DNA Hypermethylation Analysis in Sputum of Asymptomatic Subjects at Risk for Lung Cancer Participating in the NELSON Trial

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ABSTRACT

Introduction
Lung cancer is the major contributor to cancer mortality, due to metastasized disease at time of presentation. Low-dose spiral CT scan (LDCT) screening showed a high sensitivity for lung cancer diagnosis in the National Lung Screening Trial (NLST). However, many false-positive results were reported.

The current study investigates hypermethylation of biomarkers \textit{RASSF1A}, \textit{APC}, cytoglobin, \textit{3OST2}, \textit{FAM19A4}, \textit{PHACTR3} and \textit{PRDM14} in sputum of asymptomatic high risk individuals to detect lung cancer at preclinical stage.

Methods
A subset of participants of the NELSON lung cancer LDCT screening trial prospectively collected sputum at baseline. For DNA hypermethylation assessment in sputum, subjects were selected with i) lung cancer in follow-up (cases; n=65), ii) minor cytological aberrations (controls; n=120) and iii) a random selection of subjects without cytological aberrations (controls; n=99). Median follow-up time for controls was 80 months.

Results
Of all biomarkers, only \textit{APC} was able to moderately predict lung cancer (sensitivity 59\%, specificity 59\%). \textit{RASSF1A} hypermethylation (sensitivity 11\%, specificity 92\%) was most likely to occur within two years prior to diagnosis ($P=0.03$). Sputum cytology did not detect any lung cancers.

Conclusions
In a lung cancer screening setting, the analyzed DNA hypermethylation biomarkers play no role in the detection of preclinical disease in sputum.
INTRODUCTION

Lung cancer imposes a major public health care problem as it continues to be the most common cause of cancer-related death worldwide. Metastasized disease at time of presentation and limited treatment options are main contributors to the high mortality. Importantly, prognosis improves when lung cancer is detected at operable stage I or II. Therefore, current research focuses amongst others on identification of high risk individuals and early detection of lung cancer.

Lung cancer is mainly attributable (85%) to (a dose-dependent) exposure to carcinogens in tobacco. Smoking causes persistent lung damage over time; of all smokers, 10-20% will eventually develop lung cancer. Primary prevention of lung cancer through smoking cessation interventions showed to be effective in decreasing all-cause mortality, including lung cancer, but smoking relapse is common. Secondary prevention, i.e., screening for lung cancer among asymptomatic heavy smokers, was first investigated in the 60-70s of the past century. However, combined use of chest X-rays (CXR) and sputum cytology did not result in reduction of lung cancer mortality. More promising is low-dose spiral computed tomography (LDCT) scanning. The National Lung Screening Trial (NLST) reported a 20% reduction in mortality. However, apart from costs and radiation exposure, LDCT screen in the NLST resulted in many false positive cases indicating complexity to the management of lung nodules detected by LDCT. Thus, a role exists for another detection method situated independently or complementary to LDCT to improve specificity.

The use of sputum is of interest as potential screening tool, as procurement is non-invasive and simple. Tumour DNA aberrations can be detected in sputum with advanced molecular techniques with higher sensitivity as cytology alone. DNA hypermethylation of biomarkers has shown to differ between sputum and bronchial brushes of lung cancer cases and cancer-free controls. Some of these tumour aberrations can be detected in sputum years preceding clinical diagnosis and possibly may aid in the early detection of lung cancer. Recently, a rationale was introduced to distinguish diagnostic biomarkers from risk biomarkers. In this context, diagnostic markers are, when positive, characterized by high specificity, whereas risk markers have a lower specificity.

In previous studies, we selected and validated biomarkers in sputum of patients with lung cancer. The aim of the present study was to investigate whether these sputum-based biomarkers are also able to identify high risk individuals with asymptomatic lung cancer, i.e., in preclinical phase. The NELSON trial, acronym...
for ‘Dutch-Belgian lung cancer screening trial’ and largest European randomized lung cancer screening trial, was launched in 2003.\textsuperscript{19,20} In the current study, DNA hypermethylation was examined in baseline sputum from participants of the NELSON trial and compared with sputum cytology.

