Testing for faecal calprotectin (PhiCal) in the Norwegian Colorectal Cancer Prevention trial on flexible sigmoidoscopy screening: comparison with an immunochemical test for occult blood (FlexSure OBT)

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Background: Screening for colorectal cancer (CRC) using guaiac based faecal occult blood tests (FOBT) has an estimated programme sensitivity of >60% but <30% for strictly asymptomatic CRC in a single screening round. In search for improved non-invasive tests for screening, we compared a test for faecal calprotectin (PhiCal) with a human haemoglobin immunochemical FOBT (FlexSure OBT).

Methods: In the Norwegian Colorectal Cancer Prevention (NORCCAP) trial, screeners in one screening arm were offered screening with combined flexible sigmoidoscopy (FS) and FlexSure OBT. They were also requested to bring a fresh frozen sample of stool for the PhiCal test which was performed on samples from screeners with CRC (n = 16), high risk adenoma (n = 195), low risk adenoma (n = 592), and no adenoma (n = 1518) (2321 screeners in total). A positive PhiCal test was defined by a calprotectin level ≥50 μg/g.

Results: The PhiCal test was positive in 24–27% of screeners whether they had no adenoma, low risk adenoma, or high risk adenoma. Ten (63%) of 16 CRCs gave a positive PhiCal test. The total positivity rate in this population was 25% for the PhiCal test compared with 12% for FlexSure OBT, with a sensitivity for advanced neoplasia of 27% and 35%, respectively. Specificity for ‘any neoplasia’ was 76% for the PhiCal test and 90% for FlexSure OBT.

Conclusions: In colorectal screening, the performance of the PhiCal test on a single spot from one stool sample was poorer than a single screening round with FlexSure OBT and cannot be recommended for population screening purposes. The findings indicate a place for FlexSure OBT in FOBT screening.

Colorectal cancer (CRC) is one of the leading causes of cancer death in Western societies. Randomised screening studies using guaiac based faecal occult blood tests (FOBT) show a 15–33% reduction in CRC mortality. Although FOBT programme sensitivity for CRC has been estimated to be more than 60%, the sensitivity for strictly asymptomatic CRC is less than 30% for a single screening round. Additionally, FOBT frequently fails to detect premalignant neoplasia, allowing very limited possibilities for intervention along the adenoma-carcinoma sequence of progression to cancer. Thus there is a need for improved non-invasive screening tools. Although screening programme sensitivity is of prime importance, test sensitivity (single round sensitivity) may give an approximate indication as to what time intervals may be allowed between screening rounds.

Calprotectin, a calcium binding protein in granulocytes, macrophages, and epithelial cells, has shown increased levels in stools from patients with bowel inflammation and CRC. Calprotectin is stable in refrigerated storage. It is also poorly degraded during passage through the gastrointestinal tract, and swallowed sputum during respiratory tract infections may possibly influence faecal calprotectin values. Although information on faecal calprotectin in asymptomatic CRC is limited, the sensitivity of a commercially available calprotectin kit (PhiCal; Eurospital Spa, Trieste, Italy) may be >60% but poorer specificity may require work up colonoscopy in 30% of screeners compared with 4–5% of screeners after screening with unhydroyzed FOBT (Hemoccult-II).

FlexSure OBT (Beckman-Coulter Inc, Primary Care Diagnostics, Palo Alto, California, USA) is an immunochemical test for human haemoglobin which is presently not marketed. Another test, based on similar membrane technology and immunolabelled colloidal gold to detect haemoglobin, but with a different sampling procedure, is available on the market (Insure, Enterix Inc., Portland, Maine, USA).

In the Norwegian Colorectal Cancer Prevention (NORCCAP) screening trial, we used once only flexible sigmoidoscopy (FS) alone or in combination with FOBT. A low threshold for a positive FS (biotically verified adenoma at FS or a positive FOBT) created work up colonoscopy in 20% of screeners, approaching the proportion expected when using the PhiCal test. We therefore wished to explore the performance of the PhiCal test in this large scale population screening study by analysis of fresh frozen specimens of stool delivered by screeners on attendance for FS.

