Blood based colon cancer screening in Europe

Methylated Septin 9:
Biomarker of malignant development in the colon
Measured in Tissue and Plasma

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Continuous colorectal epithelial proliferation is the basis of CRC development.

**Red:** PCNA, divided cell nucleus

**Green:** Tunnel reaction, apoptotic cell nucleus

**Blue:** Calm cell nucleus
The genetic alterations in the colorectal adenoma-dysplasia-carcinoma sequence

Colorectal cancer development: a multistep, multiparameter process.
Screening of colorectal cancer by imaging

<table>
<thead>
<tr>
<th>Type of screening</th>
<th>Method Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| **Endoscopic exams** | **Flexible sigmoidoscopy:** Visual examination of the rectum and lower third of the colon by insertion of a flexible tube into the colon. | • Requires minimum preparation  
• No sedation required | • Invasive  
• Tumors in the upper colon may not be detected |
| | **Colonoscopy:** Direct visual examination of the entire colon and rectum with removal of polyps | • Visualizes entire bowel  
• Allows biopsies and polypectomies and therefore may be preventive | • Invasive  
• Requires bowel preparation  
• Sedation required |
| | **Colon capsule endoscopy:** Visualization of colon through swallowed pill-sized camera | • Complete evaluation of the gastrointestinal tract | • Requires bowel preparation  
• Capsule retention  
• After ingesting until excretion powerful electromagnetic fields should be avoided |
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<th>Disadvantages</th>
</tr>
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</table>
| Radiologic examinations | **Computed tomographic colonography (CTC):** 2- or 3-dimensional x-ray views of entire colon and rectum | - Minimally invasive  
- Procedure performed within 10 mins  
- No recovery time  
- No Sedation required | - Requires bowel preparation  
- Colonoscopy may be performed afterwards to remove any suspicious polyps  
- May require drinkable contrast solution  
- Radiation exposure |
|                     | **Double contrast barium enema (DCBE):** Usually view of entire colon; complete radiological examination of the colon | - Minimally invasive  
- Detects cancers and majority of polyps  
- Evaluates entire colon | - Requires bowel preparation  
- Colonoscopy may be performed afterwards to remove any suspicious polyps  
- Radiation exposure |
## Screening of colorectal cancer by stool test

<table>
<thead>
<tr>
<th>Type of screening</th>
<th>Method Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool tests</td>
<td>Fecal occult blood tests (FOBT): Guaiac-based (gFOBT) Immunochemical (iFOBT) Detection of tumor cell DNA shed into large bowel M2-PK: Detection of specific enzyme M2-PK</td>
<td>• Non-invasive • Inexpensive • Can detect large bleeding polyps</td>
<td>• Non-bleeding tumors not detected • Dietary restrictions for higher accuracy • Repeat samples needed • High costs for stool test • High rate of false-positives</td>
</tr>
</tbody>
</table>
Issues of the screening programs in Europe:
- Capacity of the organisations
- Costs of the programmes
- Sensitivity/specificity of the methods
- Patients’s compliance and acceptance, attitude


Blood based cancer screening markers: clinical and routine aspects:
- Simplicity
- Patient’s acceptance
- Patient’s compliance towards existing methods
Tumor (protein) markers in peripheral blood

CA19-9  Combination markers of serum tumor markers
CEA
AFP

No Screening application of today
Recurrence detection, follow-up examinations only
mRNA expression based colorectal cancer peripheral blood test

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Method</th>
<th>Test</th>
<th>Producer</th>
<th>Sensitivity [%]</th>
<th>Specificity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANXA3, CLEC4D, LMNB1, PRRG4, TNFAIP6, VNN1, IL2RB</td>
<td>mRNA expression of 7 gene biomarker combination</td>
<td>ColonSentry™</td>
<td>GeneNews (Canada)</td>
<td>72</td>
<td>70</td>
</tr>
</tbody>
</table>
Potential plasma biomarkers for future CRC screening:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Type of marker</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>microRNA in serum</td>
<td>83-90</td>
<td>90-91</td>
</tr>
<tr>
<td>BIRC5</td>
<td>mRNA in serum</td>
<td>84.8</td>
<td>80</td>
</tr>
<tr>
<td>SDC2</td>
<td>Methylated biomarker in serum</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td>KIAA1199</td>
<td>qRT-PCR for measurement of mRNA in plasma</td>
<td>77.5</td>
<td>95</td>
</tr>
<tr>
<td>ONC107</td>
<td>Mass spectrometry assay of serum proteins</td>
<td>94</td>
<td>83</td>
</tr>
</tbody>
</table>
Circulating free DNA in peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>IBD</th>
<th>Adenoma</th>
<th>CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (ng/ml)</td>
<td>176,882</td>
<td>250,217</td>
<td>193,525</td>
<td>566,568</td>
</tr>
<tr>
<td>SD</td>
<td>82,008</td>
<td>156,97</td>
<td>111,369</td>
<td>231,012</td>
</tr>
</tbody>
</table>

Healthy vs. IBD 0,3143
Healthy vs. Adenoma 0,9254
Healthy vs. CRC 0,0391
Source of plasma circulating tumor cells and tumor derived DNA
Why is methylated Septin 9 a good CRC biomarker?