FIGURE 1. Flowchart sputum DNA methylation examination in NELSON participants.
METHODS

Subjects

15,822 subjects were enrolled in the NELSON trial, between September 2003 and October 2005 (Figure 1). Inclusion criteria were current and former (quitted within the past 10 years) smokers with a smoking history of ≥15 pack years, and age 50-75 years. Subjects were randomized for LDCT scanning during four rounds in one arm versus no screening in the other arm. Total follow-up of the main study is scheduled for 10 years. Study design was approved by the Dutch Ministry of Health (2000/04WBO) and the institutional review boards (Commissie Mensgebonden Onderzoek Regio Arnhem Nijmegen; CWZ-103-2000). All subjects provided written informed consent.

Prospective collection of sputum at baseline LDCT screening was added in the study protocol at a later time-point and started from July 2005 in the Dutch regions. Spontaneous sputum was sampled at home during three days in a single canister containing Saccomanno’s fixative and sent by surface mail to a central pathology laboratory (Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands). Information on smoking history, among other things, was available for all subjects.

For this study, subjects were included who were i) cases, i.e., diagnosed with lung cancer at baseline screening or during follow-up, or ii) control, i.e., subjects without cancer at time of sputum analysis, selected based on a) (minor) abnormalities in sputum cytology results and b) random selection.

Sputum processing

Upon arrival, sputum samples were stored at 4 degrees Celsius until further processing. First the samples were overnight treated with 10% dithiotreitol, after which the sputum was centrifuged and supernatant discarded. Cell pellets were washed with 50% ethanol for several times. Of each cell pellet, 3.7 μl were used for sputum cytology; the remainder of the cell pellet was stored (at -20 degrees Celsius) until further molecular analysis (see below).

Sputum cytology

Single layer slides were prepared using the Hettich Cytology Centrifuge System. Sputum cytology samples were screened by experienced cytotechnologists (CWZ) and diagnosed by a pathologist (ET). A sputum sample was considered representative for the respiratory tract if alveolar macrophages, bronchial epithelial cells or Curschmann spirals were present. Sputum cytology was categorized as i) normal; ii) minor
cytological changes; or iii) (suspicious for) malignancy (according to the Colorado group\textsuperscript{21}). Minor cytological changes comprised: mild/moderate atypical metaplastic epithelium; atypical cylinder epithelium.

**DNA hypermethylation analysis**

DNA was isolated and modified at VU University Medical Center, Amsterdam, the Netherlands, as previously described\textsuperscript{22}. DNA hypermethylation analysis was performed for the following biomarkers, selected from previous research: RASSF1A, APC, cytoglobin (CYGB)\textsuperscript{22}, 3OST2\textsuperscript{11}, PRDM14, FAM19A4 and PHACTR3\textsuperscript{23}. Multiplex quantitative methylation-specific PCRs (qMSPs) were performed as published before\textsuperscript{18,22}. Cutoff values for diagnostically relevant hypermethylation of the biomarkers (based on Youden’s J index) were determined in previous research\textsuperscript{18}. Because of its diagnostic value, RASSF1A hypermethylation was assessed in combination with sputum cytology to examine a complementary effect. A risk classification model based on post-test probabilities on lung cancer that was composed in previous research\textsuperscript{18} is evaluated in the current study.

**Statistical analysis**

Chi-square tests were performed to assess the discrimination ability of biomarker hypermethylation and sputum cytology between cases and controls. Chi-square tests were also used to investigate whether time between sputum collection and lung cancer diagnosis influenced biomarker sensitivity, for three different timespans: \( \leq 6 \) months versus \( >6 \) months; \( \leq 12 \) months versus \( >12 \) months; and \( \leq 24 \) months versus \( >24 \) months, respectively. Student’s t tests were applied for continuous variables to assess differences between cases and controls.

A two-sided \( P \)-value of \( \leq 0.05 \) was considered significant. SPSS Statistics version 20 (IBM Corp., Armonk, NY) was used for all analyses.

**RESULTS**

**Subjects**

In total, sputum was collected by 1,548 subjects of the LDCT screening arm. Follow-up revealed lung cancer in 65 subjects (i.e., cases; Figure 1). Nine cases were excluded for the following reasons: cancer diagnosis of other primary than lung cancer (n=4), sputum collection after tumour resection (n=3), insufficient DNA available for
molecular analysis (n=2). In 5 cases, no histological diagnosis could be obtained. In the control group, 219 subjects were selected for the current study, being 120 with minor cytological aberrations and a random selection of 99 subjects with normal sputum cytology. In both subgroups, one controls were excluded from molecular analysis, because of insufficient amount of DNA available.