METHODS
In the NORCCAP trial, 20 780 men and women, aged 50–64 years and living in Telemark County or the City of Oslo, were obtained by randomisation from the National Central Person Registry to be offered a colorectal screening examination. The design of the trial has been presented in detail elsewhere. A letter of invitation suggesting a screening appointment on a given date was posted to those randomised for screening. Altogether, 777 (4%) persons were excluded according to exclusion criteria. Thus 20 003 individuals remained eligible for screening, of which 12 960 attended (65%).

Abbreviations: OR, odds ratio

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Colorectal Cancer Prevention trial on flexible sigmoidoscopy: comparison with an immunochemical test for occult blood (FlexSure OBT)

In colorectal screening, the performance of the PhiCal test on a single spot from one stool sample was poorer than a single screening round with FlexSure OBT and cannot be recommended for population screening purposes. The findings indicate a place for FlexSure OBT in FOBT screening.
Those invited were randomised (1:1) to screening with either FS alone or a combination of FS and FOBT using three stool samples for an immunochemical test for human blood, FlexSure OBT. In addition, screeners in the combined FS-FOBT arm were asked to bring a fresh frozen stool specimen for research purposes. In the letter of invitation, addition of FOBT to FS was advocated as an add on screening option, the value of which should be explored through the trial. Participants were not given an option of FOBT screening without FS. Screeners collected their single stool sample at home less than one week before FS and kept it in a 20 ml vial in their home deep freeze (−20°C) until attendance for FS.

“Any biopsyically verified neoplasia” at FS or a positive FOBT qualified for colonoscopy. Thus screen negative individuals had an FS examination only and no colonoscopy. The outcome of faecal calprotectin analysis was not used as a criterion for work up colonoscopy.

In the present study of faecal calprotectin, only screeners in the FS-FOBT group were included (that is, individuals who had also been asked to bring a frozen stool sample). All participants with neoplasia at FS and/or colonoscopy who had brought a stool sample (n = 929) were selected for faecal calprotectin analysis in addition to a random sample of screeners with no neoplasia (n = 1518, representing 29% of screeners with no neoplasia, compared with 75% of individuals with neoplasia) (table 1). Also, as “any adenoma at FS” or “a positive FOBT” was a threshold for colonoscopy, only 4.8% of individuals with “no adenoma” in table 1 had colonoscopy after FS compared with 94%, 98%, and 100% in the low risk, high risk, and CRC groups, respectively. Thus this substudy on calprotectin did not reflect the prevalence of neoplasia in the overall screened population. Analysis of absolute test performance was therefore limited to test sensitivity and specificity as the predictive values of a test are influenced by the prevalence of the condition to be tested for. A total of 126 (14%) of the 929 stool samples from screeners with neoplasia could not be analysed due to sample destruction (thawing) or inadequate labelling of stool vials for identification.

A subgroup of FS positive screeners subjected to colonoscopy was analysed separately to further compare the sensitivity of one round of the FlexSure OBT (three stool samples) and the PhiCal test (single stool sample). This group consisted of screen positive individuals who had delivered a valid FOBT (all three test windows used) and a faecal calprotectin analysis had been performed and colonoscopy work up had been accepted and performed with successful caecal intubation.

Nurses and nurse assistants at each screening centre were trained and certified for FlexSure OBT analysis by the manufacturer before being allowed to develop and read the test. They were re-certified after six months. For faecal calprotectin analysis, we used the PhiCal test. Fully trained laboratory technicians at the Research Institute of Internal Medicine (IIF), Rikshospitalet, Norway, and specially trained staff in NORCCAP performed all of the calprotectin analyses.

Calprotectin values were recorded in μg/g of stool. A positive PhiCal test was defined as faecal calprotectin ≥50 μg/g in a single stool specimen, as recommended by the manufacturer. Median time from attendance to calprotectin analysis of stored stool sample was 13 months (range 0–35). Being a remarkably stable protein, this should not pose a problem. The oldest age groups were screened during the first two years of the trial, predisposing for longer sample storage before a PhiCal test was performed at leisure. FOBT was performed on attendance. A total of 6266 (63%) out of 9990 eligible for screening attended the FS-FOBT arm of the study.