Septin 9 an absolute (100%) colorectal cancer biomarker

How is methylated Septin 9 a suitable CRC screening marker?

NGS sequencing of Plasma circulating free DNA
Methylated Septin9 in plasma

EpiProColon is a good CRC screening test?

Ver.1 ; Ver. 2.0
DNA methylation: an alternative mechanism to mutation for gene silencing

- DNA methylation of CpG islands located in the promoter region can lead to repression of transcription
- Dysregulation of DNA methylation is proven to contribute to the formation of colorectal cancer
- DNA methylation is reversible, and can be influenced by demethylation agents (e.g. 5-aza-2'-deoxycytidine)
Methylation of Septin 9 in CRC at the tissue level

Wasserkort et al. BMC Cancer 2014
Septin 9 methylation in CRC tissue

PMR for "SEPT9 in tissue"

PMR >1% found in

NED 7.7% (1/13)
Adenoma 100% (10/10)
CRC biopsies 100% (16/16)

PMR = percent methylation reference
Septin9 RNA levels in CRC laser micro-dissected samples

1. Cutting of the tissue compartments by focused laser ray
2. Removal of the samples from slide
3. RNA and DNA isolation from the cells
4. Affymetrix whole genomic microarray analysis array (>47000 transcripts)

SEPTIN9 protein expression correlates with methylation and RNA expression in the N-Ad-Dvsp1-CRC sequence.
Septin 9 in colorectal cancer

1. Methylated Septin 9 correlated with decreased protein – IHC
2. Methylated Septin 9 correlated with decreased mRNA transcription
3. Zero/0 levels of methylated Septin 9 in normal epithelia
4. Laser capture studies confirm results
NGS sequencing (Solid) of plasma, free DNA, Septin 9 gene

Adenoma | CRC | Normal | IBD
50-50 pooled specimen, 1000 base pairs resolution
NGS sequencing data of circulating free DNA: Septin 9

\[ \downarrow \text{Cpg island} \]
Application of the Epi proColon plasma Methylated Septin9 assay in the clinical workflow
Worksteps and components of the Epi proColon kit

Sample Preparation: 4.5 hrs
Epi proColon 2.0 CE Plasma Quick Kit

BisDNA Duplex PCR: 3.5 hrs
Epi proColon 2.0 CE Sensitive PCR Kit

Plasma → DNA Extraction → Bisulfite Conversion → DNA Purification → PCR1 → PCR2 → PCR3

ACTB Internal Control

Pause Point

Epi proColon 2.0 CE Control Kit

External Negative Control

Pause Point

External Positive Control

Time to Results: 8 hrs
# Semi-Automated Septin 9 PCR workflow

<table>
<thead>
<tr>
<th>Manual sample preparation</th>
<th>Prepared PCR plate</th>
<th>Automated reaction preparation</th>
<th>Quantitative PCR measurements 3x</th>
</tr>
</thead>
</table>
Methods

1. Sample collection

2. Total DNA isolation
   - plasma: Epi proColon 2.0 plasma DNA preparation kit (Epigenomics AG, Berlin, Germany).

3. Bisulfite conversion
   - non-methylated cytosines are converted to uracil

4. Quantitative determination
   - Epi proColon 2.0 Real time-PCR kit

mSEPT9 duplex real-time PCR analysis
# Plasma mSEPT9 results in the development phase

<table>
<thead>
<tr>
<th>Publication</th>
<th>Test Version</th>
<th>Algorithm</th>
<th>Sensitivity, 95% CI</th>
<th>Specificity, 95% CI</th>
<th>CRC I, 95% CI</th>
<th>CRC II, 95% CI</th>
<th>CRC III, 95% CI</th>
<th>CRC IV, 95% CI</th>
<th>Adenoma</th>
<th>Polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>deVos, 2009</td>
<td>Research Kit</td>
<td>1 of 3</td>
<td>72% [ND]</td>
<td>86% [ND]</td>
<td>53% [ND]</td>
<td>75% [ND]</td>
<td>78% [ND]</td>
<td>100% [ND]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Grützmann, 2008</td>
<td>Research Kit</td>
<td>2 of 3</td>
<td>58% [49-67%]</td>
<td>90% [85-93%]</td>
<td>36% [17-59%]</td>
<td>56% [38-72%]</td>
<td>65% [51-77%]</td>
<td>73% [39-94%]</td>
<td>18% [4-43%]</td>
<td>9% [2-34%]</td>
</tr>
</tbody>
</table>
## Plasma mSEPT9 results Epi proColon 1.0

<table>
<thead>
<tr>
<th>Publication</th>
<th>Test Version</th>
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<th>Sensitivity, 95% CI</th>
<th>Specificity, 95% CI</th>
<th>CRC I, 95% CI</th>
<th>CRC II, 95% CI</th>
<th>CRC III, 95% CI</th>
<th>CRC IV, 95% CI</th>
<th>Adenoma</th>
<th>Polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weiss, 2010</td>
<td>Epi proColon 1.0</td>
<td>1 of 2</td>
<td>67% [57-76%]</td>
<td>88% [82-92%]</td>
<td>44/66*</td>
<td>25/37*</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Church, 2014</td>
<td>Epi proColon 1.0</td>
<td>1 of 2</td>
<td>51% [32-64%]</td>
<td>91% [90-93%]</td>
<td>36% [13-60%]</td>
<td>57% [33-88%]</td>
<td>58% [17-85%]</td>
<td>80% [24-100%]</td>
<td>10% [7-16%]</td>
<td>8% [4-11%]</td>
</tr>
<tr>
<td>Church, 2014</td>
<td>Epi proColon 1.0 plus additional PCR</td>
<td>1 of 3</td>
<td>64% [48-80%]</td>
<td>88% [87-90%]</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14% [ND]</td>
<td>ND</td>
</tr>
</tbody>
</table>
# Plasma mSEPT9 results: Epi proColon 2.0

<table>
<thead>
<tr>
<th>Publication</th>
<th>Test Version</th>
<th>Algorithm</th>
<th>Sensitivity, 95% CI</th>
<th>Specificity, 95% CI</th>
<th>CRC I, 95% CI</th>
<th>CRC II, 95% CI</th>
<th>CRC III, 95% CI</th>
<th>CRC IV, 95% CI</th>
<th>Adenoma</th>
<th>Polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tóth, 2012</td>
<td>Epi proColon 2.0</td>
<td>1 of 3</td>
<td>95.6% [89.2-98.8%]</td>
<td>84.8% [75.8-91.4%]</td>
<td>84% [ND]</td>
<td>100% [ND]</td>
<td>100% [ND]</td>
<td>100% [ND]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tóth, 2012</td>
<td>Epi proColon 2.0</td>
<td>2 of 3</td>
<td>79.3% [69.6-87.1%]</td>
<td>98.9% [94.1-100%]</td>
<td>60% [ND]</td>
<td>92.8% [ND]</td>
<td>88.6% [ND]</td>
<td>77.8% [ND]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Potter, 2014</td>
<td>Epi proColon US</td>
<td>1 of 3</td>
<td>68% [53-80%]</td>
<td>80% [78-82%]</td>
<td>64% [48-77%]</td>
<td>100% [57-100%]</td>
<td>22% [18-24%]</td>
<td>20% [16-24%]</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Johnson, 2014</td>
<td>Epi proColon US</td>
<td>1 of 3</td>
<td>73% [64-81%]</td>
<td>82% [76-86%]</td>
<td>62% [43-78%]</td>
<td>80% [58-92%]</td>
<td>65% [45-81%]</td>
<td>92% [67-100%]</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Detection of CRC stages by plasma mSEPT9 assay

Positive detection rate of SEPT9 assay at each colorectal cancer stage

- Grutzmann et al., 2008
- Lofton-Day et al., 2008
- deVos et al., 2009
- Church et al., 2010
- Warren et al., 2011
- Toth et al., 2012
- Church et al., 2013
- Lee et al., 2013
## Laboratory derived tests for Septin 9

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Sens.</th>
<th>Spec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septin 9</td>
<td>LDT</td>
<td>RealTime mS9 ColoRectal Cancer assay</td>
<td>Abbott Molecular</td>
</tr>
<tr>
<td>Septin 9</td>
<td>LDT (US)</td>
<td>ColoVantage</td>
<td>Quest Diagnostics</td>
</tr>
<tr>
<td>Septin 9</td>
<td>LDT (US)</td>
<td>SEPT9 LDT</td>
<td>ARUP Laboratories</td>
</tr>
<tr>
<td>Septin 9</td>
<td>LDT (Canada)</td>
<td>SEPT9 LDT</td>
<td>Gamma Dynacare</td>
</tr>
</tbody>
</table>
Septin 9 methylation in plasma of adenoma/cancer patients

PMR for "SEPT9 in plasma"

PMR > 0.01% found in

- NED 10% (2/20)
- Adenoma 25% (5/20)
- CRC 85% (17/20)

PMR = percent methylation reference
Septin 9 colon cancer: test limitations

- Pregnancy: always positive 100%!
- Inflammatory conditions:
  as normals in the sens. and specs.
Technical advances can influence the SEPTIN9 methylated DNA based plasma testing

- DNA isolation: automation for higher plasma volume, higher DNA yield (adenoma patients)
- Bisulfite conversion: decrease in loss of DNA
- Digital PCR: increased sensitivity and single copy analysis
- DNA release induction: Proapoptotic induction therapy NSAIDs
Methylated Septin9 and the Epi ProColon test:

- Clear scientific support for a role in colorectal cancer
  - mRNA, IHC, DNA studies on Septin9 in tissue
- Clinically validated biomarker for colorectal cancer screening
  - Real-Time PCR, Next Gen Sequencing - plasma
- Epi proColon – a CE marked IVD for colorectal cancer screening
  - Complete kit for IVD use – plasma based screening for colorectal cancer