Early detection of colorectal cancer:
towards better surveillance

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ISBN: 9789463804882

Cover: Douwe Hoendervanger
Drukwerk en layout: Proefschriftmaken.nl

The printing of this thesis was financially supported by Nederlandse Vereniging voor Gastroenterologie, Sysmex Europe GmbH and NKI Antoni van Leeuwenhoek.

This research was financially supported by Alpe d'Huzes, Dutch Cancer Society.

Early detection of colorectal cancer:
towards better surveillance

Vroeg detectie van darmkanker:
op weg naar betere surveillance

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad doctor aan de Universiteit van Utrecht
op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolgde het besluit van het college voor promoties in het openbaar te verdedigen op
dinsdag 29 oktober 2019 des ochtends te 10.30 uur

door

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geboren op 22 september 1988 te Boston,
Verenigde Staten van Amerika

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voor P & M

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GENERAL INTRODUCTION

“To see what everybody else has seen,
and to think what nobody else has thought”

Albert Szent-Gyorgyi

1.

General introduction and outline of this thesis

General introduction

Burden of colorectal cancer

Colorectal cancer (CRC) is a common and deathly disease. It has a high incidence of 1.7 million annually, and is the second most common cause of cancer death worldwide comprising 9.2% of total cancer cases.[1] In the Netherlands, its incidence has increased gradually over the past years with an average annual rate of 1.3%, while CRC-related mortality has decreased in the same period with an average annual rate of 0.5%.[2] This trend is partly the result of improved management and treatment regimens for CRC,[3,4] but also related to efforts to improve its early detection.[5,6] As the 5-year survival rate of CRC is highly dependent on stage, ranging from 90% when diagnosed at stage I to a disappointing 14% at stage IV, early detection is worthwhile.[7]

Development of colorectal cancer

CRC develops from at least two types of precursor lesions, adenomas and serrated polyps, together referred to as polyps. Although the importance of adenomas in CRC development was described already in the 1960s,[8] the molecular events underlying the neoplastic initiation and progression towards cancer were presented only decades later.[9] The adenoma-to-carcinoma sequence is typically initiated by an inactivating mutation of the *APC* gene[9] and characterised by chromosomal instability.[10] Chromosomal instability accelerates the rate of gains and losses of whole or larger portions of chromosomes, leading to somatic DNA copy number alterations. Approximately 85% of sporadic CRCs develop through this adenoma-to-carcinoma sequence.[11] The remaining 15% of CRCs are thought to develop through a serrated neoplasia pathway.[12] Here, mutation of the *BRAF* gene is a typical early event, leading to CpG-island methylation phenotype (CIMP) and inactivation of mismatch repair (MMR) genes.[13,14] As a consequence, many mutations accumulate. Short repeated nucleotide sequences are especially prone to sequencing mistakes, becoming evident as microsatellite instability (MSI).[15,16]

Besides their molecular differences, adenomas and serrated polyps also differ morphologically and histologically. Serrated polyps are often flat or flat elevated lesions, have a subtle endoscopic appearance and are more frequently located in the proximal colon.[17] Progression from both adenomas and serrated polyps to CRC seems to take 10 to 15 years.[18,19]

Early detection of colorectal cancer

Due to both the stage-related survival and long dwell time of polyps, CRC lends itself well for early detection strategies.[20] Advanced neoplasia is generally used as target for early detection and includes CRC and advanced adenomas.[21] Advanced adenomas are defined based on phenotypical features that were previously associated with a higher risk of malignancy, *i.e.* size ≥10mm, villous histology or high grade dysplasia.[22,23] Since the discovery of serrated polyps as precursor lesions of CRC, also advanced serrated polyps, defined as ≥10mm or dysplastic serrated polyps, are proposed to be included in the definition of advanced neoplasia.[24,25]

In the Netherlands, the national colorectal cancer screening program started in 2014. The program aims to reduce CRC morality by diagnosing cancers in an asymptomatic, curable stage, as well as by preventing cancers through the resection of polyps. All individuals between the age of 55 and 75 are biennially invited to perform faecal immunochemical test (FIT) sampling at home. In case of a positive test colonoscopy is offered. Of the 2 million Dutch individuals that are invited each year, approximately 5% tests positive.[21] At subsequent colonoscopy, CRC is found in 7% and advanced adenomas in 39%.[21] Because these patients remain at risk even after these lesions are cleared, they are recommended to have regular follow-up colonoscopies. The monitoring of the high-risk population is referred to as surveillance. It is estimated that at present 50,000 individuals are enrolled in the Dutch surveillance program, consuming around 25% of the total colonoscopy capacity.

Surveillance of colorectal cancer in the Netherlands

The Dutch surveillance guideline[26] concerns all patients that have an indication for surveillance due to an increased risk of advanced neoplasia and include:

1. Patients with prior polypectomy
2. Patients with prior curative CRC resection
3. Patients with a familial CRC risk (here we only focus on familial CRC; hereditary forms of CRC are outside the scope of this thesis)

The guideline only applies to patients with a previous high-quality colonoscopy with radical resection of all lesions.

Patients with prior polypectomy constitute the largest part of the surveillance population. Multiple studies have shown that the risk of metachronous CRC is dependent on the polyp characteristics at index colonoscopy.[27,28] Therefore, patients are stratified into different risk categories. Based on associations between index polyp characteristics and recurrent advanced neoplasia,[29] a scoring system was developed to decide the optimal surveillance interval for individual patients (Table 1 and Table 2).

Also, the cancer risk of patients diagnosed with CRC remains elevated after intended curative resection. Recent studies reported that this risk is most pronounced in the first years after resection.[30,31] Therefore, the Dutch guideline recommends the first surveillance colonoscopy to take place one year after resection. In some instances, tumour stenosis may hamper a complete preoperative colonoscopy. In those cases, either a preoperative CT colonography, or a postoperative surveillance colonoscopy within 3 months should be performed.

The term familial CRC is reserved for families that do not have hereditary CRC, but do have a clinically relevant increased risk of CRC based on family history.[32] The prevalence of individuals that fulfil the criteria of familial CRC[32] in the general population is 0.5-1%.[26] Because the life-time risk for patients that fulfil the criteria of familial CRC is >10%, they are advised surveillance colonoscopies every 5 years from the age of 45 onwards.

Table 1 | Score table for the presence of polyp characteristics

Adenoma characteristics	Value	Score
Number of adenomas	0 - 1 2 - 4 ≥ 5	0 1 2
Presence of at least one adenoma ≥10mm and/or one serrated polyp* ≥10mm	No Yes	0 1
Presence of at least one villous** adenoma	No Yes	0 1
Presence of at least one proximal‡ adenoma	No Yes	0 1
Total score		

Table 2 | Surveillance interval, based on score from Table 1

Score during index colonoscopy	Interval after index colonoscopy
0	No surveillance*
1 – 2	5 years
3 – 5	3 years
Score at subsequent colonoscopy	**Interval after subsequent colonoscopy**
0	5 years**
1 – 2	5 years
3 – 5	3 years

The Dutch guideline illustrates that the surveillance population is made up by a mix of patients with varying histories and risk profiles. This is of importance when considering alternative surveillance strategies.

Problems associated with the surveillance strategy

Discussion exists on the added value of the current surveillance program. On the one hand, colonoscopy surveillance has been reported to reduce CRC three- to four fold compared to no surveillance.[33,34] On the other hand, these data are generated in the pre-screening period. The question has arisen whether the added value of surveillance is maintained on top of screening. Besides this effectiveness issue, the surveillance program is associated with several other drawbacks. First, the criteria defining the different risk categories, as well as the length of the surveillance intervals, are different for guidelines around the world.[24,26,30,35–37] For instance, according to the European guideline a patient with a 15mm villous adenoma in the distal colon is categorised as high-risk and should be examined after an interval of 3 years,[24] whereas the Dutch guideline recommends a 5 year surveillance interval.[26]

In the same way, the British guideline recommends the first surveillance colonoscopy after curative resection of CRC to take place after an interval of five years,[37] while the Dutch guideline recommends a one-year interval.[26] These differences are due to a lack of randomised trials that have examined the effect of different surveillance intervals on long-term outcomes per risk

group. Second, due to implementation of screening program, surveillance colonoscopies are an increasing burden for patients as well as society. For patients, drawbacks are that colonoscopy is an invasive and uncomfortable procedure, associated with a risk of complications, such as bleeding or perforation.[38] From a society's point of view, surveillance is expected to consume a larger proportion of endoscopy and health care resources. This results in high costs and may lead to longer waiting times for other indications. Third, with colonoscopies generally all polyps are detected and removed, while only an estimated 5% would have progressed to cancer.[23] As a consequence, many patients are being treated for lesions that would never have done any harm. The molecular alterations associated with progression may provide a way to more precisely pinpoint premalignant lesions that are at high risk to become malignant.

Alternative surveillance strategies

In short, there is a need for alternative and less invasive surveillance strategies. An alternative could come from a two-step program like stool-based surveillance strategies, selecting patients for treatment with colonoscopy. Similar to screening, FIT may be an appropriate triage tool to use in surveillance. However, with this test, one in four CRCs and two in three advanced neoplasia are missed, which is especially undesirable in the high-risk surveillance population.[39] The multi-target stool DNA test (mt-sDNA test) combines the detection of human haemoglobin with several DNA markers and was found to detect advanced neoplasia with significantly higher sensitivity than FIT.[40] The lower specificity, higher costs and more complicated stool collection could provide less of a problem in the surveillance population, compared to the screening population.

The studies presented in this thesis aimed to improve current surveillance strategies for the early detection of CRC, by studying more closely the molecular alterations associated with colorectal carcinogenesis and evaluating the applicability of alternative surveillance strategies in the clinic.

Outline of thesis

In **part I** of this thesis, biological concepts that could facilitate early detection strategies of CRC were explored. In **chapter 2** we aimed to describe how the identification of molecular signatures that define different stages of progression have enabled early detection of CRC. Certain somatic DNA copy number alterations have been found to be major drivers in CRC development. In **chapter 3** a novel diagnostic assay was validated to detect these copy number alterations in a simple and low-cost manner. The novel method was compared with low-coverage whole-genome sequencing (LC WGS), which is one of the methods commonly used for DNA copy number profiling. In **chapter 4**, an exceptional cohort of longitudinal observed small polyps was analysed for DNA alterations, including copy number alterations, mutations, methylation status and MMR proficiency, in order to enhance our understanding of their natural behaviour.

In **part II** of this thesis, several alternative surveillance strategies were evaluated in the clinic. **Chapter 5** explains the role of colonoscopy as primary screening method and the way surveillance colonoscopies may add to the protective effect. It is illustrated how the 'high-detection paradox' likely leads to overuse of surveillance colonoscopies. Stool-based testing in surveillance could reduce colonoscopy use by preselecting those patients with advanced neoplasia. In **chapter 6** the protocol of a large cross-sectional study is outlined, which aimed to evaluate whether stool-based surveillance could be an alternative to colonoscopy-based surveillance. The interim results of this study are presented in **chapter 7**, in which we determined the accuracy for the detection of advanced neoplasia of the mt-sDNA test, as well as FIT. It is described how compared to screening, lower test cut-offs could be applied in surveillance to increase sensitivity and reduce miss rates for advanced neoplasia. One drawback of the mt-sDNA test is that it is also less practical and more costly than the FIT, which could lead to reduced participation and a higher number of analytical drop-outs. For this reason, we sought in **chapter 8** whether the performance of FIT could be increased by adding clinicopathological risk factors into a diagnostic prediction model. In this analysis, patients with recent CRC were excluded, because of their previously demonstrated pronounced CRC risk in the first few years after resection. For this group of patients, it could appear reasonable to maintain colonoscopy surveillance. In **chapter 9** the risk of CRC after resection was examined more closely, to evaluate whether this risk justifies the colonoscopy burden that is associated with the one-year surveillance interval currently recommended.

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PART I.

Biological concepts for early cancer detection

“The cleverest of all, in my opinion, is the man who calls himself a fool at least once a month”

Fyodor Dostoevsky

2.

Early detection: the impact of genomics

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Virchows Archiv 2017

Abstract

The field of genomics has shifted our view on disease development by providing insights in the molecular and functional processes encoded in the genome. In the case of cancer, many alterations in the DNA accumulate that enable tumor growth or even metastatic dissemination. Identification of molecular signatures that define different stages of progression towards cancer can enable early tumor detection. In this review the impact of genomics is addressed using early detection of colorectal cancer (CRC) as an example. Increased understanding of the adenoma-to-carcinoma progression has led to the discovery of several diagnostic biomarkers. This combined with technical advancements, has facilitated the development of molecular tests for non-invasive early CRC detection in stool and blood samples. Even though several tests have already made it to clinical practice, sensitivity and specificity for the detection of precancerous lesions still need improvement. Besides the diagnostic qualities, also the accuracy of the intermediate endpoint is an important issue on how the effectiveness of a novel test is perceived. Here progression biomarkers may provide a more precise measure than the currently used morphologically-based features. Similar developments in biomarker use for early detection have taken place in other cancer types.

Introduction

In the mid of the 19th century Virchow postulated alterations at the cellular level as the basis of disease. Since then, wide spread analysis of molecular alterations has increased our understanding of disease causes tremendously, and resulted in large scale genomic annotations of disease phenotypes, in particular cancer.[1, 2] Many of these DNA alterations are not simply random noise, but play causal roles in the disruption of critical biological processes in the cell, referred to as the hallmarks of cancer.[3] This disruption takes place in a stepwise manner during the pathogenesis of cancer. In the vast majority of cases this concerns epithelial cancers, in which specific molecular changes occur at different stages of tumor development from normal, through stages of intra-epithelial neoplasia to cancer. Consequently, the signature of one or more molecular changes in a tissue sample can mark the stage of cancer development, hence the term biomarker. Disruption in gene function can be caused in several different ways, including DNA mutations, copy number changes, structural rearrangements, promoter-methylation, and multiple post-transcriptional mechanisms such as microRNA (miRNA) regulation. As a result, a range of different molecular biomarkers is needed to identify such changes. The scope of the present review is to highlight the impact of genomics on the early detection of diseases, using colorectal cancer (CRC) as an example.

CRC lends itself well for early detection strategies as it fulfills a number of classic screening criteria formulated by Wilson and Jungner (Box 1).[4] As the third most common cancer in the world, with over 1.2 million new cancer cases and an estimated 600.000 deaths in 2008,[5] CRC imposes an important health problem. When diagnosed in an early stage, surgical or endoscopic resection results in an average five-year survival rate of more than 90%, whereas this rate decreases to less than 10% for stage IV CRC.[5] Colorectal adenomas are recognized as the precursor lesions of CRC and have a long dwell-time, providing an excellent window of opportunity for early detection. Furthermore, the natural history of the progression from normal epithelium, through the adenoma stage and further to cancer has been widely studied, including the underlying biology.

This illustrates already one way in which genomics has impacted early diagnostics. In addition, the application of genomic technologies to detect biomarkers depicting the presence of cancer or precursor lesions has provided new, less invasive strategies for early diagnosis compared to the current standard, *i.e.* colonoscopy. Population based screening programs are complex logistic operations, in which a trade-off needs to be made between aspects of different dimensions like under and over diagnosis, costs and patient preference.[6] Frequent evaluation of the programs' effectiveness is needed to ensure continuous improvement. The intended effect of screening is to reduce death from the cancer type screened for. This endpoint, however, can easily take one to two decades to be reached. As an alternative, intermediate endpoints are used, the precision of which in case of CRC is suboptimal. Also for this purpose genomics can be of help.

Box 1 | Wilson and Jungner classic screening criteria

1.	The condition sought should be an important health problem
2.	There should be an accepted treatment for patients with recognized disease
3.	Facilities for diagnosis and treatment should be available
4.	There should be a recognizable latent or early symptomatic stage
5.	There should be a suitable test or examination
6.	The test should be acceptable to the population
7.	The natural history of the condition, including development from latent to declared disease, should be adequately understood
8.	There should be an agreed policy on whom to treat as patients
9.	The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole
10.	Case-finding should be a continuing process and not a "once and for all" project

Genomics and the natural history of colorectal cancer

A full description of the molecular biology of colorectal cancer is beyond the scope of this review. Until recently, alternatives to the adenoma carcinoma progression pathway were hardly considered. Since then the serrated pathway has attracted considerable attention.[7] Yet, for demonstrating the impact of genomics for early detection of colorectal cancer, the focus will be on the adenoma-to-carcinoma concept that was postulated in the '70s of the last century by Morson and colleagues.[8]

Colorectal adenomas are a very common finding in elderly people with a prevalence of 18–35% reported in screening series.[9,10] Only about 5% of adenomas progress to cancer.[11] Presence of a focus of cancer in adenomas was found to be associated with their size, grade of dysplasia and histological type.[8] Extrapolating from observations in familial adenomatous polyposis (FAP), a hereditary disease with hundreds to thousands of adenomas, one or more of which ultimately become cancer, progression time from adenoma to cancer was estimated to be on average 10–15 years. The mechanism behind this hereditary disorder, *i.e.* disruption of the WNT signaling pathway by mutations in the *APC* gene, turned out to be the mechanism that is responsible for adenomagenesis in the sporadic setting (Figure 1).[12,13] When the APC gene is mutated, the formation of the destruction complex that normally leads to degradation of β-catenin, is prevented. As a consequence, β-catenin can accumulate and translocate to the nucleus where it activates transcription of TCF-regulated genes, resulting in proliferation of the cells.[14] In this way, APC mutations or other mechanisms that disrupt the WNT signaling pathway stimulate the growth of dysplastic cells that first take over the crypt and then slowly produce a polyp. As proposed by seminal work from Vogelstein *et al.* development into a larger polyp requires additional mutations in *KRAS* or *BRAF* oncogenes, and will only in some cases lead to cancer progression when also genes in the TGF-β and TP53 pathways are affected.[15] This first genomic underpinning of the stepwise adenoma to carcinoma progression has led to research that further unraveled the pathways in which the key mutated genes are involved *i.e.* WNT, RAS-MAPK, PI3K, TGF-β and TP53 pathways (Figure 1).

