Applied Biosystems) at 10 ng/mL with appropriate serial dilutions at 50, 2.5, and 0.5 ng, and 250, 50, and 10 pg. Linear amplification down to the last dilution point representing 10 pg of target DNA was obtained in each experiment (correlation coefficient, 0.999 to 0.999; slope, 3.25 to 3.35).

All of the data were analyzed using the Sequence Detection System software (Applied Biosystems) to interpolate the standard amplification curve of DNA at a known quantity with amplification cycle threshold of the unknown target sample, thus obtaining the relative amount of DNA in the experimental sample.

For the follow-up study, all of the consecutive plasma samples for each patient were simultaneously analyzed in the same real-time PCR experiment to allow comparative quantification of samples along the observation time.

**Pathologic and Immunohistochemical Methods**

Clinicopathologic data were available for all patients. There were 58 adenocarcinomas, 34 squamous cell carcinomas, three large-cell carcinomas, three pleomorphic carcinomas, and two adenoid cystic carcinomas. According to the WHO classification of lung adenocarcinoma,12 19 (32.6%) showed an acinar growth pattern, 19 were papillary, 17 (29.3%) were solid, and three (5.2%) were bronchioloalveolar. According to the revised lung cancer staging system,10 tumor stage was pT1 in 18%, pT2 in 55%, pT3 in 21%, and pT4 in 6% of patients; 47% of patients were pN0, 20% were pN1, and 33% were pN2 or N3. Pathologic stage distribution was IA in 16, IB in 18, IIB in 25, IIIA in 33, IIIB in 5, and IV in three patients. In three patients, the analysis was performed only on mediastinal node metastases.

In every patient, the occurrence of either tumor necrosis or lymphoid infiltrate was evaluated semiquantitatively on a scale from absent to 2+ (1+ if ≤ 50% and 2+ if > 50% of the whole tumor). For immunohistochemical
RESULTS

Quantitative Analysis of Circulating Plasma DNA in Cancer Patients and Controls

Figure 1 shows amplification plots of fluorescence intensity against the PCR cycle from plasma samples of cancer patients and matched controls. Each plot corresponds to the initial target DNA quantity present in the sample. Calculation of the amounts of plasma DNA is based on the cycle number, where fluorescence of each reaction passes the cycle threshold, which is set to the geometric phase of the amplification above the background. The x-axis denotes the cycle number of a quantitative PCR reaction. The y-axis denotes the log of fluorescence intensity over the background (ΔRn). The relative amount of plasma DNA is much higher in patient samples (left plots) compared with those of controls (right plots). The amplification curves that are shifted to the right, representing reduced target DNA quantity, clearly discriminate controls from cancer patients. In Figure 2, the distribution of plasma DNA concentration in patients and matched controls describes two distinct populations of values, despite some overlap. The box is bounded below and above by the 25% and 75% percentiles, the median is the solid line in the box, and the lower and upper error bars indicate 90% of values. Median concentration in patients (24.3 ng/mL) was almost eight times the value detected in controls (3.1 ng/mL). High concentrations were observed only in patients, whereas at the other end of the distribution there were few patients with low concentrations of DNA (ie, 0.5 ng/mL). A greater variability of circulating DNA was observed in patients than in controls (Fig 2).

Plasma DNA Concentration As a Risk Factor for NSCLC

An elevated concentration of circulating plasma DNA was associated with a higher risk of NSCLC. Tertile stratification showed that the risk increased exponentially when study participants with plasma concentrations in the second and third tertile were compared with those in the first tertile using conditional logistic regression (up to 85-fold; Table 1). When analyzed as a continuous distribution, a unit increase in plasma DNA (nanograms per milliliter) was associated with a 21% increase in NSCLC risk (OR, 1.21; 95% CI, 1.11 to 1.31).
Diagnostic Performance of Real-Time Quantitative PCR Assay

The area under the ROC curve shown in Figure 3 was 0.94 (95% CI, 0.907 to 0.973), suggesting a strong discrimination power of the molecular assay. The curve and AUC were estimated using the logistic procedure in SAS software. Table 2 lists a few of the DNA concentration cutpoints used to generate the curve with their sensitivity, specificity, positive predictive value, and negative predictive value. The 95% CIs around sensitivity estimates overlapped between the successive concentrations shown in Table 2, except for the last two concentrations (20 and 25 ng/mL).