Statistics

Individuals were categorised according to the single most significant lesion found at screening or baseline work up of screen positives (that is, cancer; high risk adenoma (adenoma ≥10 mm diameter and/or adenoma with severe dysplasia and/or adenoma with villous components); low risk adenoma (adenoma not fulfilling the criteria for high risk adenoma or carcinoma); or no neoplasia (no adenoma or carcinoma)). The χ² test was used for comparative analysis of categorical data, and non-parametric analysis of variance (Kruskal-Wallis test) for quantitative data. A non-parametric test was chosen due to highly right skewed data distributions. When the overall analyses resulted in significance (p<0.05), the subgroup or category which tended to differ was identified by pairwise comparisons employing the χ² test and Mann-Whitney test for categorical data and quantitative data, respectively. To avoid spurious statistical significance due to multiple comparisons, the level of significance was reduced to 0.01 for each of the three comparisons being performed in those cases (table 1).

A logistic regression model was applied using a positive or negative PhiCal test as the dependent binary variable. Age of screenee, month of the year for stool sampling, and duration of stool sample storage before calprotectin analysis (in months) were included as continuous covariates, with sex, CRC, and CRC groups as categorical variables. The SPSS statistical software, version 11.0, was used (SPSS Inc, Chicago, Illinois, USA).

Ethics

The regional ethics committee and the National Institute of Data Inspection approved the study protocol. Written informed consent was obtained from all participants before entering the trial.

Table 1 Relationship between the selected fraction (n = 2321) analysed for faecal calprotectin and the total flexible sigmoidoscopy-faecal occult blood test (FS-FOBT) group (%)

<table>
<thead>
<tr>
<th>Most significant lesion, if any, at FS and/or colonoscopy</th>
<th>No of screeners examined with FS-FOBT</th>
<th>No of screeners delivering a frozen stool specimen</th>
<th>No of screeners examined with calprotectin analysis</th>
<th>Calprotectin (μg/g) (median [range])</th>
<th>Positive PhiCal test</th>
</tr>
</thead>
<tbody>
<tr>
<td>No adenoma</td>
<td>5192</td>
<td>4413 (85)</td>
<td>1518 (29)</td>
<td>21.5 [55.2] (15.6–2092.2)</td>
<td>363 (24)</td>
</tr>
<tr>
<td>Low risk adenoma</td>
<td>796</td>
<td>681 (86)</td>
<td>592 (74)</td>
<td>21.0 [46.7] (15.6–772.0)</td>
<td>152 (26)</td>
</tr>
<tr>
<td>High risk adenoma</td>
<td>258</td>
<td>231 (90)</td>
<td>195 (76)</td>
<td>24.0 [51.7] (15.6–616.8)</td>
<td>52 (27)</td>
</tr>
<tr>
<td>CRC</td>
<td>20</td>
<td>17 (85)</td>
<td>16 (80)</td>
<td>66.1 [156.5] (15.6–1245.0)**</td>
<td>10 (63)**</td>
</tr>
<tr>
<td>Total</td>
<td>6266</td>
<td>5342 (85)</td>
<td>2321 (37)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CRC, colorectal cancer.

**p<0.01 compared with the three other categories, assessed by the Mann-Whitney test and the χ² test for quantitative and categorical data, respectively. Overall significance (p<0.05) was initially achieved by the Kruskal-Wallis test and the χ² test for the two types of data, respectively.
RESULTS

In the FS-FOBT group, 6266 (63%) of 9990 attended for FS, 5098 (51%) with a valid FOBT test, and 5342 (53%) individuals brought a fresh frozen stool sample for calprotectin analysis.

Faecal calprotectin was analysed in 2321 individuals (49% men) with a mean age of 58 years (range 50–64). There was no difference in faecal calprotectin concentrations in the three groups with no adenoma, low risk adenoma, and high risk adenoma, respectively (p = 0.19), whereas the group with CRC showed significantly higher calprotectin values than each of the other groups (table 1). Similarly, there was no difference between these first three groups in the frequency of a positive PhiCal test (p = 0.55). Again, the CRC group had a higher positivity rate than any of the other groups (table 1). Thus only individuals with cancer (all asymptomatic) demonstrated a significantly increased odds ratio (OR) of having a positive PhiCal test (p < 0.01) (table 2).

There was no difference in calprotectin levels or in the OR for having a positive PhiCal test in any one month of the year in which stool was sampled.