Sociodemographic details of cases and controls are shown in Table 1. Median follow-up of subjects at time of hypermethylation data analysis was for cases 24 months (range 0-72 months) and for controls 80 months (range 71-84 months).
### Table 2. Sputum cytology of sputum samples of lung cancer cases (n=54) and controls (n=1,418) at baseline of the NELSON trial.

<table>
<thead>
<tr>
<th></th>
<th>Lung cancer (n=54)</th>
<th>Sensitivity</th>
<th>Controls (n=1,418)</th>
<th>Specificity</th>
</tr>
</thead>
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<tr>
<td></td>
<td>pos</td>
<td>neg</td>
<td>%</td>
<td>pos</td>
</tr>
<tr>
<td>Squamous cell atypia</td>
<td>2</td>
<td>52</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Cylinder cell atypia</td>
<td>2</td>
<td>52</td>
<td>4</td>
<td>30</td>
</tr>
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<td>2</td>
<td>31</td>
</tr>
<tr>
<td>Suspicious for malignancy</td>
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<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malignant cells</td>
<td>0</td>
<td>54</td>
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### Table 3. DNA hypermethylation analysis of biomarkers in sputum of lung cancer cases and controls at baseline of the NELSON trial.

<table>
<thead>
<tr>
<th></th>
<th>Lung cancer (n=56)</th>
<th>Sensitivity</th>
<th>Controls (n=217)</th>
<th>Specificity</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pos</td>
<td>neg</td>
<td>%</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>6</td>
<td>56</td>
<td>11</td>
<td>17</td>
<td>200</td>
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<tr>
<td>APC</td>
<td>33</td>
<td>23</td>
<td>59</td>
<td>90</td>
<td>127</td>
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<td>CYGB</td>
<td>34</td>
<td>22</td>
<td>61</td>
<td>113</td>
<td>104</td>
</tr>
<tr>
<td>3OST2</td>
<td>8</td>
<td>48</td>
<td>14</td>
<td>29</td>
<td>188</td>
</tr>
<tr>
<td>FAM19A4</td>
<td>19</td>
<td>37</td>
<td>34</td>
<td>62</td>
<td>155</td>
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<tr>
<td>PHACTR3</td>
<td>48</td>
<td>8</td>
<td>86</td>
<td>181</td>
<td>36</td>
</tr>
<tr>
<td>PRDM14</td>
<td>20</td>
<td>36</td>
<td>36</td>
<td>79</td>
<td>138</td>
</tr>
<tr>
<td>RASSF1A, 3OST2 &amp; PRDM14</td>
<td>24</td>
<td>32</td>
<td>43</td>
<td>79</td>
<td>138</td>
</tr>
</tbody>
</table>

<sup>a</sup> Comparison between lung cancer cases and controls.

### Table 4. Sputum cytology and RASSF1A hypermethylation analysis combined for sputum samples of lung cancer cases and controls. Cytology threshold comprises minor cytological aberrations.

<table>
<thead>
<tr>
<th></th>
<th>Lung cancer (n=54)</th>
<th>Sensitivity</th>
<th>Controls (n=217)</th>
<th>Specificity</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pos</td>
<td>neg</td>
<td>%</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>Cytology&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7</td>
<td>47</td>
<td>13</td>
<td>85</td>
<td>132</td>
</tr>
<tr>
<td>Cytology&lt;sup&gt;b&lt;/sup&gt; &amp; RASSF1A hypermethylation</td>
<td>12</td>
<td>42</td>
<td>22</td>
<td>93</td>
<td>124</td>
</tr>
</tbody>
</table>

<sup>a</sup> Comparison between lung cancer cases and controls.

<sup>b</sup> Presence of squamous cell atypia, cylinder cell atypia or squamous metaplasia.
Sputum cytology

Sputum cytology details were available for 1,458 participants of the LDCT arm who collected sputum at baseline (Table 2). None of the cases showed malignant cells or cells suspicious for malignancy at baseline. Minor cytological aberrations were detected in 7 cases and 120 subjects without lung cancer, including squamous metaplasia, mild or moderate squamous cell atypia and/or cylinder cell atypia.