<table>
<thead>
<tr>
<th>Percentage distribution of plasma DNA concentration in ng/mL by smoking and disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
</tr>
<tr>
<td>DNA (ng/mL)</td>
</tr>
<tr>
<td>10&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>25&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>median</td>
</tr>
<tr>
<td>75&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>90&lt;sup&gt;th&lt;/sup&gt;</td>
</tr>
<tr>
<td>max</td>
</tr>
<tr>
<td>mean</td>
</tr>
<tr>
<td>sd</td>
</tr>
</tbody>
</table>

Table 1. Plasma DNA Concentration As a Risk Factor for NSCLC

<table>
<thead>
<tr>
<th>Tertile Distribution of DNA (ng/mL)*</th>
<th>Patients (n = 100)</th>
<th>Controls (n = 100)</th>
<th>Conditional† Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 4</td>
<td>4</td>
<td>62</td>
<td>1†³</td>
<td></td>
</tr>
<tr>
<td>4.1-20</td>
<td>27</td>
<td>36</td>
<td>5.5</td>
<td>1.9 to 16.3</td>
</tr>
<tr>
<td>&gt; 20</td>
<td>69</td>
<td>2</td>
<td>85.5</td>
<td>16.5 to 445</td>
</tr>
</tbody>
</table>

Abbreviation: NSCLC, non–small-cell lung cancer.

*Obtained from the pooled distribution of plasma DNA values in cases and in controls.
†Adjusted for age.
‡Reference group.

Fig 3. Receiver-operating characteristic curves for the diagnosis of non–small-cell lung cancer using plasma DNA values. Curve and area under the curve (AUC) estimated using the logistic procedure in SAS software (Cary, NC). R<sub>n</sub>, level of fluorescence detected; ROC, receiver operating characteristic curve.
Correlation of Plasma DNA Levels With Clinicopathologic Features

Plasma DNA was significantly associated with age, and increased with increasing age after adjusting for remaining variables in the model (Table 3). No association was observed between plasma DNA levels and smoking intensity or duration, cell type, pathologic stage, or other features such as necrosis, lymphoid infiltration, or growth patterns.

The association between plasma DNA and microvessel density was modified by the age of the patient (significant interaction): plasma DNA increased with increasing microvessel density in younger and not in older patients. Ki67 and EGFR expression were not statistically associated with plasma DNA after controlling for differences in the other variables in the model. Addition of number of cigarettes smoked did not introduce any relevant change in the model.

Regression of all variables explained 29% of the observed variability in plasma DNA.

Change in Plasma DNA Levels During Follow-Up

In 35 cancer patients, a second plasma sample was collected, 3 to 15 months after surgery (median elapsed time, 8 months), and analyzed to monitor changes in DNA levels during clinical follow-up. The overall median DNA concentration in follow-up plasma samples was 8.4 ng/mL, showing a clear trend toward reduction, compared with median baseline levels of 24.5 ng/mL ($P < .0001$). When these patients were tested according to their clinical status, median DNA concentration at follow-up was significantly lower in 30 disease-free individuals as compared with the five cancer patients with proven cancer relapse (7.1 vs 24.7 ng/mL; $P = .002$). Figure 4 shows the reduction in DNA levels of each patient, stratified by relapse status.

DISCUSSION

Previous studies have reported significantly higher concentrations of serum DNA in patients with various types of cancers by a radioimmunoassay method, and have suggested the use of serum DNA in cancer patients as a prognostic tool to monitor the effect of cancer therapy.$^{37,38}$ By using a simple colorimetric assay in a representative series of lung cancer patients and controls, we have demonstrated that a quantitative plasma DNA test is a valuable diagnostic tool to discriminate patients from healthy individuals and to detect early recurrence during follow-up. A recent study performed in a group of miscellaneous tumors confirmed these results by using a fluorometric assay and supported a digital single nucleotide polymorphism analysis of allelic imbalances as a sensitive and specific tool for ovarian cancer screening.$^{26}$

### Table 2. Screening Performance of Plasma DNA Concentration

<table>
<thead>
<tr>
<th>Cutpoint (ng/mL)*</th>
<th>Sensitivity</th>
<th>95% CI†</th>
<th>Specificity</th>
<th>95% CI†</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>97</td>
<td>91.5 to 99.4</td>
<td>60</td>
<td>49.7 to 69.7</td>
<td>71</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>92</td>
<td>84.8 to 96.5</td>
<td>77</td>
<td>67.5 to 84.8</td>
<td>80</td>
<td>91</td>
</tr>
<tr>
<td>10</td>
<td>88</td>
<td>80.0 to 93.6</td>
<td>86</td>
<td>77.6 to 92.1</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>15</td>
<td>78</td>
<td>68.6 to 85.7</td>
<td>95</td>
<td>88.7 to 98.4</td>
<td>94</td>
<td>81</td>
</tr>
<tr>
<td>20</td>
<td>69</td>
<td>58.9 to 77.9</td>
<td>98</td>
<td>93.0 to 99.8</td>
<td>97</td>
<td>76</td>
</tr>
<tr>
<td>25</td>
<td>46</td>
<td>35.9 to 56.3</td>
<td>99</td>
<td>94.5 to 100</td>
<td>98</td>
<td>65</td>
</tr>
</tbody>
</table>

*Contrasting concentrations lower than the cutpoint against concentrations equal to or greater than the value specified.

†Sensitivity and specificity estimates were treated as binomial parameters to calculate 95% CIs using Statxact (Cambridge, MA).