For those delivering valid samples for FlexSure OBT and having a PhiCal test, the overall positivity rate was 12% and 25%, respectively (p<0.01) (table 3). The OR for “any neoplasia” giving a positive FlexSure OBT was 1.72 (95% confidence interval (CI) 1.32–2.24) (p<0.01) compared with 1.11 (95% CI 0.91–1.36) for the PhiCal test. The PhiCal test was also poorer in differentiating between advanced lesions (high risk adenoma or CRC) on the one hand and no neoplasia or low risk lesions on the other (table 3). The OR for advanced lesions giving a positive test compared with low risk or no adenoma detected at FS was 1.25 (95% CI 0.91–1.72) for PhiCal and 5.16 (95% CI 3.72–7.14) (p<0.01) for FlexSure OBT. Although there was a considerable difference between the high and low risk adenoma groups in size distribution (76% >10 mm v none, respectively), the PhiCal positivity rate was 26% in both groups and similar to the group with no neoplasia (24%) (table 3). Thus the sensitivity for “any neoplasia” was 27% (204 of 766) with a specificity of 76% (1090 of 1427) for the PhiCal test. Corresponding values were 16% (121 of 766) and 90% (1289 of 1427) for FlexSure OBT (table 3).

In total, 666 individuals with neoplasia had a valid FOBT, faecal calprotectin analysis, and successful caecal intubation at colonoscopy. The difference in test performance observed in the total material was verified in this subgroup subjected...
to full colonoscopy. Again, the ORs for advanced lesions giving a positive test compared with low risk adenomas was 1.07 (95% CI 0.73–1.57) for PhiCal and 4.86 (95% CI 3.84–7.34) for FlexSure OBT (p < 0.01). Sensitivity for advanced neoplasia (CRC or high risk adenoma) was 27% (51 of 186) and 35% (65 of 186), respectively, for PhiCal and FlexSure OBT (table 4). Collectively, both tests failed to identify 88 (51%) patients with high risk adenoma but only one of 12 CRCs.

**DISCUSSION**

The overall positivity rate was 25% for the PhiCal test and 12% for FlexSure OBT, with poorer sensitivity for advanced neoplasia and poorer specificity when using the former test alone.

The present study on calprotectin in stool samples from 2321 individuals is, as far as we are aware, the largest ever and the only one with recruitment from a large-scale screening study of a population at average risk for CRC. A sensitivity of 67% for CRC is very much in accordance with that observed by Johne et al. (64%) when using one sample from one stool and the recommended 50 µg/g as the cut-off for the PhiCal test. Similar to our study, Kronborg and colleagues found no difference in calprotectin levels when comparing one spot samples from adenoma patients and patients with no polyps at colonoscopy. They however find increased levels in stools from adenoma patients when performing two spots in each of 1–2 stools. But independent of the number of spots tested, they found no reduction in faecal calprotectin levels 6–12 weeks after polypectomy, suggesting that increased faecal calprotectin may be due to a general mucosal defect rather than the mere presence of adenomas. Differences in possible sources of marker protein cannot be recommended for population screening purposes.

**Table 4** Sensitivity (%) of PhiCal and FlexSure OBT for asymptomatic neoplasia in 666 screenees delivering a valid FlexSure OBT test card, having a faecal calprotectin analysis, and being examined to the caecum at colonoscopy

<table>
<thead>
<tr>
<th></th>
<th>Low risk adenoma (n = 480)</th>
<th>High risk adenoma (n = 174)</th>
<th>CRC (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive PhiCal</td>
<td>125 (26)</td>
<td>43 (25)</td>
<td>8 (67)</td>
</tr>
<tr>
<td>Positive FlexSure OBT</td>
<td>48 (10)</td>
<td>56 (32)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Any one test positive</td>
<td>131 (27)</td>
<td>77 (42)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Both tests positive</td>
<td>21 (4.4)</td>
<td>13 (7.3)</td>
<td>6 (50)</td>
</tr>
</tbody>
</table>

In conclusion, the performance of a single spot from one stool sample using the PhiCal test in colorectal screening was poorer than a single screening round with FlexSure OBT and cannot be recommended for population screening purposes.

**CONCLUSIONS**

We owe thanks to the Norwegian Cancer Society and the Ministry of Health and Social Affairs for financial support and to the Norwegian Gastrointestinal Cancer Group (NGICG) for initiating the study. The technical assistance of Kristin Hoimyr, Jorunn Bratlie, Tove Hamborg, and Marianne Nilsen was greatly appreciated, as were the samples for certification and re-certification of nurses supplied by Beckman-Coulter Inc.
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