DNA hypermethylation analysis

Ratio values of each biomarker did not significantly differ between cases and controls, apart from \( APC \) (Table 3). Based on the cutoff values defined in previous research, \( APC \) was able to discriminate between cases and controls, detecting 33 of the 56 cases (59%) at a specificity level of 59% \((P=0.02)\). The diagnostic marker \( RASSF1A \) identified 11% of cases (at a specificity of 92%).

Time between sputum collection and lung cancer diagnosis did not influence biomarker sensitivity for none of the defined time categories. However, we observed that cases with positive DNA hypermethylation for \( RASSF1A \) were most likely to present with lung cancer within two years (5 of 6 cases; \( P=0.03)\).

For 54 cases and 217 controls, both cytological and hypermethylation results were available. A separate analysis was performed regarding the combination of cytological aberrations and \( RASSF1A \) hypermethylation (Table 4). This yielded a sensitivity of 22% at a specificity level of 57% \((P=0.005)\). The low specificity is mainly attributable to the frequent presence of minor cytological aberrations in controls.

The risk classification model (Table 5) was constructed in previous research which

<table>
<thead>
<tr>
<th>( RASSF1A )</th>
<th>( 3OST2 ) &amp; ( PRDM14 )</th>
<th>Lung cancer</th>
<th>Control</th>
<th>( P )-value (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{n/56} )</td>
<td>( % )</td>
<td>( \text{n/217} )</td>
<td>( % )</td>
</tr>
<tr>
<td>( \geq 60% )</td>
<td>4</td>
<td>7.1</td>
<td>13</td>
<td>6.0</td>
</tr>
<tr>
<td>40 - 60%</td>
<td>1</td>
<td>1.8</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>20 - 40%</td>
<td>9</td>
<td>16.1</td>
<td>33</td>
<td>15.2</td>
</tr>
<tr>
<td>0 - 20%</td>
<td>42</td>
<td>75.0</td>
<td>170</td>
<td>78.3</td>
</tr>
</tbody>
</table>

\(^a\) Comparison between lung cancer cases and controls.
included diagnostic marker \textit{RASSF1A} and risk markers \textit{3OST2} and \textit{PRDM14}.\textsuperscript{18} Evaluation of the model in the current study showed that 7\% of cases are defined as ‘high risk’ (i.e., \textasciitilde60\% chance on lung cancer) and 75\% of cases were classified as having low risk. The distribution of risk proportions between cases and controls was not different ($P=0.74$).

\section*{DISCUSSION}

In this study, we investigated DNA hypermethylation of biomarkers in sputum of high risk individuals for lung cancer development, participating in the NELSON trial. This is the first time that DNA hypermethylation is investigated in sputum from LDCT screening participants including both subjects with and without lung cancer in the follow-up. The presence of \textit{RASSF1A} hypermethylation showed high specificity (92\%), but low sensitivity (11\%) compared to higher sensitivity in patients with clinically diagnosed lung cancer.\textsuperscript{17,18} Cases positive for \textit{RASSF1A} hypermethylation were most likely to be diagnosed with lung cancer within two years. The \textit{RASSF1A} data may denote presence of invasive cancer because of its high specificity, but sensitivity is too low for useful application in screening. All risk markers demonstrated moderate sensitivity and specificity, of which \textit{APC} was significantly able to distinguish subjects with LDCT detected lung cancer from cancer-free subjects (59\%) with specificity of 59\%. The relatively well performance of this marker is not in line with previous results,\textsuperscript{17,18} and might be explained as coincidental finding or require further validation. Overall, the risk markers are not suitable for first line markers in the screening setting, which is also illustrated by the outcomes of the risk classification model.

Previously, one other study examined molecular alterations in sputum samples from a screening cohort, the Italian DANTE trial.\textsuperscript{24} In this study, spontaneous sputum samples were examined from participants who were not diagnosed with lung cancer at baseline LDCT screen; affected participants were not tested.\textsuperscript{25} Participants with lung cancer were excluded. Mutations in \textit{KRAS}, \textit{p53}, and hypermethylation of \textit{p16}, \textit{NOR1A} and \textit{RASSF1A} were investigated in this study. Despite different study design and different set of selected markers, a similarity between this and our study results is recognized, being the low sensitivity of the tested markers in an at risk population, including the in our opinion diagnostic marker \textit{RASSF1A} at the time symptoms arise in patients with lung cancer.

We did not find a significant association with time to diagnosis. For biomarkers \textit{RASSF1A} and \textit{3OST2}, the number of positive lung cancer cases showing hypermethylation
in relation to time may possibly be too low for reliable assessment. Still, the diagnostic potential of \textit{RASSF1A} in previous research\cite{11,14} and the observation in this study that \textit{RASSF1A} hypermethylation was mostly found in cases with lung cancer diagnosis within two years, supports the rationale as proposed in a review before\cite{16} that a diagnostic marker is expected to have a positive test result two years prior to diagnosis. This is in line with observations in the study of Honorio \textit{et al}\cite{26} who reported that three of five controls with positive \textit{RASSF1A} hypermethylation developed metastatic lung cancer within one year. In our study, five of six \textit{RASSF1A} positive cases were detected with LDCT screen. Interestingly, the sixth case was detected at the second interval and was one of the two SCLC tumours in the whole set. \textit{RASSF1A} hypermethylation is thus able to detect SCLC cancer that goes undetected at LDCT screen. Although it concerns one case, a complementary value of DNA hypermethylation analysis in sputum is not excluded.

The sensitivity of the hypermethylation analysis is much lower than in our previous studies in symptomatic patients. Especially \textit{RASSF1A}, \textit{3OST2} and \textit{PRDM14} were lower than expected for prediction of lung cancer in cases. One explanation may be that in preclinical phase concentration of methylated tumour DNA in sputum is below analytical detection limit of the assay, as in symptomatic patients the tumour related DNA content was already close to the detection limit. Remarkably, 4 of 6 \textit{RASSF1A} positive cases initially had an ‘intermediate’ LDCT scan result and were found positive after follow-up scan (within 6 months). In total, 32 cases were diagnosed after such a follow-up scan, of whom 14 (44\%) showed positive DNA hypermethylation in their sputum for \textit{RASSF1A}, \textit{3OST2} or \textit{PRDM14}. Although sensitivity and specificity of the panel is too low for application in screening setting, this observation supports the hypothesis that concurrent biomarker analysis may be helpful, but that additional diagnostic markers are required.

The limited role of sputum cytology in this study (no malignant cells detected) differs from sputum cytology performance in screening trials in the past, examining CXR in combination with sputum cytology.\cite{6,27} In the Memorial Sloan Kettering study, sputum cytology detected 29\% of lung cancer cases, of whom 10\% were also detected by CXR. Tumours detected on CXR have a size of at least 2 centimeters, and chance on positive sputum cytology increases with tumour size.\cite{28} Thus, as LDCT-detected tumours are usually smaller (nodules from size 2 mm can be detected), this may explain that sputum cytology is less likely to identify tumour cells.

One of the study strengths is the study design and another the use of a rationale to distinguish diagnostic markers from risk markers. While \textit{RASSF1A} hypermethylation
in symptomatic patients is a diagnostic marker, in the screening setting it performs as a risk marker. Still, even in this setting, this marker shows a high specificity (92%). Additional markers of this type are highly desirable for application in screening. New technologies have emerged for biomarker selection, such as genome-wide profiling, which may yield more diagnostic biomarkers. Considering technical issues, one of our strengths is the use of the qMSP assay which is a reliable and robust method, suitable for high-throughput screening. Furthermore, procurement of sputum has shown to be a patient-friendly approach. Sputum can be stored for years, provided that cooling temperature is low and that sputum is preserved after initial handling with DTT and stored in a fixative, such as Saccomanno solution (2% carbowax and 50% ethanol) in this study.29

Taking all results into consideration, we conclude that the studied hypermethylation risk markers are not suitable for lung cancer risk stratification both in the clinical and screening setting. Risk markers may possibly play a complementary role to other marker(s), provided that specificity is maintained. This implies that also after our study most of investigated biomarkers so far are not appropriate for lung cancer screening.16 Given insufficient sensitivity, thus far, additional diagnostic markers are needed for reliable lung cancer prediction. As the NELSON screening strategy is related to growth rate of the lung nodule, it is not excluded that with another lung nodule management policy (e.g., as referred to30–32) sputum analysis may play a role.

In conclusion, in the screening setting DNA hypermethylation in sputum has limited sensitivity. RASSF1A is a potential marker with high specificity, but complementary markers are needed to improve sensitivity.

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