### Table 3. Correlation of Plasma DNA Concentration With Clinical and Pathologic Parameters: Multiple Linear Regression of the Logarithm of Plasma DNA (ng/mL)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>3.429</td>
<td>0.974</td>
<td>.001</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 60$ (1)</td>
<td>$-6.262$</td>
<td>1.682</td>
<td>.0003</td>
</tr>
<tr>
<td>$61-71$ (2)</td>
<td>$-2.280$</td>
<td>1.198</td>
<td>.06</td>
</tr>
<tr>
<td>$\geq 72$ (3) Reference</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log mean MVD, continuous distribution</td>
<td>0.0001</td>
<td>0.262</td>
<td>.99</td>
</tr>
<tr>
<td>Log mean MVD $\times$ age (1)</td>
<td>1.596</td>
<td>0.469</td>
<td>.001</td>
</tr>
<tr>
<td>Log mean MVD $\times$ age (2)</td>
<td>0.475</td>
<td>0.346</td>
<td>.17</td>
</tr>
<tr>
<td>EGFR $&lt; 70%$</td>
<td>0.379</td>
<td>0.227</td>
<td>.09</td>
</tr>
<tr>
<td>$\geq 70%$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki67 %, continuous distribution</td>
<td>0.007</td>
<td>0.005</td>
<td>.17</td>
</tr>
<tr>
<td>Smoking, No. of cigarettes/d</td>
<td>$-0.009$</td>
<td>0.006</td>
<td>.15</td>
</tr>
</tbody>
</table>

NOTE. Numbers in parentheses (1, 2, and 3) represent the three age classes used for linear regression analysis of log mean MVD, as presented in this Table.

Abbreviations: MVD, microvessel density; EGFR, epidermal growth factor receptor.
We report here the results of a large case-control study, the first to our knowledge, for validation of free circulating DNA in plasma as a potential lung cancer diagnostic marker. Our results show that real-time quantitative PCR assay, using the hTERT gene as a target sequence for quantification of circulating DNA in plasma, has high sensitivity and specificity, as estimated by AUC ROC curves, by analyzing values either as continuous distribution or as selected cutpoints. Furthermore, median concentration in patients (24.3 ng/mL) was almost 8 × the concentration detected in controls (3.1 ng/mL). Although the highest sum of sensitivity (90%), specificity (86%), positive predictive value (90%), and negative predictive value (90%) was obtained with a DNA concentration value of 9 ng/mL (not shown), the CIs around these diagnostic indicators overlap with those of adjacent concentrations. The selection of the optimal cutpoint will therefore have to acknowledge this variability. The value of 25 ng/mL is the only cutpoint shown with sensitivity that does not overlap with that of other cutpoints, although it shows the lowest sensitivity (46%; 95% CI, 36% to 56%). The magnitude of reported ORs proves the strong association between plasma DNA concentration and NSCLC risk, despite wide confidence limits. To our knowledge, similar OR values never were reported previously for any biologic marker and could be of substantial benefit in clinical practice.

We found increased amounts of circulating plasma DNA in samples from any stage and tumor size. This is particularly relevant for small lesions, the systematic detection of which could help reduce lung cancer morbidity and mortality.

One important aspect of our quantitative analysis was the ability to follow longitudinal changes after cancer resection. The data available on 35 cancer patients showed a rapid decrease of circulating DNA values after lung resection. Conversely, no decreasing or increasing levels of plasma DNA identified individuals with recurrence of their disease (24.7 v 7.1 ng/mL in cancer-free patients; P = .002), suggesting that quantification of plasma DNA might represent a novel approach to monitor surgical patients or assess treatment efficacy after chemoradiotherapy.

To explore the possible modulation of free DNA release by smoking exposure, we analyzed 20 never smokers older than 55 years of age: their median DNA value was 0.61 ng/mL, indicating low amounts of free circulating DNA in unexposed groups.

The origin and mechanism of circulating DNA are not fully understood. In addition to cell lysis, apoptosis, necrosis, and active DNA release have been advanced as possible sources of circulating DNA. To explore the potential mechanisms of tumor DNA release into the bloodstream, we evaluated necrosis, angiogenesis, and proliferation features in all primary tumor samples. Plasma DNA levels in patients were not associated with necrosis, lymphoid infiltration, or growth patterns; or Ki67 or EGFR expression. These data suggest that the mechanism of release of tumor DNA into the bloodstream is not related to the necrotic rate or tumor cell proliferation. Of interest, a significant association with microvessel density suggests a link with tumor angiogenic status. Because angiogenesis appears to be an early event in lung carcinogenesis, plasma DNA quantitative assay could be effective in identifying early but nevertheless angiogenic lung cancers.

In summary, these results highlight a potential value of this DNA-based plasma test for early detection of lung cancer in high-risk individuals, and particularly former heavy smokers. A large study is currently under way with more than 1,000 smoking volunteers to determine whether quantitative detection of plasma DNA might increase the accuracy of spiral CT scan for early detection of lung cancer. Moreover, levels of plasma DNA could help identify high-risk individuals for chemoprevention trials, and could be tested as a potential intermediate biomarker of the efficacy of intervention.

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES