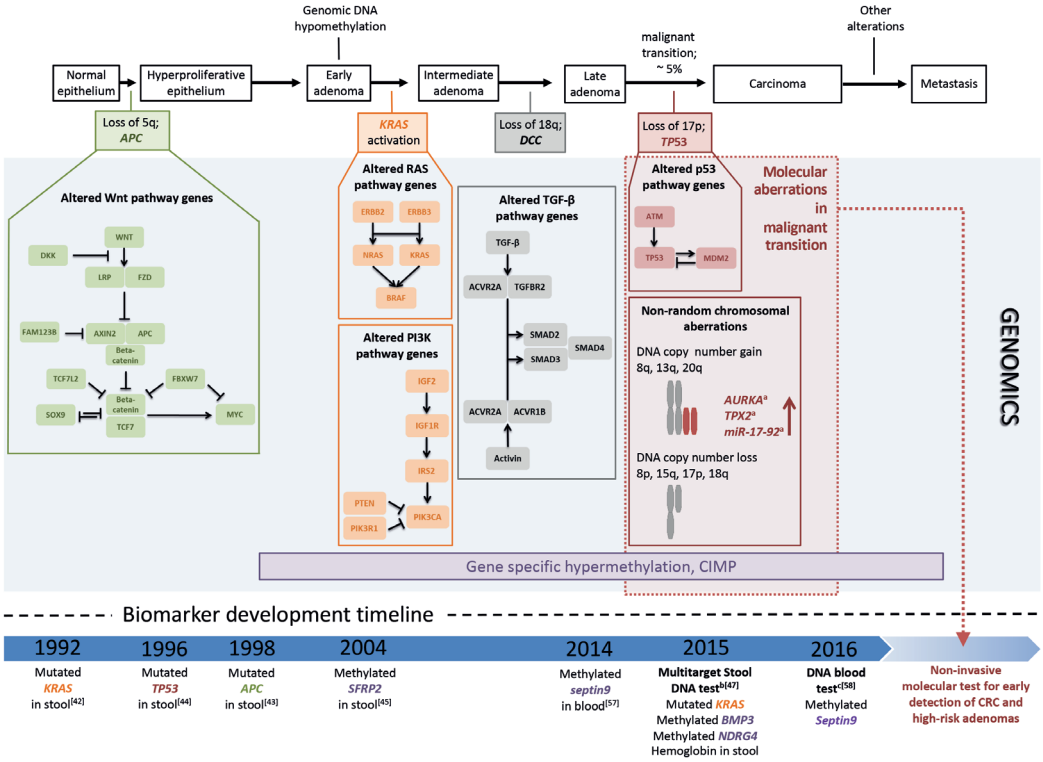

Figure 1 | The impact of genomics on the development of biomarkers for early detection of colorectal cancer

Schematic presentation of the genomic alterations underlying colorectal cancer development through the adenoma stage, also known as the 'Vogelgram',[15] and the impact on biomarker development over time. APC, KRAS and TP53 mutations have subsequently been identified as detectable in stool, as well as many hypermethylated genes of which SFRP2 was the first to be described. Further developments have led to the first FDA approved biomarker tests in 2015 and 2016. Genomics impacts early detection by increasing the knowledge of the underlying processes and disrupted molecular pathways,[2] leading to new and better biomarkers. Genomics further impacts early detection through the application of advanced genomic technologies to read out biomarkers. Molecular aberrations that contribute to the malignant transition of adenoma to carcinoma are of particular interest for the development of biomarkers that detect those adenomas with a high risk of progression in addition to detecting CRC.

a Examples of genes upregulated as a result of DNA copy number gain on chromosome 13q and 20q [24,25,33] b Cologuard test, FDA approved, developed by Exact Sciences corp. c Epi ProColon test, FDA approved, developed by Epigenomics AG

Next to mutations, also other mechanisms that contribute to disruption of gene function are relevant to adenoma-to-carcinoma progression. These are in particular promoter hypermethylation and chromosomal instability (CIN). Of these, promoter hypermethylation occurs as a more early event in adenoma to carcinoma progression.[16] The transcription of many genes is dependent on the methylation status of the CpG islands localized in their promoter region. When these CpG islands are hypermethylated, the binding of transcription factors to the promoter regions is inhibited, leading to direct inactivation of the gene. Hypermethylation of the WNT antagonist *SFRP* or the mismatch repair gene *MLH1* are examples of how

inactivation may initiate cancer related events.[17] Because alterations in DNA methylation can be detected by PCR-based assays even against large amounts of background DNA, they are excellent targets for early detection of CRC.

Chromosomal instability (CIN) occurs at a late stage in adenomas and is critically associated with progression to cancer. CIN occurs in approximately 85% of sporadic CRCs, whereas the residual 15% are hypermutated as a results of a deficiency of the DNA mismatch repair system, leading to microsatellite instability (MSI).[18] MSI is associated with the serrated pathway.[7] The exact factors that underlie the CIN phenotype are less well understood, but may be caused by defects in genes that regulate formation of the mitotic spindle and segregation of chromosomes at mitosis.[19] Instead of being random noise, the chromosomal alterations occur in specific patterns and are associated with different clinical behavior.[20] Of the alterations frequently reported in CRC,[21-23] especially 8q, 13q and 20q gains and 8p, 15q, 17p and 18q losses are associated with the transition from adenoma to carcinoma.[24] The chromosomal changes may provide growth advantages by affecting the average expression level of genes residing on these regions, as has been described for multiple genes on the 13q and 20q amplicon.[25,26] For a number of the reported genes also functional relevance in progression towards cancer was demonstrated, including the genes *DIS3*, *LNX2* and *CDK8* on the 13q amplified region[26-28] and *TPX2* and *AURKA* at the 20q amplicon (Figure 1).[29]

The gene dosage effects caused by DNA copy number changes associated with CIN not only affect expression of coding genes, but also that of miRNAs.[30] MiRNAs are small non-coding RNA molecules of 18–25 nucleotides that regulate the expression of genes through the degradation of messenger RNA (mRNA) or blockage of mRNA translation. By targeting genes that control cell cycle progression and cell death, the miR-17–92 cluster localized on 13q31 was found to regulate oncogenic activities.[31,32] Recent data show that the expression of the miR cluster was positively related to the 13q gain that is present in 40–60% of all colorectal cancers and is associated with adenoma to carcinoma progression (Figure 1).[26,33] In addition, miR-135a and miRNA-135b have been described to regulate APC by decreasing the translation of the APC transcript leading to stimulation of the WNT signaling.[34] Given the regulatory effects on important cancer related genes, miRNA might prove useful biomarkers for cancer detection.

Molecular classifications of colorectal cancer

Multiple approaches have been taken to molecularly classify colorectal cancer. Based on the type of genomic instability, CRC is classified as chromosomal instable or MSI.[35] Also the extent of promoter hypermethylation in colorectal cancers has been used for the distinction between CpG island methylation phenotype (CIMP) high and CIMP low cancers[35] and recently a consensus molecular subtypes (CMS) classification based on mRNA expression patterns has been proposed.[36] These different systems show important overlaps in the classes they identify. For instance a large proportion of CIMP high cancers are MSI, and also one of the CMS classes predominantly contains MSI cancers.[36] The genomic classification of CRC may

accelerate the understanding of its development and optimize the clinical approach towards prevention and treatment.[37]

Genomics and early detection of colorectal cancer

For early detection through screening, the availability of a suitable and acceptable test is indispensable according to the criteria of Wilson and Jungner (Box 1).[4] In terms of test characteristics, the test should have adequate sensitivity and specificity to detect the disease in an early, treatable stage. This means that it should most likely be based on a panel of biomarkers to account for tumor heterogeneity. Also, the test should ideally be non-invasive to minimize patient burden and maximize participation.

Markers in stool

In the case of CRC, stool provides a representative and readily available sample that contains certain molecules indicative of the presence of a tumor. These end up in the stool through leakage of disturbed lymph- or blood vessels, active secretion by the tumor cells and cell exfoliation.[38] A well-known leaked biomarker is the protein hemoglobin, which is bound by antibodies for the detection of human occult blood in the fecal immunochemical test (FIT). The FIT is currently the most widely used CRC screening test. However, because leakage may occur intermittent and bleeding is not specific for neoplastic lesions, performance of the FIT is suboptimal, especially for the detection of precursor lesions.[39] Exfoliated biomarkers, on the other hand, are directly derived from neoplastic cells and colonocytes are continuously being shed to the fecal stream during all stages of tumorigenesis.[38] Therefore, DNA markers, RNA markers and protein markers may provide more appropriate alternatives for CRC detection.

DNA markers in stool

Tumor DNA is detectable in relative abundance compared to the normal colonocytes due to increased proliferation and cell viability, increased folded surface and reduced adhesion of the cells to other cells or the basement membrane.[40] Still, it is technologically challenging to trace the aberrant tumor DNA amongst the vast majority of non-human DNA (99.99% of total stool) and wild type human DNA copies (99.5% of human DNA).[41] New approaches such as BEAMing (beads, emulsion, amplification and magnetics technology) and digital melt curve assays have substantially improved the sensitivity of analytical assays and can now detect <0.1% of mutant copies in stool required for the detection of precursor lesions.[38,41]

The detection of mutations associated with the development of colorectal tumors was first demonstrated in 1992 by Sidranksy *et al.* (Figure 1).[42] They found that in the stool of eight out of nine patients with benign or malignant neoplasms from proximal and distal colonic epithelium, RAS mutations were present. This stimulated the exploration of other genetic alterations in stool, such as mutations in the *TP53* and *APC* gene,[43,44] as well as epigenetic alterations. In 2004 Müller

et al. identified *SFRP2* methylation as a single epigenetic marker with a sensitivity of 77–90% and specificity of 77% for CRC (Figure 1).[45] More research into single marker tests was pursued, but their performance remained insufficient for clinical use because high sensitivities were associated with low specificities and vice-versa.[46] Combining methylation and genetic markers achieved higher sensitivities of 70–96% for CRC with specificities of 72–96%.[46] However it was not until the multitarget stool DNA test was developed that the first DNA marker test was officially approved for screening (Figure 1). The test consists of a molecular assay for mutant *KRAS* and the hypermethylation markers *BMP3* and *NDRG4*, as well as an immunochemical assay for human hemoglobin. In a large clinical trial it showed a sensitivity of 92.3% for CRC and 42.4% for advanced precancerous lesions, against a specificity of 86.6%.[47] Especially the improved sensitivity for serrated lesions compared to the FIT screening tool appears a striking benefit.[48]

Protein and RNA markers in stool

Because the detection of proteins in stool is possible with relatively easy methods and in small sample volumes, these markers have gained increasing attention. Such examples are human hemoglobin and calprotectin. which are non-cancer specific markers released from bleeding and/or inflamed lesions. The most widely used screening test FIT is based on human hemoglobin detection and shows a sensitivity of 79% for CRC at a specificity of 94%,[39] but much lower sensitivities around 31% for the detection of advanced adenomas.[49] In a clinical trial, testing for calprotectin in stool was found to be inferior to FIT and it was therefore not recommended for screening purposes.[50] Of the tumor-derived markers, M2 pyruvate kinase (M2P) has been most extensively studied. Evaluation in a systematic review and meta-analysis showed a pooled sensitivity and specificity for CRC of 79% and 80%, respectively.[51] Performance in the detection of precursor lesions was not specified.

Next to proteins, also RNA markers are detectable in stool. Because high amounts of mRNA transcripts may be present when a gene is (over)expressed, these molecules are an interesting target. One study assessing the expression profile of nine genes showed a sensitivity of 78% with a specificity of 100% for CRC in fecal samples.[52] More recently, *ITGA6* as a single mRNA marker detected CRC with 81% sensitivity and 88% specificity and advanced adenomas with 75% sensitivity and 88% specificity.[53] An advantage of miRNA compared to mRNA is that these molecules are remarkably well-protected from endogenous degradation due to their small size. It has been shown that miRNAs can be extracted from stool easily and reproducibly and that the expression patterns of miRNA are different between CRC patients and healthy individuals.[54] This suggests they could be appropriate markers for noninvasive screening tests. However, more clinical investigations are needed to establish the performance of the RNA markers.

Markers in blood

Next to biomarkers from samples in the proximity of the tumor, *i.e.* stool, also biomarkers in blood have been evaluated for early detection of CRC. In terms of practicality blood samples might have several advantages over stool, because of potentially greater patient acceptance

and the absence of disturbing micro flora. Nevertheless, blood-based markers are still generally detectable only in advanced stages of cancer but not in those with early-stage or pre-cancerous lesions,[38] because entry into the systemic blood stream requires blood vessel invasion or phagocytosis of neoplasia-derived cells.

DNA markers in blood

Tumor DNA may circulate in blood as intact cells (circulating tumor cells or CTCs) or as small DNA fragments (cell-free circulating tumor DNA or ctDNA). Although the mechanisms by which these components end up in the circulation are not yet fully understood, CTCs are most likely shed into the circulation directly, whereas ctDNA is released indirectly from phagocytosis of necrotic or apoptotic cells including CTCs.[55] The detection of DNA mutations, as well as hypermethylation markers in plasma was demonstrated only a few years after their detection in stool.[56] Methylated SEPTIN9 is the most extensively studied blood marker for early detection of CRC (Figure 1). In a recent clinical study the SEPTIN9 blood test reported sensitivities of 48.2% for CRC and 11.2% for advanced adenomas against a specificity of 91.5%.[57] Despite the relative low sensitivities, the test was FDA approved as a screening tool for CRC in 2016.[58] Also other hypermethylation markers and marker panels are under development, but none of them are yet suitable for use in the clinic.[59]

Protein and RNA markers in blood

The first protein blood marker linked to the presence of CRC was CEA.[61] This marker is mostly used for disease monitoring, but not for early detection of CRC, because of the low sensitivity and reliability. Recent proteomics studies have generated new candidate protein markers.[61] In particular the level of the protein CD24 in peripheral blood leukocytes seems a promising marker for detection of CRC neoplasia, showing high sensitivity for CRC (71–80%) and for adenomas (84–89%) with specificities of 70–84%.[62]

Also various types of RNA (such as mRNA and miRNA) have been studied in blood. Use of mRNAs gained more attention when it was reported that an important fraction of the mRNA detected in plasma is not degraded, but highly preserved due to protection from vesicle-like structures.[63] One blood-based mRNA panel of seven genes distinguished CRC patients from controls with a sensitivity of 78% and a specificity of 66% in a large case-control study.[64] Most mRNA and miRNA sequences evaluated for early detection are still in the proof-of-principle and pilot stage.[65]

The road ahead

The advances in genomics and proteomics technologies have led to the identification of many novel biomarkers potentially relevant for CRC screening. However, only a fraction of these have been implemented in clinical tools, due to technical challenges, inadequacies in test sensitivities and specificities or user-unfriendliness. Evidently, the merits of biomarker based tests need to be compared to established non-genomic early detection methods such

as colonoscopy and the widely used FIT.[66] Also validation of novel biomarkers in large patient cohorts has proven to be a bottleneck for the implementation of such biomarkers in clinical practice. Innovative approaches hold promise to accelerate the discovery and verification of biomarker candidates in stool as well as in plasma directly. Assays with higher analytical sensitivity for (epi)genetic alterations are under development, thereby facilitating reduction of the sampling volume and improved user-friendliness. An example of this is the methylation on beads procedure, that enhances the yield of methylated DNA in blood and sputum samples.[67] At the same time ongoing developments in increasing the sensitivity of "liquid biopsies" may allow the detection of ctDNA already at a precursor stage, when only small amounts of tumor DNA fragments are present against a high background of normal circulating DNA fragments. On the protein level, development of multiplex antibody assays and targeted mass spectrometry[68] and antibody suspension bead arrays[69] have become available for large scale and multiplexed detection of protein biomarkers.

Genomics and Intermediate endpoints in CRC screening

The aim of the CRC screening program is to reduce CRC-related mortality by detecting CRC in an early or, preferably, premalignant stage. The perceived effectiveness of new screening tests on reducing death from colorectal cancer depends on the diagnostic qualities of the new candidate test, but also on the accuracy of the intermediate endpoint used. While substantial efforts are being made in discovering new diagnostic biomarkers, finding appropriate biomarkers reflecting the adenoma progression risk is under-addressed in present CRC research. Based on the prevalence of focal cancer in removed adenomas it is estimated that only about 5% of adenomas ever progress to cancer.[11]

Currently, advanced adenomas (*i.e.* adenomas larger than 10mm, and/or with a villous component and/or with high grade dysplasia) are widely used as intermediate endpoints in studies evaluating new screening tests. Yet, this is a rather poor reflection of the risk of dying from CRC, and has been introduced and adopted in literature without sufficient evidence.[70] For this reason, there is need for alternative intermediate endpoints that more precisely reflect the natural course of the disease, and more specifically identify adenomas at high risk of progressing to cancer.[71]

Definitive studies identifying the proportion and specific category of adenomas progressing to cancer cannot be performed, as their design would be unethical (*i.e.* leaving adenomas in place and follow them over time until they progress to cancer). Evidence therefore must come from our general understanding of (colorectal) cancer[2,3] and testing of the molecular alterations in model systems like cell lines and organoids.[13,72] This knowledge can then be combined with cross-sectional observations of genomic alterations in adenomas at different stages of progression.[24] Specific DNA copy number aberrations were able to distinguish simple adenomas without invasion, from adenomas with a cancer focus as well as later stage

cancers with a sensitivity of 78% and a specificity of 78%.[24] Also, multiple putative oncogenes have been identified at the gained regions that contribute to adenoma-to-carcinoma progression.[25,26] These specific DNA copy number aberrations were found in only 23% of the advanced adenomas and can occur in 1.5% of non-advanced adenomas.[73] Moreover, in functional approaches modeling CRC development, by using engineered human intestinal organoids, it was observed that in fact only adenoma-organoids that carried chromosomal instability were able to progress to invasive carcinomas in mice.[72] These studies lend support to the role of these copy number alterations in the risk of progression to cancer, reason why these molecular features may be better intermediate endpoints than the advanced adenoma phenotype. Incorporating molecular knowledge to assess the risk of progression in the tissue samples of adenomas detected and removed during colonoscopy, could improve the evaluation of screening programs and development of new tests. While a similar approach could be considered for serrated lesions, this is hampered by the low prevalence of dysplastic serrated lesions, possibly reflecting a short time frame involved once they do progress to (MSI) cancers. If this rapid progression indeed is the case, then there hardly is an opportunity to identify molecular high-risk serrated lesions to serve as intermediate endpoints in screening studies.

In theory, intermediate endpoint biomarkers would be suitable for early detection as well. However, many of these biomarkers, such as DNA copy number alterations, are very challenging to measure in stool and blood samples where a high background of normal and/or bacterial DNA is present.

Discussion

Molecular medicine has been boosted over the past decades by increasing awareness of the central role of genomics in pathophysiology. This awareness has driven the scientific community to rapidly expand the knowledge in this field and search for new techniques to do so. The most effective way of reducing mortality for a given cancer type through prevention and early detection of the disease or even the precursor lesions. The progress made in early detection of colorectal cancer is an example of how genomics-derived understanding may impact clinical practice.

Research into the biology of adenoma-to-carcinoma progression has confronted us with the complexity of the underlying molecular mechanisms. Yet, the concept of using this information for biomarker development and the accomplishment of applying these biomarkers for early detection is already innovative.

Similar transitions towards biomarker use for early detection have taken place in other cancer types, such as cervical cancer and esophageal adenocarcinoma (EAC). Both of these cancer types are preceded by a premalignant condition, *i.e.* cervical intra-epithelial neoplasia (CIN) and dysplasia in Barrett's esophagus (BE) respectively. Already since the 1950s cytology was

introduced as a primary screening method for cervical cancer. Because the clinical sensitivity of a single Pap smear is modest (60–70%), multiple efforts have been taken to improve accuracy and cost effectiveness of cytology based screening protocols through biomarker discovery. Specific chromosomal alterations (3q, 5p) induced by infection of the human papillomavirus are already present at precancerous stages and have been studied as potential biomarkers.[74] Yet, presence of HPV DNA itself has emerged as the most effective biomarker and is currently being implemented in the Netherlands as the primary test in population based screening.[75] In the case of BE, biomarkers for early detection as well as surveillance are important targets to increase effective mortality reduction. Several copy number alterations were found to identify patients with an increased risk of progression, such as loss of 5q, 13q, 18q and 17q in EAC patients.[76] The use of chromosomal instability as a biomarker for esophageal cancer risk still needs validation in larger cohorts before clinical implementation.

The expectation is that in the upcoming years novel technology will be developed to target high-risk premalignant lesions and carcinomas more sensitively and specifically using patient-friendly methods. Making sense out of these 'big genomic data' is pivotal for a meaningful development. For comprehensive data management, infrastructures are needed that link clinical, imaging, biobanking and experimental data. In the Netherlands, the Translational Research IT (TraIT) project is developing and implementing a long-lasting IT infrastructure for translational research projects that facilitates the collection, storage, analysis, and archiving of data generated in the biomedical research projects. The need for this type of platforms will further increase in the light of global collaborations and the wide range of available experimental techniques.

Acknowledgements

The authors were supported by the Dutch Cancer Society (reference numbers: KWF Fellowship 2013-5885 and NKI 2013-6338) and a SU2C-DCS International Translational Cancer Research Dream Team Grant (MEDOCC). Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

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“The true sign of intelligence is not knowledge but imagination”

Albert Einstein

3.

Low-cost and clinically applicable copy number profiling using repeat DNA

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Submitted for publication

Abstract

Large-scale cancer genome studies suggest that tumors are driven by somatic copy number alterations (SCNAs) or single-nucleotide variants (SNVs). Due to the low-cost, the clinical use of genomics assays is biased towards targeted gene panels, which identify SNVs. There is a need for a comparably low-cost and simple assay for high-resolution SCNA profiling. Here we present our method, conliga, which infers SCNA profiles from a low-cost and simple assay.

Somatic copy number alterations (SCNAs) are common in cancer. On average, cancer samples see SCNAs in 34% of the genome, with 17% of the genome amplified and 16% deleted.[1, 2, 3] Certain SCNAs, particularly amplifications of oncogenes and deletions of tumor suppressor genes, have been found to be major drivers in tumor development, associated with prognosis and response to therapy.[1] SCNA burden varies considerably between cancer types.[3] For example, oesophageal adenocarcinoma (OAC) has relatively high levels of SCNAs,[4, 5, 6, 7] and generally develops from Barrett's oesophagus. Patients with OAC tend to be diagnosed at a late stage, when spread has occurred to lymph nodes and distant organs. This makes treatment more difficult and leads to poor prognosis.[8] Although most patients with Barrett's do not progress, early stage disease (high grade displasia or intramucosal adenocarcinoma) can be successfully treated, usually obviating the need for surgery. There is a critical need to develop technologies that can detect early disease and distinguish between patients at low versus high risk for progression. Since most mutations in OAC driver genes are already present in pre-malignant disease,[9] but an increased SCNA load distinguishes OAC,[10,11,12] low-cost SCNA profiling would be a valuable research and clinical tool.
SCNAs have been identified using a number of methods, including comparative genomic hybridisation (CGH),[13] array-based CGH,[14] single nucleotide polymorphism (SNP) arrays,[15] and whole-genome sequencing (WGS).[16] Recently, low-coverage (LC) WGS has gained popularity due to its reduced cost and strong performance.[17] However, while LC WGS reduces the cost of sequencing, standard WGS library preparation is required with its associated fixed expense and time needed to produce each sample. A technically simple, fast, easily automated, high-resolution and inexpensive alternative method for SCNA detection, with clinical potential, would be extremely valuable.

Recent studies have shown the genome can be amplified at multiple (>10,000) genomic loci with the use of a single non-specific primer pair, using the FAST-SeqS method.[18,19] With this approach, two polymerase chain reaction (PCR) rounds replace the complicated and expensive library preparation steps associated with WGS. The amplified regions are sufficiently short such that the assay can be performed on cell-free DNA as well as DNA extracted from tissue biopsies. The resulting amplicons can be sequenced, with samples multiplexed on the same sequencing lane. With this method, we maintain a similar sequencing depth to 30-50X high-coverage (HC) WGS while sequencing only specific loci. This is in contrast to LC WGS which samples the whole genome but at reduced sequencing depth (Supplementary Fig. 1). The cost involved in sample preparation and sequencing combined is approximately £14 per sample compared with approximately £52-72 for LC WGS, depending on the library preparation kit used (Supplementary Note 1). The sample preparation can be performed in less than an hour with minimal hands-on time, compared to approximately 3 hours or greater for LC WGS.

Until now, the use of FAST-SeqS data has been limited to the detection of whole chromosome gains[18] and entire chromosome arm gains and losses.[19,20] This means that chromosome segment

(focal) alterations are not detected, or perhaps falsely considered as whole chromosome or chromosome arm alterations. Moreover, in these methods SCNAs are not quantified and regions are simply classified as amplified, deleted or normal.
Here we present a method (and associated tool: 'conliga') that uses a fully probabilistic approach to infer relative copy number (RCN) alterations at each locus from FAST-SeqS data. conliga provides a RCN profile per sample and therefore enables this low-cost sequencing approach to be used as a SCNA assay.

Based on observations of raw data (Supplementary Note 2, Supplementary Fig. 1), we created a probabilistic model (Methods, Supplementary Note 3). The model takes account of the observed bias in loci counts, which predominantly results from unequal PCR efficiencies between loci. Since neighboring loci are likely to share the same copy number, we use a hidden Markov model (HMM) to model the spatial dependence between loci. This allows loci with high counts to share statistical strength with neighboring loci, enabling us to infer contiguous regions of copy number more accurately. Moreover, we use a Bayesian nonparametric approach (sticky HDP-HMM)[21] to address the issue of the unknown number of copy number levels present in a given sample a priori (Methods). We use Markov Chain Monte Carlo (MCMC) methods to infer the RCN of each locus, plus all other latent variables in the model (Methods, Supplementary Table 1, Supplementary Notes 4, 5 and 6). This enables us to provide the uncertainty of the RCN estimates, summarized by credible intervals, in conliga's standard output.

To test our method, we analysed 11 oesophageal adenocarcinoma tumors (Methods, Supplementary Tables 2 and 3), which had been sequenced using HC WGS (>50X) and FAST-SeqS. In addition, we downsampled the WGS data of each sample to nine million reads to simulate typical LC WGS (~ 0.1X coverage) samples (Methods). We compared the copy number calls derived from ASCAT[22] (applied to HC WGS data) with the RCN calls from QDNAseq[17] (LC WGS data) and conliga (FAST-SeqS data). conliga and QDNAseq achieved a median Pearson correlation coefficient with ASCAT of 0.95 and 0.98 respectively (Methods, Supplementary Table 4).

In figure 1a-d we demonstrate that similar RCN profiles are obtained with the three methods for an example sample (OAC2) and that high-resolution SCNA information is maintained by sampling genomic loci using FAST-SeqS. Figure 1e and 1f show the performance of conliga and QDNAseq, both obtaining similar Pearson correlation coefficients with ASCAT's RCN calls across all 11 OAC samples (conliga: 0.953, QDNAseq: 0.987) and residual distributions when compared to ASCAT (Methods). It should be noted that by downsampling reads from the same WGS sample, this analysis is potentially biased in favor of QDNAseq's results.

From the literature[23,12] we selected a set of 36 genes that have been observed to be recurrently amplified or deleted in OAC (Supplementary Table 5, Methods). We determined the

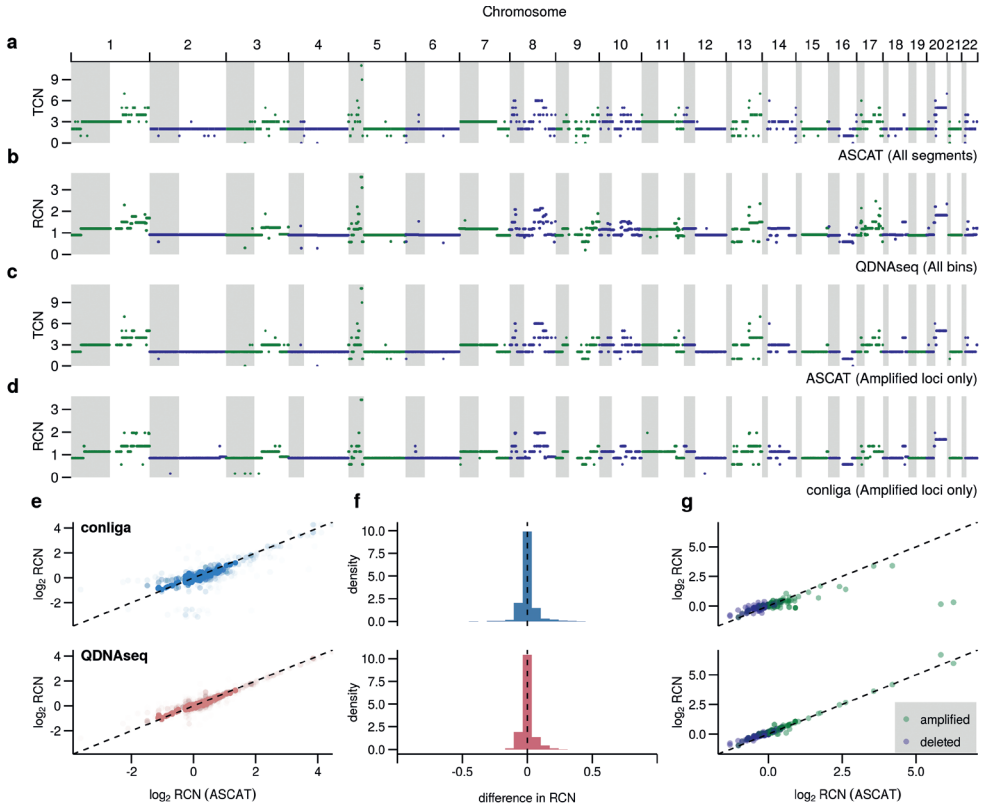

Figure 1 | Comparison of conliga method with ASCAT and QDNAseq.
(a) Total copy number profile determined by ASCAT from HC WGS data for sample OAC2, showing all copy number segments. (b) Relative copy number profile determined by QDNAseq from LC WGS data for sample OAC2, showing all 15 Kbp bins. (c) Total copy number profile determined by ASCAT from HC WGS data for sample OAC2, showing ASCAT's copy number calls at the intersection of ASCAT's called regions and FAST-SeqS loci. (d) Relative copy number profile determined by conliga from FAST-SeqS data for sample OAC2, at the intersection of ASCAT's called regions and FAST-SeqS loci. (e) Comparison of $\log_2$ relative copy number calls from 11 samples between conliga and ASCAT (top) and QDNAseq and ASCAT (bottom). All RCN calls at the intersection of ASCAT's called regions, QDNAseq 15Kb bins and FAST-SeqS loci in all 11 OAC samples are shown as points. (f) Distribution of differences between ASCAT RCN calls and conliga RCN estimates for 11 OAC samples (top) and ASCAT RCN calls and QDNAseq RCN estimates for 11 OAC samples (bottom). (g) Comparison of performance at gene level resolution between ASCAT and conliga (top) and ASCAT and QDNAseq (bottom). The values represent the weighted mean of RCN calls at each gene for each of the 11 OAC samples (Methods).

weighted mean of the RCN calls for these genes for each sample via each method (Methods, Supplementary Tables 6 and 7). While FAST-SeqS/conliga would not be the assay of choice if only interested in a small gene panel, in Figure 1g we see that there are only two instances from 396 comparisons (36 genes x 11 samples) where a substantially different result would be achieved. Naturally if an SCNA is so narrow as to fall between two FAST-SeqS loci then it will not be detected in this way, but the detection of many highly-localized events demonstrates how informative FAST-SeqS/conliga can be. Even within this panel of 36, it is notable that

some genes harbour FAST-SeqS loci (Supplementary Tables 8 and 9), providing evidence of intra gene SCNAs in some cases, such as the focal deletions observed in FHIT, PARK2, and MACROD2 (Supplementary Fig. 2). Focal deletions such as these may be functionally relevant, potentially rendering tumor suppressor genes inactive.

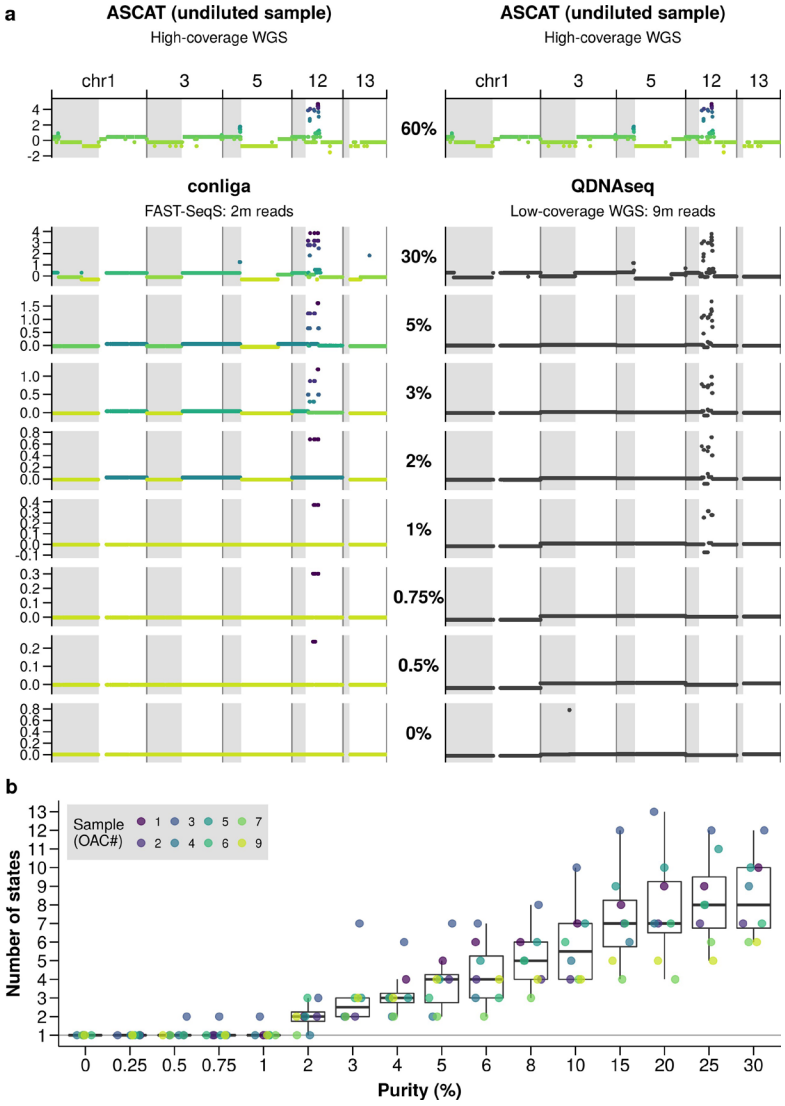

Figure 2 | Comparing the performance of SCNA detection in low tumor purity samples and determining the limit of detection.

(a) left column: relative copy number calls by conliga at different dilutions of sample OAC3, compared to ASCAT relative copy number profile (top left), discrete copy number states are colored with a gradient (light green to purple), highlighting regions with differing SCNAs. right column: relative copy number calls by QDNAseq at different dilutions of sample OAC3, compared to ASCAT relative copy number profile (top right). (b) The number of copy number states detected by conliga in each of eight OAC samples at differing purity levels. The limit of detection is determined by the lowest purity level in which more than one copy number state is detected.

The purity of tumor samples obtained by dissection can vary widely,[24] as can samples obtained non-invasively, e.g ctDNA from plasma.[25] As tumor purity reduces, the copy number signal to noise ratio decreases. To determine the performance of conliga and QDNAseq under different purity conditions, we generated samples with varying purity by mixing sequencing reads from normal and OAC samples (Methods). FAST-SeqS samples were generated with two million reads and LC WGS samples were generated with nine million reads.

Figure 2a shows the performance of both methods for sample OAC3. At 30% purity, both conliga and QDNAseq recapitulate the copy number profile as determined by ASCAT. At 5%, other than the focal amplification on chromosome 12, QDNAseq fails to detect sub chromosomal SCNAs, whereas conliga shows evidence of chromosome arm and sub-chromosomal arm changes. At 2% purity, conliga is able to distinguish some of the more prominent chromosomal arm SCNAs. The focal amplification on chromosome 12 is identified by conliga at 0.75% and 0.5% purity and not detected by QDNAseq below 1%. At 0.75%, 0.5% and 0% purity, it is hard to distinguish whole chromosome SCNAs from noise generated by segmentation in the QDNAseq profiles. This highlights the advantage of conliga's ability to assign loci to discrete states, meaning we can easily distinguish when SCNAs are and are not different between loci. Despite using 4.5 fold fewer reads, conliga appears to be more sensitive than QDNAseq.

In Figure 2b, we show that conliga is able to detect SCNAs at 3% purity in all samples (eight), five at 2% and one at 0.5%. The limit of detection is dependent on the amplitude and lengths of SCNAs present in the sample. Long chromosomal arm amplifications can be detected at 2-3% purity, while some focal amplifications (particularly those occurring at loci with a bias towards obtaining a high number of counts) can be detected at <1% purity (e.g. chr12 in OAC3, Figure 2a). The limit of detection also depends on the technical variability of the protocol and the total number of reads per sample. Increasing the total number of reads beyond two million and reducing technical variability would further improve the limit of detection.

These data demonstrate the potential clinical utility of FAST-SeqS coupled with conliga. Ciriello et al. identified that either somatic single nucleotide variants (SNVs) or SCNAs[3] can drive oncogenesis. Currently, there is a bias towards screening for SNVs using targeted gene panels[26] meaning SCNA-driven cancers may not be detected. To this end, we analyzed samples with pre-malignant disease (Barrett's oesophagus) and were able to detect clinically relevant copy number alterations, such as evidence for focal gains of PRKCI, ERBB2 and GATA6 and deletions of regions containing CDKN2A, PTPRD, SMAD4 and TP53 (Supplementary Fig. 2). This suggests that there is potential for FAST-SeqS to be used alongside existing low-cost gene panels to detect SCNAs, in addition to SNVs, to screen and surveil patients for the development of cancer.

In addition to use as a detection tool, inexpensive production of FAST-SeqS data allows for large cohorts of patients to be studied to find relationships between SCNA profiles and response to therapies, for example. With this in mind, we looked at the average SCNA profiles across small cohorts of patients with OAC, Gastric cancer and Barrett's oesophagus (Supplementary Fig. 2,

Methods) which highlighted amplifications of known oncogenes such as EGFR, MYC, GATA4, and MDM2, some with known drug targets, and deletions of tumor suppressor genes, e.g. FHIT, TP53, SMAD4 and RUNX1. Other potential uses include low-cost screening of samples in large-scale cancer genomes studies, such as ICGC or TCGA projects, prior to further genomic analyses. Furthermore, due to the low-cost and low-input DNA required, several spatially or temporally related samples can be analyzed for the purposes of determining how SCNAs accumulate in normal tissues and contribute to tumor evolution, in a similar fashion to previous studies on somatic mutations in the eyelid epidermis.[27]
Areas for future study could include determining an acceptable number of reads which balances the cost and limit of detection, finding ways to minimise the technical variability, and altering the number of reads obtained at specific loci to increase statistical power in regions of interest.

We have shown that FAST-SeqS data can be used as a viable, inexpensive, and simple alternative to LC WGS for the purpose of SCNA detection and quantification. conliga provides accurate and high-resolution SCNA profiles across the genome and at regions of interest such as oncogenes and tumor suppressors. conliga (applied to FAST-SeqS data with two million reads per sample) is particularly useful in detecting and discriminating SCNAs in low purity samples and our results suggest it to be more sensitive than QDNAseq (using LC WGS, nine million reads) for this purpose. We believe that conliga makes FAST-SeqS data a clinically valuable diagnostic assay to detect and monitor patients for the development of cancer, as well as a useful research tool, enabling inexpensive and fast SCNA profiling of cancer samples.

Methods

conliga: statistical model

Statistical model for sample counts

We model the sample counts, in L selected loci, by assuming that the count at locus l in chromosome arm r in sample j is distributed:

$$y_{r,l,j} \sim \text{Binomial}(n_j, \theta_{r,l,j}) \tag{1}$$

Here, n_j is the total number of sequencing reads aligned to the L loci in sample j, $\theta_{r,l,j}$ represents the probability of observing an aligned read at locus l in chromosome arm r in sample j. We model $\theta_{r,l,j}$ as follows:

$$\theta_{r,l,j} \sim \text{Beta}(s_j \hat{c}_{r,l,j} m_{r,l}, s_j(1 - \hat{c}_{r,l,j} m_{r,l})) \tag{2}$$

Here, s_j is the inverse dispersion variable for sample j where $s_j > 0$, $m_{r,l}$ represents the probability of an aligned sequencing read originating from locus l in chromosome arm r in a control sample, where $\Sigma_r \Sigma_{l=1}^{Lr} m_{r,l} = 1$ and $\hat{c}_{r,l,j}$ is the relative copy number at locus l in chromosome arm r in sample j. The number of loci in each chromosome arm is denoted as L_r and so the total number of loci, $L = \Sigma_r L_r$.

We can interpret m as defining the bias in observing aligned read counts from the FAST-SeqS protocol. This bias can be explained by unequal PCR efficiencies between loci in addition to biases in aligning reads uniquely to FAST-SeqS loci, among other factors. Note that:

$$\mathbb{E}\left[\theta_{r,l,j}\right] = \hat{c}_{r,l,j} m_{r,l} \tag{3}$$

We can be interpret this equation intuitively; the relative copy number scales the probability of reads to align to a locus. For example, if the relative copy number of a locus is 2 we expect the proportion of reads at the locus to double. This fits with our observations shown in Supplementary Fig. 1.

The inverse dispersion variable, s_j, is sample specific and reflects our observations that the level of dispersion varies between samples. This variation in dispersion between samples might be due to varying levels of DNA degradation and/or varying quantities of starting material between samples, among other factors. s_j relates to the variance and the mean of $\theta_{r,l,j}$ in the following way:

$$\mathrm{Var}\left(\theta_{r,l,j}\right) = \frac{1}{s_j + 1}\left(\mathbb{E}\left[\theta_{r,l,j}\right] - \mathbb{E}\left[\theta_{r,l,j}\right]^2\right) \tag{4}$$

The expected count, $y_{r,l,j}$, in chromosome arm r at locus l in sample j is:

$$\mathbb{E}\left[y_{r,l,j} \mid \theta_{r,l,j}\right] = \mu = n_j \hat{c}_{r,l,j} m_{r,l} \tag{5}$$

The variance of $y_{r,l,j}$ can be written as a quadratic function of μ with the coefficients being a function of n_j and s_j:

$$\mathrm{Var}\left(y_{r,l,j} \mid \theta_{r,l,j}\right) = \left(1 + \frac{n_j - 1}{s_j + 1}\right)\mu - \left(\frac{1}{n_j} + \frac{n_j - 1}{s_j + 1}\right)\mu^2 \tag{6}$$

Note that in the limit sj → ∞, a Binomial noise model is recovered.

Probabilistic generative model of loci counts for control samples

We assume that the loci within a control sample, k, have equal copy numbers (diploid). This means that the RCN for each locus is 1. By setting $\hat{c}_{r,l,k} = 1$, we model the generative process of counts from a control sample as follows:

$$
\begin{aligned}
s_k \mid \psi &\sim \mathrm{Gamma}(\psi_{\mathrm{shape}}, \psi_{\mathrm{scale}}) \\
m_{r,l} \mid \phi &\sim \mathrm{Beta}(\phi_{c,r,l}, \phi_{d,r,l}) \\
\theta_{r,l,k} \mid s_k, m_{r,l} &\sim \mathrm{Beta}(s_k m_{r,l}, s_k(1-m_{r,l})) \\
x_{r,l,k} \mid \theta_{r,l,k}, n_k &\sim \mathrm{Binomial}(n_k, \theta_{r,l,k})
\end{aligned}
\tag{7}
$$

Here, Gamma(ψ_{shape}, ψ_{scale}) represents the prior distribution over the sample specific inverse dispersion parameter, s_k, and Beta($\varphi_{c,r,l}$, $\varphi_{d,r,l}$) defines the prior distribution over $m_{r,l}$.

Linking FAST-SeqS loci using a hidden Markov model

We assume that chromosome arms are independent. By that we mean, the RCN of the first locus in arm q is independent of the RCN of the last locus in arm p from the same chromosome (and all other chromosome arms). As such, we model each chromosome arm as an independent Markov chain for each sample *j*. We denote (note that for simplicity we have dropped the sample index *j*):

- $z_{r,l}$ as the *hidden state* (or *copy number state*) of the Markov chain at locus *l* in chromosome arm *r*
- π^0 as the *initial distribution* of the first locus ($l = 1$), in chromosome *r*
- π_u as the *transition distribution* for hidden state, *u*
- $\hat{c}_u$ as the *relative copy number* associated with hidden state, *u*.

The first locus of a chromosome arm ($l = 1$) is distributed:

$$
z_{r,1} \sim \pi^0 \tag{8}
$$

For all other loci ($l > 1$):

$$
z_{r,l} \mid z_{r,l-1} \sim \pi_{(z_{r,l-1})} \tag{9}
$$

The count, $y_{r,l}$, at locus *l* in chromosome arm *r* is conditionally independent of the hidden states and observations of other loci:

$$
\begin{aligned}
\theta_{r,l} \mid \hat{\boldsymbol{c}}, z_{r,l}, m_{r,l}, s &\sim \mathrm{Beta}(s\hat{c}_{z_{r,l}} m_{r,l}, s(1-\hat{c}_{z_{r,l}} m_{r,l})) \\
y_{r,l} \mid \theta_{r,l}, n &\sim \mathrm{Binomial}(n, \theta_{r,l})
\end{aligned}
\tag{10}
$$

The joint density for L_r loci in chromosome arm r is:

$$\begin{aligned} p(z_{r,1:L_r}, y_{r,1:L_r}, \theta_{r,1:L_r}) &= p(y_{r,1} \mid z_{r,1}, \theta_{r,1}) p(\theta_{r,1} \mid z_{r,1}) p(z_{r,1}) \\ &\quad \prod_{l=2}^{L_r} p(y_{r,l} \mid z_{r,l}, \theta_{r,l}) p(\theta_{r,l} \mid z_{r,l}) p(z_{r,l} \mid z_{r,l-1}) \\ &= \pi^0_{z_{r,1}} p(y_{r,1} \mid z_{r,1}, \theta_{r,1}) p(\theta_{r,1} \mid z_{r,1}) \\ &\quad \prod_{l=2}^{L_r} \pi_{z_{r,l-1}, z_{r,l}} p(y_{r,l} \mid z_{r,l}, \theta_{r,l}) p(\theta_{r,l} \mid z_{r,l}) \end{aligned} \tag{11}$$

where, $z_{r,1:Lr}$ denotes the sequence $\{z_{r,1}, \ldots, z_{r,Lr}\}$, $y_{r,1:Lr}$ denotes $\{y_{r,1}, \ldots, y_{r,Lr}\}$, and $\theta_{r,1:Lr}$ denotes $\{\theta_{r,1}, \ldots, \theta_{r,Lr}\}$. The joint density for all L loci in the genome is given by:

$$p(\boldsymbol{z}, \boldsymbol{y}, \boldsymbol{\theta}) = \prod_r p(z_{r,1:L_r}, y_{r,1:L_r}, \theta_{r,1:L_r}) \tag{12}$$

Probabilistic generative model of a sample's relative copy number profile

The number of copy number states present in a sample is unknown a priori. In samples that have equal copies of each locus, only one copy number state is present. Conversely, it is possible (although unlikely) that each locus has its own unique copy number, meaning that there could be up to L copy number states in a sample.

Additionally, we expect neighboring loci to share the same copy number given their genomic distance from each other (Supplementary Fig. 1). To address these two features of the data, we used the sticky hierarchical Dirichlet process hidden Markov model (sticky HDP-HMM)[21] as a framework to model the generative process of a sample's relative copy number profile. By doing so, we adequately model the spatial persistence of copy number states and allow for countably infinite numbers of states within a sample. The generative model is as follows:

$$\begin{aligned} \beta \mid \gamma &\sim \mathrm{GEM}(\gamma) \\ \pi^0 \mid \alpha, \beta &\sim \mathrm{DP}\,(\alpha, \beta) \\ \pi_u \mid \alpha, \kappa, \beta &\sim \mathrm{DP}\left(\alpha + \kappa, \frac{\alpha\beta + \kappa\delta_u}{\alpha + \kappa}\right) \\ \hat{c}_u \mid H, \lambda &\sim H(\lambda) \\ z_{r,1} \mid \pi^0 &\sim \pi^0 \\ z_{r,l} \mid \{\pi_u\}_{u=1}^{\infty}, z_{r,l-1} &\sim \pi_{z_{r,l-1}}, \text{ for } l > 1 \\ \tilde{s} \mid \omega &\sim \mathrm{Gamma}(\omega_{shape}, \omega_{scale}) \\ \tilde{\theta}_{r,l} \mid \{\hat{c}_u\}_{u=1}^{\infty}, z_{r,l}, \hat{m}_{r,l}, \tilde{s} &\sim \mathrm{Beta}(\tilde{s}\hat{c}_{z_{r,l}}\hat{m}_{r,l}, \tilde{s}(1 - \hat{c}_{z_{r,l}}\hat{m}_{r,l})) \\ y_{r,l} \mid \tilde{\theta}_{r,l}, \tilde{n}, &\sim \mathrm{Binomial}(\tilde{n}, \tilde{\theta}_{r,l}) \end{aligned} \tag{13}$$

Note that we use $\tilde{n}$, $\tilde{s}$, $\tilde{\theta}_{r,l}$ to distinguish these variables from those in the probabilistic model of control counts (equation 7) and denote them as specific to the sample with copy number profile. Here, GEM denotes the stick-breaking construction of the Dirichlet Process as described in Fox *et al.*.[21] γ is a hyperparameter of the sticky HDP-HMM and represents our prior on the number of copy number states in the sample; the greater the value of γ, the greater number of copy number states we expect in the sample. Each row of the transition matrix, π_u, is drawn from a Dirichlet Process and depends on β, α and κ. It can be shown that:

$$\mathbb{E}\left[\pi_{u,v} \mid \alpha, \beta, \kappa\right] = \frac{\alpha\beta_v + \kappa\delta_{u,v}}{\alpha + \kappa} \tag{14}$$

where $\delta_{u,v}$ represents the discrete Kronecker delta function. If we define $\rho = \frac{\kappa}{\alpha + \kappa}$ (as in Fox *et al.*[21]) and by noting that α = (1 − ρ)(α + κ), we obtain:

$$\mathbb{E}\left[\pi_{u,v} \mid \beta, \rho\right] = (1-\rho)\beta_v + \rho\delta_{u,v} \tag{15}$$

As such, we see that ρ defines how much weight is placed on self-transition within a copy number state.

The vector, β, itself drawn from a Dirichlet Process, represents the global transition distribution and holds information about the proportion of loci expected in each copy number state. The variance of the transition probability from copy number state *u* to *v* is given by:

$$\mathrm{Var}(\pi_{u,v} \mid \alpha, \beta, \kappa) = \frac{\mathbb{E}\left[\pi_{u,v} \mid \alpha, \beta, \kappa\right]\left(1 - \mathbb{E}\left[\pi_{u,v} \mid \alpha, \beta, \kappa\right]\right)}{\alpha + \kappa + 1} \tag{16}$$

We see that α + κ is inversely proportional to the variance of the state transition probabilities.

H is the prior base distribution of the Dirichlet Process and represents a parametric distribution, which in this case is a Gamma distribution, with parameters λ. It can be viewed as our prior probability distribution on the relative copy number values of the hidden states.

Note that $\hat{m}_{r,l}$ refers to the maximum a posteriori (MAP) value of $m_{r,l}$ and is such assumed to be a known quantity in equation 13. For simplicity, the hyperparameters (α, κ, γ, λ, ω and *n*) are shown as fixed quantities in the model. In practice, γ, λ, ω and *n* are treated as fixed, while the model is parameterized in terms of ρ and (α + κ), with a Beta prior placed on ρ and a Gamma prior placed on (α + κ) as in Fox *et al.*.[21] See the section on inference for further details of prior distributions used and Supplementary Note 3 for further discussion on the model.

Inference

Inference of loci count proportion bias (m)

Given a set of *K* control samples, and their loci counts, x_k, we used our model defined in equation 7 and Markov Chain Monte Carlo (MCMC) methods to infer the latent variables *m* and *s* (the vector of sample specific inverse dispersion parameters). A Metropolis-Hastings MCMC algorithm was used to obtain a sample of the posterior probability of $m_{r,l}$ for all r and l,

and s_k, for each sample k. Full details of the algorithms are provided in Supplementary Notes 4 and 5. Count data for samples analyzed in this study, processed by the pipeline described, are provided in Supplementary Table 10.

For each sequencing experiment, a suitable set of controls samples were used (see Supplementary Table 11 for the list of samples used in each experiment). As described in equation 7, control samples were assumed to have a relative copy number of one at each locus. In all experiments described in this paper, we used the following values for the hyperparameters:

- $\psi_{shape} = 1.5$, $\psi_{scale} = 10^6$; where ψ_{shape} and ψ_{scale} define the shape and scale of the Gamma prior distribution on s_k, respectively.
- $\varphi_{c,r,l} = 1$ and $\varphi_{d,r,l} = 1$ for all r and l; *i.e.* we used a flat Beta(1, 1) prior for all $m_{r,l}$

In each sequencing experiment, 20,000 iterations of the MCMC were run and the first 5,000 iterations were discarded (burn-in). Maximum a posteriori (MAP) estimates of m (denoted as $\hat{m}$) were obtained by determining the mode of the sampled posterior densities for each locus using the KernSmooth R package.[28] Note that the MAP estimates are unlikely to sum to 1 exactly, and as such we enforced this by setting $\hat{m}_{r,l} = \frac{\hat{m}_{r,l}}{\sum_r \sum_l^{L_r} \hat{m}_{r,l}}$.

Inference of relative copy number profile

Given $\hat{m}$ and the loci counts (y) for a sample with unknown copy number profile, we used the generative model defined in equation 13 and MCMC methods (based on algorithm 3 in Fox *et al.* [21]) to infer the latent variables in our model. MCMC methods were used to obtain a sample of the posterior probability of the hidden state of each locus ($z_{r,l}$ for all r and l), the relative copy number of each hidden state ($\hat{c}_u$), the sample specific inverse dispersion ($\tilde{s}$), along with other latent variables in our generative model. Full details of the MCMC algorithms can be found in Supplementary Notes 4 and 6. In all experiments described in this paper, we used the following values for the hyperparameters:

- $\gamma = 1$
- Gamma(2000, 10) prior distribution (defined by shape and scale) was placed on $(\alpha + \kappa)$
- Beta(100000, 100) prior was placed on ρ
- Gamma(3, 1) prior distribution (defined by shape and scale) was placed on the relative copy number value of the hidden states; the shape and scale parameters are defined by λ in equation 13
- $\omega_{shape} = 1.5$, $\omega_{scale} = 10^6$; where ω_{shape} and ω_{scale} define the shape and scale of the Gamma prior distribution on $s\tilde{}$, respectively

The output of the MCMC was summarized in two main ways, 1) by marginalizing out the copy number state information and computing the MAP estimate (using KernSmooth R package[28]) and credible interval of the relative copy number of each locus, 2) by making use of the copy

number state assignments in the following way:

1. we determined the MAP number of states observed in the MCMC chain (after burn-in). This was achieved by calculating the number of populated states in each iteration of the MCMC, and then choosing the most frequently observed number of populated states. Note that a state was considered populated in an iteration of the MCMC if at least one locus was assigned to it.
2. we filtered the iterations of the MCMC (after burn-in), choosing only those iterations that had the number of populated states equal to the MAP number of states.
3. we used the Stephens algorithm (algorithm 2 in the paper)[29] along with the Hungarian (Munkres) algorithm[30] to relabel the states, to resolve the label switching problem inherent in MCMC methods.
4. we calculated the MAP estimate and credible intervals for the relative copy number values of each relabeled state.
5. we assigned each locus to a relabeled state, choosing the relabeled state it was most frequently assigned to in the filtered iterations of the MCMC chain.

For the results presented in Figure 2, summarization method 2 was used. For all other results presented in the paper, summarization method 1 was used. For the oesophageal cancer, gastric cancer and Barrett's oesophagus samples, 50,000 iterations of the MCMC were run and the chain was thinned such that every 5th iteration of the MCMC was output to file. Additionally, the first 20,000 iterations of the MCMC were discarded (burn-in), to ensure the Markov chain had reached its equilibrium distribution. For the in silico diluted samples, presented in Figure 2, 30,000 iterations were run, with the chain thinned so that every 5th sample was output to file and the first 5,000 iterations of the MCMC were discarded.

Sample preparation and sequencing of samples

Sample preparation and generation of FAST-SeqS data

Sequencing libraries were prepared using two rounds of PCR, using a similar protocol to previously published methods.[18,19] Each extracted DNA sample underwent a 50 µl first round PCR reaction with 10 µl 5x Phusion HF Buffer (ThermoFisher Scientific), 1 µl 10 mm dNTP (ThermoFisher Scientific), 5 µl of both the forward and reverse primers (0.5 µm) each (Sigma-Aldrich), 0.5 µl Phusion Hot Start II DNA Polymerase 2U/µl, 5-10 µl DNA template depending on the extracted concentration, and RNAse free water to make the total reaction volume.

The cycling conditions for the L1PA7 primers were 98 °C for 120 s followed 2 cycles of 98 °C for 10 s, 57 °C for 120 s, and 72 °C for 120 s. The second round was also carried out as a 50 µl sample reaction using 20 µl taken from the first round. The rest of the reaction constituents were the same as the first round reaction with the exception of primers (Supplementary Table 12), which contained a unique index for each sample. The cycling conditions for the second round reaction were 98 °C for 120 s followed by 13 cycles of 98 °C for 10 s, 65 °C for 15 s, and 72 °C for 15 s for all the primers. After the second round, samples underwent

quantification using the 2200 TapeStation (Agilent), Agilent 2100 Bioanalyser (Agilent) and Kapa quantification (KapaBiosystems) prior to submission for sequencing. The samples were then pooled in equimolar concentrations and gel extracted according to manufacturer's instructions (Qiaquick gel extraction kit, Qiagen). Finally the samples were submitted for sequencing on a MiSeq (Illumina) platform. All samples were run with 20% PhiX to increase complexity for sequencing. Sequencing was performed as 150bp single end. Samples were run with at least three normal controls prepared at the same time and sequenced on the same platform.

Sample preparation and generation of high-coverage WGS data

WGS library preparation and sequencing was performed as previously described by Secrier *et al.*.[6]

In silico generation of low-coverage WGS data

For our purposes, LC WGS data was defined as nine million single-end 50 base pair reads per sample because this was the type of data analyzed in Scheinin *et al.*.[17] Samples are typically multiplexed together and sequenced on a single Illumina sequencing lane. After processing and alignment of the reads, we expect approximately 0.1X coverage of the genome (as per analysis described in Scheinin *et al.*). We obtained LC WGS data by down-sampling reads from HC WGS BAM files in the following way:

1. we selected a subset of the alignments, containing only reads sequenced on a single lane (chosen to be the lane from the first read in the BAM file), and trimmed the reads and Phred scores to the first 50 base pairs using a custom Bash script.
2. The resulting alignments were filtered (using samtools[31] version 0.1.18), excluding those that were secondary alignments (-F 256) and including only those that were first in a pair (-f 64) and output to a new BAM file.
3. This BAM file was down-sampled to 9 million reads/alignments using the DownsampleSam command from Picard tools (http://broadinstitute.github.io/picard, version 2.9.1) using the "Chained" strategy.
4. The resulting BAM file was converted to FASTQ by SamToFastq (Picard tools).
5. The FASTQ file was aligned to GRCh38 (GenBank accession: GCA_000001405.15, no alt analysis set) using BWA-backtrack (bwa samse and bwa aln, version 0.7.15-r1140),[32] which is more suitable for reads below 70 base pairs in length.
6. In the resulting BAM file, we removed PCR duplicates and removed alignments with mapping quality below 37 as per the analysis undertaken by Scheinin *et al.*[17] using samtools (version 0.1.18).

We performed these steps for 11 oesophageal samples and their matched normal samples along with an additional four normal samples obtained from other patients (Supplementary Table 1). This resulted in greater than seven million primary alignments per sample.

In silico generation of FAST-SeqS dilution data

We performed an in silico dilution of FAST-SeqS data by mixing sequencing reads from control samples with reads from OAC samples. Since the number of reads in the matched controls were insufficient to create samples with two million reads, we created a pool of control reads (in silico) which were used to dilute the OAC samples. This was done by sub-sampling two million reads from 12 control samples (which were prepared and sequenced in the same batch as the OAC samples). The total number of reads from these 12 control samples was 14,405,596. To obtain a pool of 2 million reads, we used the 'sample' command from seqtk (urlhttps://github.com/lh3/seqtk, version: 1.2-r101) to sample a proportion (2/14.405596) of each control sample's reads and merged these together into a single FASTQ file. The reads that were sub-sampled were removed from the control samples (using a custom python script) to avoid using the same reads to fit *m*.

We mixed the pool of control reads with the OAC samples in varying proportions to achieve a desired diluted tumor purity. The OAC samples did not have a tumor purity of 100%, instead they were themselves a mixture of tumor and normal DNA. The purity of these samples were determined by ASCAT-NGS (version 2.1).[22] Based on ASCAT's purity value, we calculated the number of reads required from the OAC sample to achieve a desired dilution and total number of reads. This was calculated as follows:

$$\text{required tumor reads} = \text{round}\left(\frac{\text{desired purity proportion} \cdot \text{required total reads}}{\text{ASCAT inferred purity proportion}}\right) \tag{17}$$

Hence, the number of control reads required were:

$$\text{required control reads} = \text{required total reads} - \text{required tumor reads} \tag{18}$$

We produced in silico dilution FASTQ files in the following way:

1. we used the 'sample' command from seqtk to sample the required number of tumor reads from the OAC FAST-SeqS FASTQ file
2. we used the 'sample' command from seqtk to sample the required number of control reads from the pooled control reads FASTQ file
3. we merged the sampled tumor and control reads into a single FASTQ file

We performed these steps for each OAC sample to create diluted samples with two million total reads and the following purity values: 0.3, 0.25, 0.2, 0.15, 0.1, 0.08, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01, 0.0075, 0.005, 0.0025 and 0. Here purity is defined as the proportion of tumor reads in the sample. Of the 11 OAC samples, 8 (OAC1-7 and 9, Supplementary Table 1) were of sufficient initial tumor purity to feasibly create all the desired dilution levels.

In silico generation of LC WGS dilution data

We produced in silico diluted LC WGS tumor samples by mixing reads from tumor and matched normal LC WGS BAM files (previously downsampled and filtered as described above). We

first calculated the number of reads in the tumor BAM and normal BAM files using samtools (samtools view -F 256 -c [BAM file]).
Next, we calculated the number of reads required using equations 17 and 18. Using the DownsampleSAM command (Picard tools) and the 'HighAccuracy' strategy, we sampled the corresponding desired proportion of reads from the tumor BAM file and normal BAM file. We used samtools to merge the resulting sampled tumor BAM file with the normal BAM file into a single file representing the diluted sample. We aimed to obtain seven million filtered primary alignments per diluted sample (as this is what we expect from nine million reads after alignment and filtering) and dilution levels which matched the diluted FAST-SeqS samples. This was performed for 8 OAC samples and their matched normals (OAC1-7 and 9).

Processing of FAST-SeqS sequencing data to counts

Each sequencing run of the Illumina MiSeq platform produced a BCL file which was converted to FASTQ format (using Illumina's bcl2fastq tool). Sequencing reads that failed the Illumina chastity filter were removed. The FASTQ file was demultiplexed into separate FASTQ files corresponding to each sample using the demuxFQ tool (https://genomicsequencing.cruk.cam.ac.uk/glsstatic/lablink/downloads/DemultiplexingGuide.html) with the default settings. The sample barcodes are provided in Supplementary Table 12. Each sample's FASTQ file was then processed through a custom pipeline which we describe below.

Identifying forward primer position

For each read in the FASTQ file, the position of the forward primer sequence was detected by searching for the sequence with the minimum hamming distance to the forward primer sequence using a sliding window. Reads with a minimum hamming distance greater than 5 were discarded.

Read trimming

The portion of the reads before and including the forward primer sequence were trimmed. The ends of the reads were also trimmed such that the length of the reads used for downstream analyses were 100 base pairs minus the forward primer length. Any reads shorter than 100 base pairs minus the forward primer length after trimming were discarded.

Quality control

After trimming, reads were discarded if they contained at least one base with a Phred quality score less than 20 and/or contained one or more ambiguous base calls (N).

Obtaining unique sequences and counts per unique sequence

To avoid aligning the same sequence multiple times, only unique read sequences were kept. For each unique read, the number of identical fragments were recorded.

Alignment of unique sequences

Unique raw read sequences were aligned with Bowtie 1.0.[33] (using the option: -r). Three mismatches were permitted (option: -v3) and reads aligning to multiple locations were discarded (option: -m1). The reads were aligned to GRCh38 (GenBank accession: GCA_000001405.15, no alt analysis set).

Counts and alignments combined

Each sample's unique read alignments and their corresponding unique read counts were combined into a single file consisting of a matrix of counts. The rows corresponded to genomic positions (the union of genomic positions from the alignments in all samples) and columns corresponded to samples. The first three columns of the matrix corresponded to the chromosome, position and strand for the locus, respectively. The matrix of counts used in this analysis can be found in the conliga R package and in Supplementary Table 10.

Selecting loci

Rows of the count matrix corresponding to genomic loci within chromosomes X, Y and within unplaced or unresolved contigs were discarded. For each batch of samples, genomic loci obtaining a zero count in any one of a set of control samples were also discarded. Depending on the sequencing batch we analyzed and the controls chosen to filter loci (Supplementary Table 11), this resulted in approximately 10,000 - 12,000 genomic loci across chromosomes 1 to 22.

Analysis of copy number from FAST-SeqS data

conliga (version 0.1.0)[34] was used to analyze all FAST-SeqS samples in this study (Supplementary Table 1) using R (version 3.2.3)[35] and RcppAramdillo (version 0.6.500.4.0).[36] Of the 15 OAC samples sequenced, four were excluded due to their obtaining fewer than 350,000 reads. Two control samples were excluded due to their inferred RCN profiles having two main hidden states incompatible with their supposed 'normal' status. The values for the priors used and MCMC settings are stated in the inference sections above. The samples used as a basis to filter loci and fit $\hat{m}$ for each experiment are listed in Supplementary Table 9.

Analysis of copy number from high coverage WGS data

High coverage WGS samples were processed and aligned using BWA-MEM[37] (version 0.5.9) and total copy number (TCN) profiles and normal contamination estimates were provided by ASCAT-NGS (version 2.1) using a pipeline previously described by Secrier *et al.*.[6] The only exception to this was that the reads were aligned to GRCh38 (GenBank accession: GCA_000001405.15, no alt analysis set) rather than GRCh37.

Analysis of copy number from low-coverage WGS data

QDNAseq (version 1.6.1) was used to obtain relative copy number calls for all LC WGS data. The bin size used was 15Kb as per the analysis performed in Scheinin *et al.*[17] for 0.1X LC WGS. The bins were created using GRCh38 (BSgenome.Hsapiens.NCBI.GRCh38) and a mappability file (bigWig format) for 50-mers was created for GRCh38 using the GEM library (GEM-binaries-Linux-x86_64-core_i3-20130406-045632) https: //sourceforge.net/projects/gemlibrary/. 15 normal LC WGS samples (Supplementary Table 1), were used to run the applyFilters and iterateResiduals functions. 11 of these 15 samples correspond to the matched normals of the oesophageal samples (Supplementary Table 1). We did not run the functions normalizeBins and normalizeSegmentedBins which scale the read counts by the median value. This was not necessary and would make the comparison between ASCAT, QDNAseq and conliga results more difficult to interpret.

Comparison of copy number between methods

ASCAT outputs total copy number (TCN) in contiguous genomic regions, QDNAseq outputs relative copy number (RCN) in 15 Kb bins across the genome and conliga outputs RCN values at specific FAST-SeqS loci. To make a fair comparison between the tools, it was necessary to convert ASCAT's TCN calls to RCN as follows:

$$\mathrm{RCN}_i = \frac{(1 - \mathrm{normal}) \cdot \mathrm{TCN}_i + \mathrm{normal} \cdot 2}{\mathrm{mean\ TCN}} \tag{19}$$

Here, normal represents the estimated normal contamination value provided by ASCAT and *i* represents a contiguous genomic region or a discrete locus or fragment. In the case of a contiguous region, the mean TCN (or ploidy) was calculated as follows:

$$\mathrm{mean\ TCN} = \frac{\sum_i (\mathrm{TCN}_i \cdot \mathrm{length}_i)}{\sum_i \mathrm{length}_i} \tag{20}$$

and in the case of discrete loci or fragments:

$$\mathrm{mean\ TCN} = \frac{\sum_i \mathrm{TCN}_i}{L} \tag{21}$$

where *L* represents the total number of loci or fragments considered.

In Figure 1e and f, we compared the RCN values at the intersection of genomic loci across ASCAT, QDNAseq and conliga. Since this intersection represented a subset of each method's genomic loci, the RCN values were rescaled considering only this subset. QDNAseq and conliga RCN values were rescaled by the sample's mean RCN of the considered loci. ASCAT's RCN was calculated using equations 19 and 21.

In figure 1g, we compared RCN values in genes of interest. Recurrently amplified and deleted genes were obtained from Dulak *et al.*[23] and Ross-innes *et al.*.[12] Here, ASCAT's RCN values were calculated using equations 19 and 20 using all called regions for each sample. For each gene in each sample, the weighted mean of the relative copy number (weighted by the length of the overlapping called region) was computed for ASCAT and QDNAseq. This was calculated as follows:

$$\mathrm{RCN}_{\mathrm{gene}} = \frac{\sum_i \mathrm{RCN}_i \cdot l_i}{\sum_i l_i} \tag{22}$$

where l_i represents the length of the overlapping portion of the called region with the gene. For conliga, if loci occurred within the gene, the mean of the RCN values within the gene was used, otherwise the loci directly upstream and downstream, *i.e.* either side, of the gene were used and a mean value was taken. See Supplementary Table 4 for the full list of genes used in the analysis.

Computing Pearson correlation

For each sample, the Pearson correlation coefficient between ASCAT and conliga was calculated. We used ASCAT's TCN and conliga RCN values at the intersection of genomic loci between ASCAT and conliga. The median value of the sample's correlation coefficients was reported (all sample correlation coefficients can be found in Supplementary Table 3).

For each sample, the Pearson correlation coefficient between ASCAT and QDNAseq was calculated. We used the intersection of QDNAseq bins with ASCAT copy number regions, using the length-weighted mean of ASCAT's overlapping TCN values.

When calculating the Pearson correlation for all calls across all samples, we used the re-scaled RCN value at the intersecting genomic loci between ASCAT, QDNAseq and conliga, using the rescaled RCN values described above for Figures 1e and f.

Code availability

conliga source code[34] is freely available under an open-source GPLv2 license at https://github.com/samabs/conliga and as Supplementary Software.

Data availability

The WGS and FAST-SeqS data can be found at the European Genome-phenome Archive (EGA) under accession EGAD00001004289. The copy number results obtained from ASCAT, QDNAseq and conliga can be found https://osf.io/bhx6f/?view_only=ed25e2fb521d46239e5274c032350f0b

Acknowledgements

Funding for sample sequencing was through the Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) Consortium as part of the International Cancer Genome Consortium and was funded by a programme grant from Cancer Research UK (RG66287). SA was funded by Wellcome Trust award [102272]. ERM was University of Cambridge, Cancer Research UK and Hutchison Whampoa Limited. In particular we acknowledge the support of the Cancer Research UK Cambridge Institute's Genomics Core Facility. We thank the Human Research Tissue Bank, which is supported by the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre, from Addenbrooke's Hospital. Additional infrastructure support was provided from the CRUK funded Experimental Cancer Medicine Centre. The authors also wish to thank Sarah Field, Wing-Kit Leung, Charlie Massie, Paul Coupland and Astrid Wendler for helpful discussions and Ginny Devonshire for her help with uploading the sequencing data to EGA.

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Supplementary Material

For supplemental (excel) tables, please visit www.biorxiv.org

Supplementary Note 1: Time and cost comparison for FAST-SeqS and LC WGS

Here we provide our calculations of the cost to produce a FAST-SeqS sample and a LC WGS sample. Note that we used the prices that were available to us and where possible this was the recommended retail price (RRP). We note that prices can vary by date, country and institution. As such, the calculations are provided as a guide and basis for relative comparison between the two sequencing approaches.

Cost of FAST-SeqS (2 million single-end 150 bp reads per sample)

The library preparation requires two rounds of PCR (see Methods) and requires deoxynucleotides (dNTPs), polymerase, RNAse free water and primers.

dNTPs

1 µl 10 mm of dNTPs are required per PCR reaction. 5 ml 10 mm (ThermoFisher Scientific) can be purchased at £317.55 at the time of writing and provides dNTPs for 5000 PCR reactions. Two PCR reactions are required per sample, equating to £0.13 per sample.

Polymerase

0.5 µl Phusion Hot Start II DNA Polymerase 2U/µl with 10 µl 5x Phusion HF Buffer is required per PCR reaction. 500 units can be purchased at £436.71 (ThermoFisher Scientific). One unit is required per PCR reaction and two PCR reactions are required per sample, equating to £1.75 per sample.

Nuclease-free water

Nuclease-free water (not DEPC-Treated) 10 x 50 ml (ThermoFisher Scientific) can be purchased at £88.28 at the time of writing. 18.5 µl - 23.5 µl is used in the first PCR reaction, depending on the amount of DNA used. This would equate to less than £0.01 per sample.

Primers

5 µl (0.5 µm) of each forward and reverse primers are required in each PCR reaction. 5 nmol of primer can be synthesized for approximately £20. Diluting with 10 ml of water gives 10 ml at 0.5 µm of primer. 5 µl of forward primer and 5 µl of reverse primer is required in each PCR reaction. Two PCR reactions are required per sample, equating to approximately £0.04 per sample.

DNA Quantification and Quality Control

Prior to pooling the samples, quantification of DNA is performed using Bioanalyzer 1000 DNA kit on a Agilent 2100 Bioanalyzer instrument, for example. 300 Bioanalyzer 1000 DNA chips can be purchased at approximately £500, which equates to £1.67 per sample.

Next-generation sequencing

We calculated the per sample cost based on using a Illumina HiSeq 4000 sequencer which, for our purposes, produces 350 million single end (SE) 150 base pair (bp) reads from a single lane of sequencing. We factored in that our library would include 20% PhiX to increase diversity for sequencing, and as such, we would expect 280 million reads per lane to originate from our FAST-SeqS amplicons. Aiming for approximately 2 million reads per sample, this would mean multiplexing 140 FAST-SeqS on a single lane.

There is considerable variation in the cost of sequencing services, depending on sector, location, and relationship with the customer. Moreover, of those services that display their costs up front, extremely few provide a direct comparison of prices for Single-End 50bp reads and Single-End 150bp reads on an Illumina HiSeq 4000 machine. Thus we will make use here of the Stanford Medicine Genome Servicing Sequence Centre prices,roughly converted to Sterling, obtained from http://med.stanford.edu/gssc/rates.html on 17/08/18. These costs do not differ substantially from our own experience. Thus, the cost of a single lane 150 bp SE sequencing on the HiSeq 4000 we take to be approximately £1400 at the time of writing. As such, the sequencing cost equates to £10 per sample.

Total cost per sample

The total cost for processing the samples, as explained above, is £0.13 (dNTPs) + £1.75 (polymerase) + £0.01 (nuclease-free water) + £0.04 (primers) + £1.67 (quantification and quality control) + £10 (sequencing) = £13.60.

Cost of LC WGS (9 million single-end 50 bp reads per sample)

DNA Shearing

Prior to library preparation, the input DNA needs to be sheared to a desired length distribution. This is often achieved by the use of sonification, for example using Covaris microTUBE strips. At the time of writing, 12 x 8 microTUBE strips (*i.e.* for 96 samples) can be purchased for £413.10 and is therefore approximately £4.30 per sample.

Library preparation

To process the DNA and prepare the library for sequencing, library preparation is required. This generally consists of end-repair, adapter ligation, and is sometimes followed by PCR amplification to generate sufficient quantities of the library for sequencing. Library preparation kits can be purchased from a variety of manufacturers with varying costs and time to prepare each sample. Examples of library preparations include:

- Illumina TruSeq DNA PCR-Free High Throughput Library Prep Kit (96 samples) can be purchased for approximately £2,100 at the time of writing and is therefore approximately £21.88 per sample.
- KAPA Hyper Prep Kit, PCR-free (96 samples) can be purchased for approximately £1,900; £19.79 per sample
- NEBNext Ultra II DNA Library Prep Kit for Illumina (E7103L, 96 reactions): £1,986; £20.69 per sample.
- SMARTer ThruPLEX DNA-seq 96D Kit (R400407, 96 reactions); £4,063; £42.32 per sample

DNA Quantification and Quality Control

Similarly to FAST-SeqS, quantification of DNA is performed using Bioanalyzer 1000 DNA kit on a Agilent 2100 Bioanalyzer instrument, for example. 300 Bioanalyzer 1000 DNA chips can be purchased at approximately £500, which equates to £1.67 per sample.

Next-generation sequencing

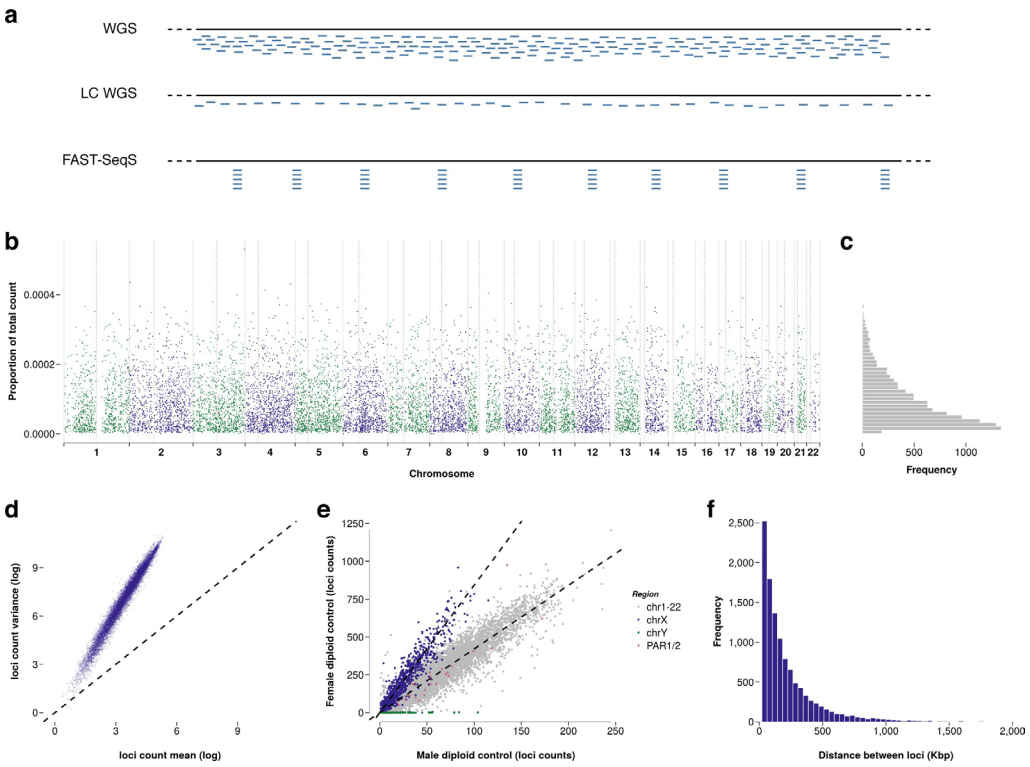
We calculated the per sample cost based on using a Illumina HiSeq 4000 sequencer which can be used lowcoverage WGS to produce 350 million single end (SE) 50 base pair (bp) reads from a single lane of sequencing. PhiX should not be required as the library should not be low complexity. To achieve approximately 9 million reads per sample in order to obtain approximately 0.1X coverage as per Scheinin et al.,[1] would mean multiplexing 38 samples on a single lane. The cost of a single lane 50 bp SE sequencing on the HiSeq 4000 we take to be £1000 (justification as in the previous section). As such, the sequencing cost equates to £26 per sample.

Total cost per sample

The total cost for processing a low-coverage WGS sample (0.1X coverage, single-end 50 bp reads), as explained above is, £4.30 (DNA shearing) + approximately £20-£40 (library preparation) + £1.67 (quantification and quality control) + £26 (sequencing) = £52-£72.

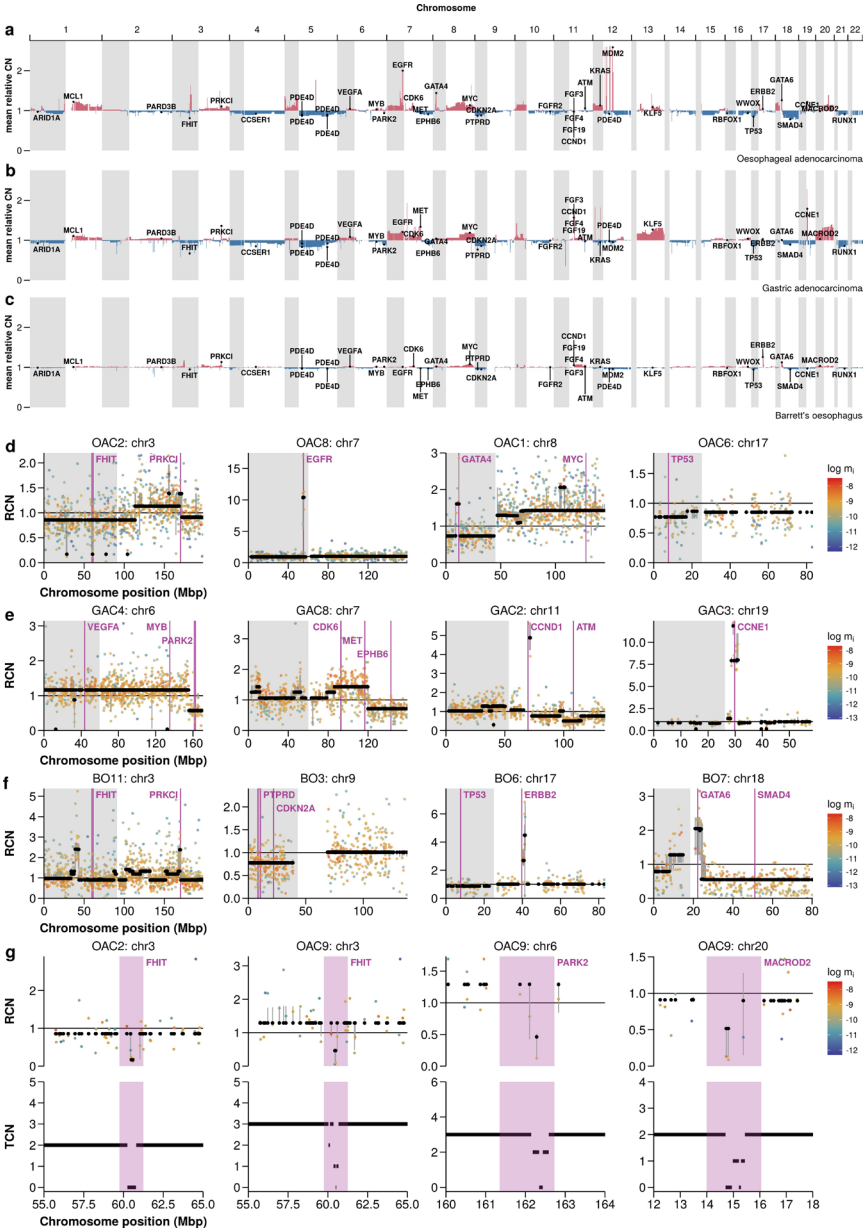
Supplementary Note 2: Aspects of FAST-SeqS data

We explored the loci counts of normal samples (which were assumed to be predominantly diploid) and observed various aspects of the data which led to the model. We observed (1) a technical bias in the number of reads aligned to each locus (Supplementary Figure 1b and 1c), (2) the variation in the data exceeds that expected from sampling variation alone (over-dispersion) (Supplementary Figure 1d), (3) this additional variation is likely to be predominantly technical and the amount of this variation varies between samples, (4) the expected proportion of reads at each locus is directly proportional to the relative copy number between loci (Supplementary Figure 1e), and (5) the genomic distance between loci implies that neighboring loci are likely to share the same copy number (Supplementary Figure 1f).



Supplementary Figure 1 | Aspects of FAST-SeqS data.

(a) a graphical representation of the different approaches to sequencing for the purposes of SCNA profiling; high-coverage WGS (top), low-coverage WGS (middle), FAST-SeqS (bottom). (b) The proportion of reads obtained at each locus in chr1-22 for control sample (NORM1). (c) Histogram of the proportion of reads obtained at each locus across in chr1-22 for control sample NORM1. (d) log mean vs log variance for each locus in control samples. (e) A male control sample (NORM2) counts plotted against a female control sample (NORM1) counts, showing a relative doubling of count proportions in chrX for the female control sample vs male and absence of counts from chrY in the female sample. (f) Histogram of distances between loci with a mean distance of approximately 200Kbp between loci.



Supplementary Figure 2 | Copy number profile summary of patient cohorts used in this study.
(a) Mean relative copy number profile for 11 oesophageal adenocarcinoma samples. (b) Mean relative copy number profile for 8 gastric adenocarcinoma samples. (c) Mean relative copy number profile for 16 Barrett's oesophagus samples, with varying levels of dysplasia. (d)-(f) Examples of relative copy number profiles for various chromosomes from different samples for OAC, GAC and BO respectively. Black points represent the maximum a posteriori (MAP) relative copy number for each locus, the colored points represent the proportion of reads expected in a control sample (log), with red representing a high proportion and blue representing a low proportion, grey lines represent 90% credible intervals. (g) Zoomed-in regions of chromosomes 3, 6 and 20 showing intra-gene deletion of FHIT, PARK2 and MACROD2. conliga results (top) with comparison to ASCAT (bottom).

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“If you don’t know, the thing to do
is not to get scared, but to learn”

Ayn Rand

4.

Molecular profiling of longitudinally observed small colorectal polyps: a cohort study

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EBioMedicine 2018

Abstract

Background

Knowledge of the natural history of colorectal adenomas is limited because these lesions are removed upon detection. The few studies in which small adenomas have been left *in situ* for a limited period of time have shown that most lesions remain stable or even completely regress. Specific DNA copy number changes ('cancer associated events' or CAEs) are associated with progression of adenomas to cancer. In this study we evaluated whether molecular features of progression correlated with growth of small polyps.

Methods

Small (6–9 mm) colorectal precursor lesions detected on CT-colonography (CTC) were left *in situ* and re-evaluated with CTC after three years. Based on volumetric change, polyps were classified as either grown, stable or regressed. Surveillance CTC was followed by colonoscopy, during which all lesions were resected. Using DNA isolated from FFPE polyp tissues, low-coverage whole genome sequencing was performed to determine DNA copy number profiles, as well as target enrichment mutation analysis and CpG island methylation phenotype (CIMP) analysis. Expression of DNA mismatch repair (MMR) proteins was determined by immunohistochemistry. Samples were marked as MMR proficient if all MMR proteins were expressed.

Findings

Out of 68 polyps resected at colonoscopy, for 65 (96%) material was available. Of these, 31 (48%) had grown, 27 (41%) remained stable and 7 (11%) regressed. Polyps with at least one CAE had higher growth rates compared to polyps without CAEs (difference 91% growth (95% CI 13% to 169%), p=.023). CAEs were absent in lesions that had partially regressed. Mutations occurred in 94% of the polyps, with higher growth rates being associated with polyps having ≥2 mutations compared to lesions with only 0–1 mutations (difference 99% growth (95% CI 9% to 189%), p=.032). All samples were MMR proficient. No relation between growth and CIMP was observed.

Interpretation

Molecular alterations associated with colorectal cancer, correlated with growth of small polyps and were absent in polyps that regressed. Therefore, this longitudinal study provides *in vivo* support in the human setting for the functional role of these molecular alterations, that have mostly been identified by cross sectional observations in tissue samples of colorectal adenomas and cancers.

Funding

Alpe d'Huzes, Dutch Cancer Society (project number NKI2013-6338).

Introduction

The development of colorectal cancer (CRC) is a stepwise process, in which normal epithelial cells transform into an adenocarcinoma through a benign intermediate lesion (*i.e.* mostly adenomas, but also serrated polyps). While the prevalence of adenomas is high, only a minority of approximately 5% eventually progresses into cancer; the remaining lesions do not convert to malignancy or even completely disappear over time.[1,2] Understanding the natural history of disease is at the basis of all strategies for early detection of cancer. In the case of colorectal adenoma to carcinoma progression, the absence of longitudinal observations, like we do have in for instance Barrett's oesophagus and cervical cancer[3], causes an evident blind spot in our knowledge of this disease. Molecular analysis of colorectal polyps that have been left *in situ* for a few years, even if this concerns small lesions only, provides a unique opportunity to fill some of these gaps.

Cross-sectional studies have shown that adenomas larger than 10 mm have a higher risk of harbouring a focus of cancer (2.1–6.9%), compared to small 6–9 mm lesions (0–0.42%).[4] During adenoma formation and subsequent malignant progression, several cell signalling pathways get disrupted. An early event is the disruption of the WNT signalling pathway by mutation of the *APC* gene, followed by mutations in the RAS-MAPK, PI3K-AKT, TGF-ß, and p53 pathways in later stages of progression.[5] The increased rate of new mutations is facilitated by the acquisition of some form of genomic instability, most commonly chromosomal instability (CIN) that is present in about 85% of sporadic CRCs.[6] DNA copy number alterations particularly associated with the transition from adenoma to carcinoma are gains in 8q, 13q and 20q and losses in 8p, 15q, 17p and 18q.[7–9] The presence of two or more of any of these seven CAEs marked adenomas at high risk of progression with high accuracy (78% sensitivity and 78% specificity).[7]

Besides adenomas, also serrated polyps have been recognised as precursors lesions. In serrated polyps a *BRAF* mutation is typically the initiating event, leading to increased gene promoter hypermethylation (CpG island methylation phenotype or CIMP). When hypermethylation affects the expression of the mismatch repair (MMR) genes, this gives rise to another form of genomic instability, known as microsatellite instability (MSI).[10]

Although the molecular events occurring during CRC development have been widely studied, most observations are from cross-sectional studies as precursor lesions are removed upon detection at colonoscopy. In a few CT-colonography (CTC) studies in which small polyps were left *in situ*, adenoma growth during follow-up was associated with an advanced adenoma phenotype at resection.[2,11] The aim of the present study was to assess whether polyp growth was related to molecular features of colorectal cancer. For this purpose, we used a unique series of patients with small (6–9 mm) colorectal lesions initially identified by CTC that were left *in situ* and ultimately resected after a surveillance interval of three years. This allowed longitudinal assessment of lesion size (*i.e.* growth) in relation to histological and molecular characteristics at time of resection.

Methods

Study design and participants

In a Dutch multi-centre, randomised controlled screening trial (COlonoscopy or COlonography for Screening (COCOS) trial, 2009–2010, trial number: 2009/03WBO and NTR1829, The Hague, Netherlands) comparing primary colonoscopy to CTC,[12] patients in the CTC-arm with one or two small 6–9 mm colorectal lesions were advised to undergo a surveillance CTC after an interval of three years. Patients with more than two 6–9mm polyps or larger polyps on baseline CTC were referred for colonoscopy directly and therefore not included in the present follow-up study. The 95 small lesions detected on index CTC in this patient subpopulation were thus left *in situ* and re-measured at follow-up to assess the percentage of volumetric change for each lesion over the entire surveillance interval.[2] Based on volumetric change on CTC over the entire surveillance interval as proportion of baseline volume, lesions were classified as either grown (>30% growth), stable (<30% regression to <30% growth) or regressed (>30% regression). Details of this method and the choice of the 30% cut-off are described elsewhere.[2] Following the surveillance CTC, all patients were offered a colonoscopy for resection of the lesions. Location and size of the polyp on colonoscopy were recorded. The distal colon was defined as rectum and sigmoid. From those lesions for which histopathology was available, formalin fixed paraffin-embedded (FFPE) material was retrieved and reviewed by an expert pathologist (GAM). Based on histopathology and size at colonoscopy, lesions were classified as non-advanced adenoma, advanced adenoma, non-advanced serrated polyp or advanced serrated polyp according to the definitions summarised in Table 1.

Ethics approval from the Dutch Health Council was obtained for COCOS, including surveillance CTC after 3 years. Patients had already given their written informed consent to be contacted for follow-up studies and consented to this study.

Table 1 | Classification of colorectal polyps

Lesion type	Abbreviation	Definition
Non-advanced adenoma	NAA	tubular adenoma (TA) <10mm with low-grade dysplasia
Advanced adenoma	AA	adenoma ≥10mm and/or with high-grade dysplasia and/or a villous component of ≥25%
Non-advanced serrated polyps	NASP	hyperplastic polyp (HP), sessile serrated lesion (SSL), or traditional serrated lesion (TSL) <10mm without dysplasia
Advanced serrated polyp	ASP	hyperplastic polyp (HP), sessile serrated lesion (SSL), or traditional serrated lesion (TSL) ≥10mm and/or with dysplasia

Procedures

DNA isolation

DNA was isolated as previously described.[13] In brief, DNA from FFPE material was isolated following micro-dissection (> 70% tumour cells). A six-day incubation period with proteinase K in lysis buffer (ATL buffer, QIAmp, DNA micro-kit, Qiagen, Venlo, The Netherlands) was performed. Every day, proteinase K (10 µl or 20 ng/µl) was freshly added. DNA was isolated

using the QIAmp DNA micro-kit (Qiagen) and concentrations and purity were measured on a Nanodrop ND-1000 spectrophotometer (Isogen, IJsselstein, The Netherlands). Isolated DNA was used as input for copy number analysis, mutation analysis and CIMP analysis, in that order specifically.

DNA copy number analysis

DNA copy number changes were analysed with low-coverage whole genome sequencing (WGS).[14] Briefly, DNA was fragmented by sonication (Covaris S2, Woburn, MA, USA) and run on the HiSeq 2500 (Illumina, San Diego, CA, USA) on a 65 basepairs single-read modus using the KAPA HyperPrepKit (KAPA Biosystems, KK8504, Wilmington, MA, USA). This yielded a coverage of 0.13 × (IQR 0.12 to 0.14) genome coverage. The WGS reads were analysed with Bioconductor R-package QDNAseq, using a published workflow.[15] For every fixed-sized region of 30 kb on the genome, the relative abundance of sequence reads was used to determine the aberration status, applying corrections for mappability and GC content and removing germline specific variations.[14] A wavy pattern seen in copy number plots, 'genomic waves', which may be caused by replication timing of proliferating cells,[16] were smoothed using NoWaves.[17] This algorithm uses a set of normal samples (in this case of patients with CRC)[18] as reference to correct bins (genomic intervals) which systematically obtain a higher or lower signal. The obtained copy number profiles were segmented into regions of constant log2-read count and aberrations were called as high-level amplification (2), gain (1), normal (0), loss (-1) or homozygous deletion (-2). When the number of called copy number segments was above 200 over the whole genome while at the same time had a very a high difference between expected and observed noise (more than 1.5 times the interquartile range of values of all analysed samples), samples were excluded for further analysis. When ≥2 CAEs were present, the lesion was marked as a high-risk adenoma.

Mutation analysis

Samples in which DNA was still available after copy number analysis, were subjected to mutation analysis. DNA libraries were prepared using the KAPA HyperPrep Kit (KAPA Biosystems, Wilmington, MA, USA) as described in the KAPA HyperPrep Kit protocol (KR0961 – v5.16). Target enrichment was performed using a custom 48 gene xGen® Predesigned Gene Capture Pools (Integrated DNA Technologies, San Diego, CA, USA), according to the Rapid Protocol for DNA Probe Hybridisation and Target Capture Using an Illumina TruSeq® Library, Version 2.1, with an extended hybridisation reaction of 24 h. The gene panel consisted of 48 cancer-related genes, including genes most often mutated in colorectal cancer such as *APC*, *KRAS*, *NRAS*, *PIK3CA*, *SMAD4*, *TP53* and *BRAF* (Supplementary table 1). Paired-end 65 bp sequencing data were generated with Illumina Hiseq 2500 (Illumina, San Diego, CA, USA), yielding a median of 89x (IQR 55 to 148) coverage in the target regions, after removal of duplicate reads. The target regions, spanning the exonic sequences of the 48 genes, covered ~3.55×10^5 bp in total.

After adapter trimming, the reads were aligned to the human reference GRCh38 with BWA-MEM.[19] Subsequently base quality scores were recalibrated and the variants were called according to the GATK HaplotypeCaller.[20] Variant effects prediction was performed using SnpEff[21] and external data sources were linked using SnpSift.[22] To exclude DNA polymorphisms present in the normal populations, variants reported in dbSNP as 'common' or 'G5' were excluded. Furthermore, variants present at ≥1% in the ExAC exome data[23] and variants affecting non-coding sequences were excluded. Variants were required to have a 'medium' or 'high' effect, according to SnpEff, which led to the exclusion of silent mutations. Mutations were summed per gene and per sample using a representation called Oncoprint, which was created using R Bioconductor, package ComplexHeatmap.

CIMP analysis

Samples in which DNA was still available after copy number and mutation analyses, underwent sodium bisulfite modification (EZ DNA methylation kit, ZYMO research Co., Orange, CA, USA) to determine CIMP status. Nested methylation specific PCR (nested-MSP) for the CIMP marker panel as defined by Weisenberger[24] was performed as described earlier.[25] Ten µl of each MSP reaction was loaded onto a 2% agarose gel, stained with GelStar and visualised using ultraviolet light. Polyps were defined as CIMP-high when ≥ 3 of the 5 markers (CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1) from the CIMP marker panel were methylated.

MMR-status analysis

Immunohistochemistry (IHC) of the FFPE tumour samples was performed on a BenchMark Ultra autostainer (Ventana Medical Systems, Oro Valley, AZ, USA). Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 min and deparaffinised with EZ prep solution. Heat-induced antigen retrieval was carried out using Cell Conditioning 1 for 32 min at 95 °C (MSH2, MSH6 and PMS2), or 64 min at 95 °C (MLH1). MLH1 was detected using clone M1 (Ready-to-Use, 32 min at 37 °C, Ventana Medical systems), MSH2 using clone G219-1129 (Ready-to-Use, 12 min at 37 °C, Ventana Medical systems), MSH6 using clone EP49 (1/50 dilution, 32 min at 37 °C, Epitomics, Burlingame, CA, USA) and PMS2 using clone EP51 (1/40 dilution, 32 min at 37 °C, Dako).

For PMS2 signal amplification was applied using the Optiview Amplification Kit (4 min, Ventana Medical Systems). Bound antibody was detected using the OptiView DAB Detection Kit and slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems, Oro Valley, AZ, USA).

The slides were scored for positivity by an expert pathologist (GAM or PS). In case of positivity of the four MMR genes, the sample was considered MMR proficient. In case expression of one or more MMR genes was lost, the sample was considered MMR deficient.

Statistics analysis

Supported by the observation that colorectal polyps originating from the same patient differed in morphology, colonic location, histopathology and/or growth, all colorectal lesions were assumed to develop independently (Supplementary table 2). For comparisons of numerical data between two unpaired subgroups, the independent t-test was used. For comparison of categorical data between unpaired subgroups the Chi-square test or Fischer's Exact test was used. For all test, two-sided p≤.05 was considered significant. For the comparison of aberration frequencies between two groups, R-package CGHtest was applied, which runs a Chi-square test and a Wilcoxon rank-sum test. A multiple testing correction to the p-values was performed according to the Benjamini and Yekutieli FDR rule, using a cut-off for significance of 0.10.[26]

Data depository

Sequence data has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001003284.[27]

Results

Histopathology

Of the 68 polyps resected at the surveillance colonoscopy, material was available for 65 (96%) polyps (Figure 1). These 65 polyps came from 46 patients (57% male, mean age 66.7 (s.d. 6·9) years) who had a mean surveillance interval of 3.3 (s.d. 0.29) years. The lesions included 47 (72%) tubular adenomas (TAs) with low grade dysplasia (LGD), 9 (14%) tubulovillous adenomas (TVAs) with LGD, 1 (2%) sessile serrated lesion (SSL) without dysplasia and 8 (12%) hyperplastic polyps (HPs) without dysplasia (Table 2). When using the 30% threshold of volumetric change,[2] 48% (31/65) of the lesions had grown, 41% (27/65) remained stable and 11% (7/65) regressed (Table 2).

Histopathological features and molecular profiles of the 65 polyps, ranked by growth rate, are summarised in Figure 2. Of the adenomas, 36% (20/56) were classified as advanced adenomas (Table 1 and Figure 2B). All nine serrated polyps were classified as non-advanced serrated polyps.

In relation to histopathology, growth rates were significantly higher in TVAs compared to TAs (difference 121% growth (95% CI 42% to 200%), independent t-test, p=.003, Figure 3A). Also growth rates were significantly higher in polyps that were advanced adenomas at resection compared to non-advanced adenomas (difference 80% growth (95% CI 18% to 142%), independent t-test, p=.012, Figure 3B).

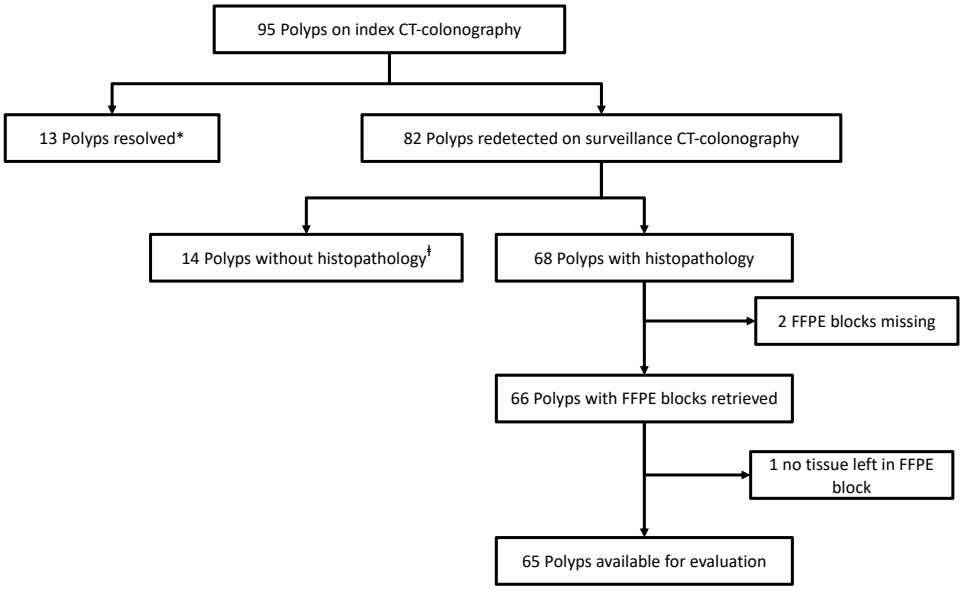

Figure 1 | Flow-chart of polyps followed longitudinally after initial CT-colonography (CTC) detection.

*13 polyps could not be redetected at surveillance CTC, despite good distention and good quality of fecal tagging in the relevant segments for 12 out of the 13 polyps. ‡14 polyps had no histopathological diagnosis, because the patient was not referred for colonoscopy (<6mm polyp at surveillance CTC) (n=5), the patient refused colonoscopy (n=5) or polyps were neither detected (n=3), nor retrieved (n=1) during colonoscopy.

Table 2 | Clinicopathological characteristics of the 65 longitudinally observed polyps per growth category

	Grown	Stable	Regressed
All polyps (n=65)	n=31	n=27	n=7
Location			
Proximal	16 (52%)	11 (41%)	5 (71%)
Distal	15 (48%)	16 (59%)	2 (29%)
Morphology			
Sessile	16 (52%)	14 (52%)	6 (86%)
Pedunculated	9 (29%)	11 (41%)	1 (14%)
Flat	6 (19%)	2 (7%)	0
Adenomas (n=56)	n=27	n=23	n=6
Histology			
Tubular	20 (74%)	21 (91%)	6 (100%)
Tubullovillous	7 (26%)	2 (9%)	0
Villous	0	0	0
Dysplasia			
Low-grade	27 (100%)	23 (100%)	6 (100%)
High-grade	0	0	0
Serrated polyps (n=9)	n=4	n=4	n=1
Histology			
Sessile serrated	1 (25%)	0	0
Hyperplastic	3 (75%)	4 (100%)	1 (100%)
Dysplasia			
Absent	4 (100%)	4 (100%)	1 (100%)
Present	0	0	0

DNA copy number analysis

Good quality DNA was obtained from 59 of the 65 retrieved lesions. The copy number profiles of 4/59 samples did not meet the quality criteria, leaving 55 lesions for analysis (Figure 4). These concerned 48 adenomas and seven serrated polyps (6 HPs and 1 SSL). Twenty percent (11/48) of adenomas, but none of the serrated polyps showed at least one CAE (Figure 2B). In these adenomas, 13q gain was the most common CAE (91%, 10/11), followed by 20q gain (4/11: 36%), 8q gain (3/11; 27%) and 17p loss (9%; 1/11) (Supplementary table 3).

The mean growth rate of lesions with CAEs was significantly higher in lesions with ≥1 CAEs, compared to lesions without CAEs (difference 91% growth (95% CI 13% to 169%), independent t-test, p=.023, Figure 5A). CAEs were absent in the five lesions that had regressed. Based on the molecular definition of having ≥2 CAEs, 10% (5/48) of the adenomas were classified as being at high risk for progression. Two of these high-risk adenomas were advanced adenomas that had grown, one was an advanced adenoma that had remained stable, one was a non-advanced adenoma that had remained stable and one was a non-advanced adenoma that had grown (Figure 2B).

Mutation analysis

Mutation analysis could be successfully completed for 34 samples (Figure 4), including 31 adenomas and three serrated polyps (2 HPs and 1 SSL). One or more mutations were observed in 94% (32/34) of the samples (median 2, range 0 to 5). Mutations of the WNT pathway were found in 74% (23/31) of adenomas, including *APC* mutation as the most common overall alteration in 61% (19/31) and *CTNNB1* mutation in 16% (5/31) (Figure 2A). Only one adenoma had a mutation in both the *APC* and *CTNNB1* genes; in all other samples these mutations were mutually exclusive. The PI3K-AKT pathway was affected in one adenoma (3%) with a mutation in the *PTEN* gene. Genetic alterations in the RAS-MAPK pathway occurred in 10% (3/31) of the adenomas, concerning two *KRAS* mutations (6%; 2/31) and one mutation in *ERBB2*. No mutations were found in the TGFβ pathway. Sixteen percent (5/31) of adenomas showed mutations in the p53 pathway 16% (5/31), occurring in *ATM* (16%; 5/31) and/or *TP53* (3%; 1/31). In the serrated polyps, *BRAF* was the only mutation detected (67%; 2/3).

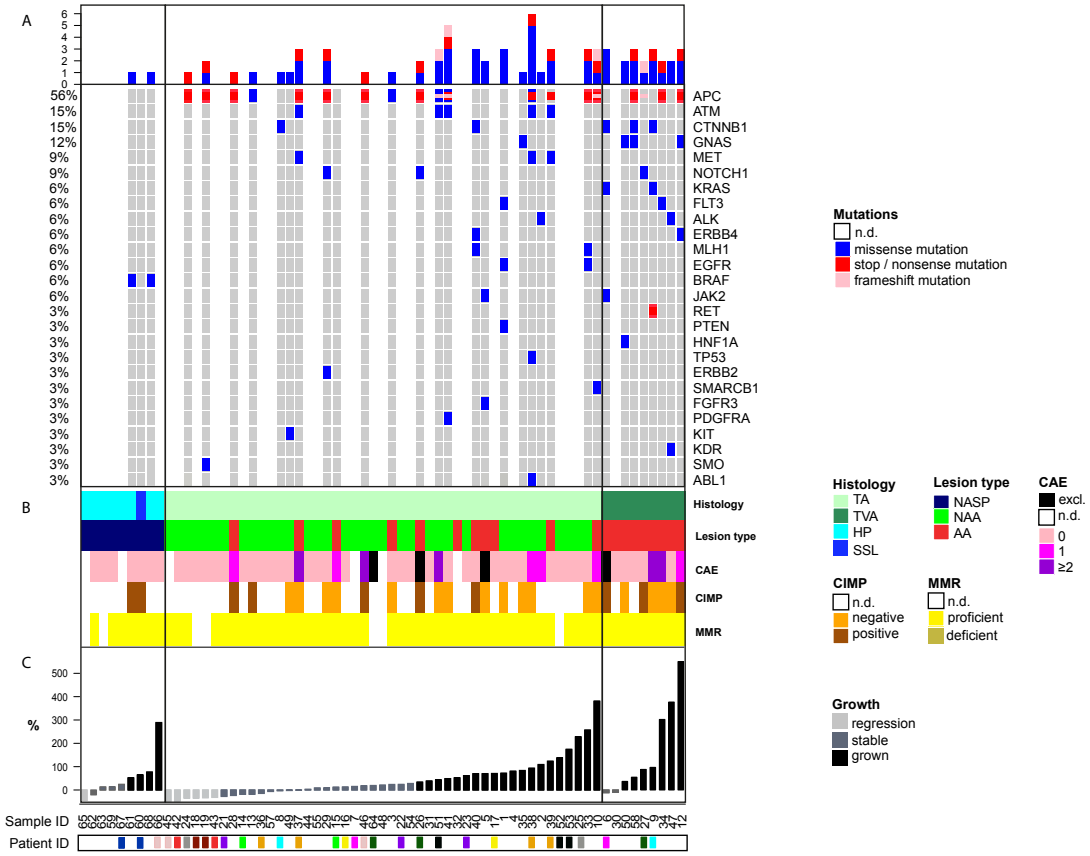

Figure 2 | Molecular profile and histopathological features ordered by growth rate for hyperplastic polyps and sessile serrated lesions (left), tubulovillous adenomas (middle) and tubular adenomas (right).

Each vertical column represents an individual lesion. A. Mutational profile. Only genes that have a mutation in at least one of the samples are shown. The top bars represent the number of mutations per sample. Percentages on the left indicate the prevalence of a specific mutation over all the analysed samples. B. Histopathological features and molecular profile resulting from copy number analysis, CIMP analysis and immunohistochemistry of the mismatch-repair genes. Several samples could not undergo the entire range of molecular analyses due to limited DNA available. Histology: TA = tubular adenoma, TVA = tubulovillous adenoma, HP = hyperplastic polyp, SSL = sessile serrated lesion; Lesion type: NASP = non-advanced serrated polyp, NAA = non-advanced adenoma, AA = advanced adenoma; CAE = cancer associated event, which includes chromosomal gains in 8q, 13q and 20q and losses in 8p, 15q, 17p and 18q. The presence of ≥2 CAEs defines high-risk adenomas. Four samples were excluded from DNA copy number analysis due to poor quality; CIMP = CpG island methylation phenotype; MMR = mismatch repair. C. Growth rates according to CT-colonography measurement during the 3-year surveillance interval. Patient ID is depicted in colour marking only for patients with ≥1 polyp.

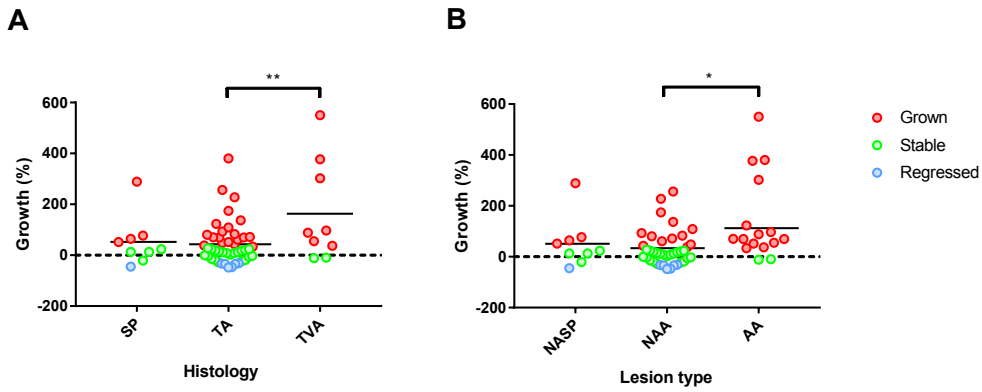

Figure 3 | Growth rates for individual polyps based on histology and lesion type.
Growth rates for individual polyps classified as A. serrated polyp (SP), tubular adenoma (TA) and tubulovillous adenoma (TVA) with corresponding means (** independent t-test, p<.01) B. non-advanced serrated polyp (NASP), non-advanced adenoma (NAA), and advanced adenoma (AA) (* independent t-test, p<.05). The red, green and blue dots represent lesions that had grown, remained stable or regressed during the 3-year surveillance interval, respectively, according to the threshold of volumetric change.

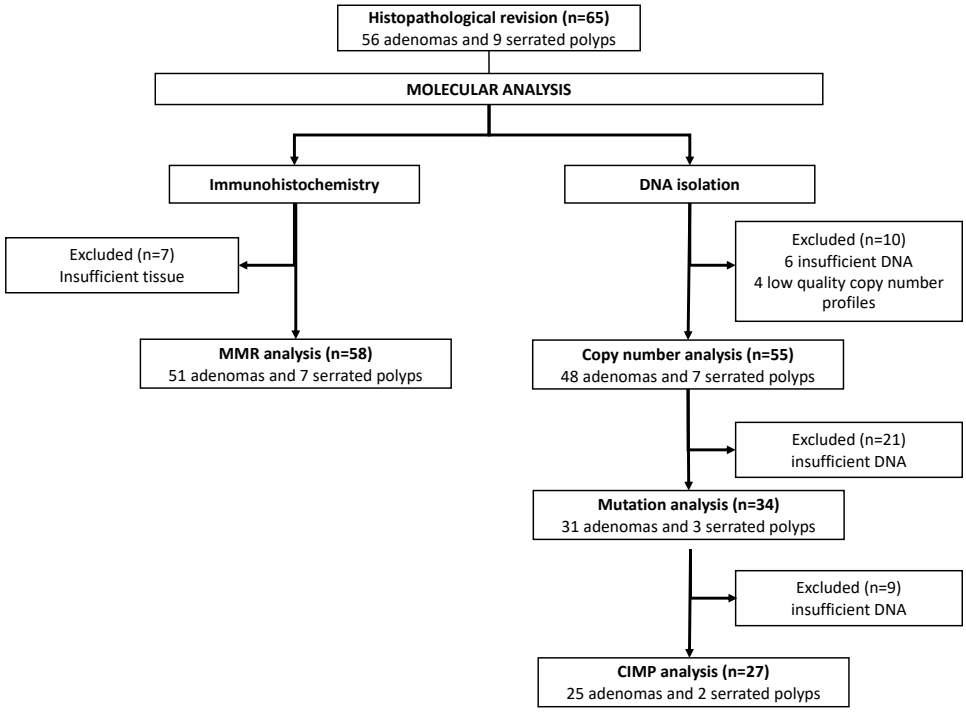

Figure 4 | Flow-chart of polyp samples used for molecular analyses.
CIMP = CpG island methylation phenotype; MMR = mismatch repair

Mutation burden correlated with growth: those lesions with ≥2 mutations had higher growth rates compared to lesions with only 0–1 mutations (difference 99% growth (95% CI 9% to 189%), independent t-test, p=.032, Figure 5B). *APC* mutations were present in all growth categories. Six of the 19 (32%) samples with *APC* mutations also carried one or more mutations in the PI3K, RAS-MAPK, or p53 pathways. Such combinations were not found in lesions that had regressed.

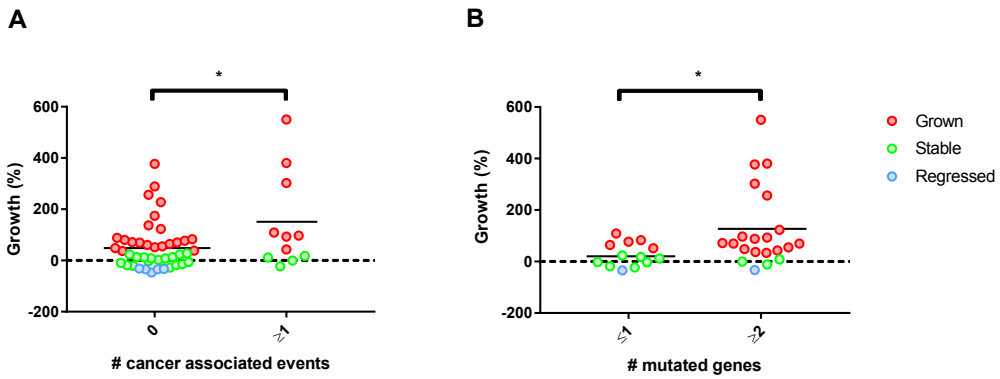

Figure 5 | Growth rates for individuals polyps based on number of cancer associated events and mutated genes.
Growth rates for individual polyps that have A. 0 or ≥1 cancer associated events, which include chromosomal gains in 8q, 13q and 20q and losses in 8p, 15q, 17p and 18q (* independent t-test, p<.05) B. ≤1 mutation or ≥2 mutations, with corresponding means (* independent t-test, p<.05). The red, green and blue dots represent lesions that had grown, remained stable or regressed during the 3-year surveillance interval, respectively, according to the threshold of volumetric change.

CIMP analysis

CIMP status was determined on all 27 samples in which DNA was still available (Figure 4). These were 25 adenomas and two serrated polyps (1 HP and 1 SSL). Eight of 25 adenomas (32%) were CIMP positive (Figure 2B). Both of the serrated polyps were CIMP positive and located in the distal colon. In the small number of polyps on which CIMP analysis was performed, no statistically significant difference could be observed.

MMR-status analysis

In all 58 samples (51 adenomas and 7 serrated polyps) that were assessed by IHC (Figure 4), expression of MLH1, MSH2, MSH6 and PMS2 proteins was present (Supplementary figure 1), hence these were all classified as MMR proficient (Figure 2B).

Discussion

In the present study, we sought to evaluate whether growing colorectal polyps showed distinct molecular features associated with progression, compared to lesions that regressed or remained stable over time. To this aim, we performed detailed molecular analyses in a set of

65 small (6–9 mm) colorectal polyps that were removed three years after initial identification by CTC.

Higher growth rates were related to presence of non-random DNA copy number alterations associated with colorectal adenoma to carcinoma progression (cancer associated events or CAEs), as well as to increased mutation burden. Regressed lesions did not show CAEs, but did have mutations in genes involved in common CRC pathways, which concerned mostly *APC* mutations. Because *APC* inactivation is such an early event in adenoma genesis, it may not prevent lesions from regressing. The only mutation besides *APC* that was present in one of the regressed lesions was in the *SMO* gene, a rare mutation occurring in 0.9% of CRCs.[28] The SMO protein is a component of the hedgehog signalling pathway, which has been shown to negatively regulate WNT signalling.[29] In gastric cancers, however, *SMO* mutations were found not to be associated with altered expression of hedgehog target genes, indicating that these are probably passenger mutations.[30]

Our observation that multiple mutations are already present in a substantial proportion of small colorectal polyps is in line with previous observations that detectable mutations occurred at an early stage of polyp development, at a mean size of only 30±35 crypts.[31,32] For perspective; lesions of 10mm^3 contain approximately 3×10^5 crypts. Many of the somatic mutations detected in tumours may occur even before morphologically recognisable tumour formation.[33,34] Recent data show that in ~1% of morphologically normal colorectal crypts driver mutations were already present.[35] In the current study, approximately one third of the adenomas (6/19; 32%) with *APC* mutations, also had mutations in the PI3K, RAS-MAPK, or p53 pathways. However, only two of these adenomas with additional mutations in the PI3K, RAS-MAPK, or p53 pathways were at high risk of progression based on the criterion of having ≥2 CAEs.[7] Accumulating evidence suggests that whereas mutations in driver genes are already present early in precancerous lesions, chromosomal instability is a late phenomenon during adenoma to carcinoma progression.[36] Therefore, copy number alterations likely play a more critical role in malignant transformation. This is functionally supported by the observation that engineered patient-derived, adenoma organoids with critical driver mutations, only obtained metastatic capacity when CIN was present.[37] The results from our study confirm that mutations, present in regressed, stable and grown lesions, do not reflect polyp risk of progression, whereas CAEs, only present in stable and grown lesions, may likely do so.

Identifying adenomas with a high progression risk is of value for clinical practice. At the current moment, adenomas ≥10mm, with villous component or high-grade dysplasia are considered high risk - or advanced - adenomas. Yet, this definition gives a suboptimal estimation of the true risk of progression and has been introduced and adopted in literature without much evidence.[38] The presence of ≥2 CAEs more precisely reflects the natural course of the disease and more specifically identifies adenomas at high risk of progressing to cancer.[7] Previous research has shown that only 25% of advanced adenomas and 3% of non-advanced adenomas presented with ≥2 CAEs,[39] suggesting that the majority of advanced adenomas should not be considered high risk, whereas a small proportion of non-advanced adenomas

should. The problem associated with the definition of advanced adenoma, is also reflected by studies that investigated the relationship between polyp growth and the risk of progression, taking advanced adenomas as the endpoint.[2,11] In the original description of our cohort,[2] the rates of advanced adenomas were 47%, 21% and zero in progressed, stable and regressed lesions, respectively. As 6–9 mm polyps that grow will easily qualify as advanced adenoma, when reaching 10 mm or more, we hypothesised that these rates were an overestimation of the actual risk of progression. Indeed, when focusing on molecular features rather than phenotypical features to define high-risk adenomas, only 13% of the adenomas that had grown, 11% of the stable adenomas and none of the regressed adenomas had ≥2 CAEs. Although numbers are small, these last rates appear to be more consistent with the actual progression risk, as it is estimated that approximately 5% of adenomas eventually progress to cancer.[1]

The application of CAEs to identify high risk adenomas could be used in the development of novel diagnostic screening tests. After all, screening programs ideally should aim to detect precursor lesions just before they transform to colorectal cancer, in order to reduce both cancer incidence and mortality. In addition, molecularly-defined high risk adenomas could impact surveillance. According to the current post-polypectomy colonoscopy surveillance guideline, the presence of advanced adenomas shortens the surveillance interval.[40] In our study a total of 18 patients had at least one advanced adenoma based on phenotypical features at follow-up colonoscopy, compared to five patients based on molecular features (of which only three overlapping between the groups), which suggests overdiagnosis is happening with the current strategy. With the technical advancements over the recent years, low cost and fast methods for copy number profiling have become available.[41] This makes the use of CAEs for risk stratified surveillance a realistic approach, although first further research is needed to assess the correlation between the presence of molecularly-defined high risk adenomas and the risk of metachronous lesions.

The use of CAEs as progression biomarker only applies to adenomas and not to serrated polyps. In the present cohort, CAEs were absent in serrated polyps irrespective of their growth category. This is not surprising, since progression of this lesion type is associated with the acquisition of MSI instead of CIN and therefore different molecular events characterise high risk serrated polyps. We found that in serrated polyps, *BRAF* mutations and CIMP positivity were present, but MLH1 was not yet affected. Because no dysplasia was present in any of the serrated polyps studied, this is in line with previous studies showing that MLH1 deficiency coincides with dysplasia in a serrated polyp.[42]

For ethical reasons, longitudinal studies leaving polyps *in situ* for some years can only be done in patients with small polyps, as these are considered low risk. As most of these polyps are still small when removed after follow-up, a practical consequence is that from some polyps only limited amounts of tumour DNA can be obtained for molecular analyses after standard diagnostic procedures. As a result, only a subset (42%) of samples could undergo the entire range of molecular assays. In addition, no paired normal tissue was available, therefore making

the analysis dependent on public databases to filter out polymorphisms. Inherent to the fact that histopathology of these small polyps can only be determined at one point in time, being the time of resection of the polyps, evaluating the morphological evolution in relation to the biological evolution of the polyps is not feasible. Despite these limitations, the present study uniquely provides a comprehensive overview of DNA copy number, mutation, CIMP and MSI profiling status of a relatively large series of polyps that were followed longitudinally.

In conclusion, molecular alterations associated with colorectal adenoma to carcinoma progression were related to growth over time, but were absent in regressed lesions. So far, these molecular alterations have been mostly identified by cross-sectional observations in tissue samples from colorectal adenomas and cancers. The present longitudinal study provides *in vivo* support in the human setting for the functional role of these molecular alterations in this process.

Acknowledgements

We thank the Genomic Core Facility at The Netherlands Cancer Institute for their sequencing service. We thank the Core Facility – Molecular Pathology and Biobank for facilitating immunohistochemical staining.

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Supplementary Material

Supplementary table 1 | Custom 48 gene panel used for mutation analysis

ABL1	FGFR3	NOTCH1
AKT1	FLT3	NPM1
ALK	GNA11	NRAS
APC	GNAQ	PDFRA
ATM	GNAS	PIK3CA
BRAF	HNF1A	PTEN
CDH1	HRAS	PTPN11
CDKN2A	IDH1	RB1
CSF1R	JAK2	RET
CTNNB1	JAK3	SMAD4
EGFR	KDR	SMARCB1
ERBB2	KIT	SMO
ERBB4	KRAS	SRC
FBBXW7	MET	STK11
FGFR1	MLH1	TP53
FGFR2	MPL	VHL

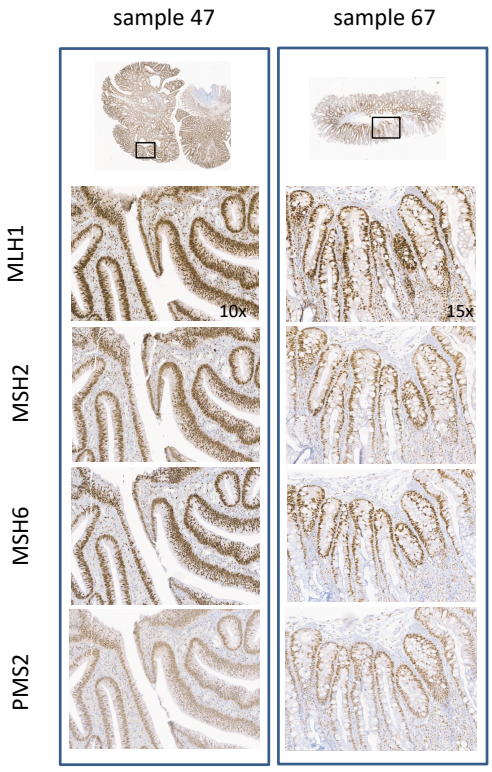
Supplementary table 2 | Characteristics of lesions originating from patients with multiple polyps. Lesions were assumed to develop independently based on differing morphology, colonic location, size, histopathology and/or growth. The copy number alterations of the polyps confirmed this assumption.

Patient	Sample ID	Morphology	Location	Endoscopy size	Histo-pathology	Dysplasia	Volumetric change	Total # CNA	8q gain	13q gain	20q gain	8p loss	15q loss	17p loss	18q loss	Total # CAE
10284	6	pedunculated	proximal	8	TVA	LGD	-11.67	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.
10284	7	flat	distal	8	TA	LGD	14.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10375	9	sessile	proximal	10	TVA	LGD	97.02	4	Yes	Yes	No	No	No	No	No	2
10375	8	sessile	proximal	5	TA	LGD	-2.92	0	No	No	No	No	No	No	No	0
10482	15	pedunculated	distal	10	TA	LGD	11.92	1	No	Yes	No	No	No	No	No	1
10482	14	sessile	distal	7	TA	LGD	-18.53	0	No	No	No	No	No	No	No	0
10916	16	pedunculated	distal	7	TA	LGD	12.65	0	No	No	No	No	No	No	No	0
10916	17	pedunculated	distal	15	TA	LGD	70.51	0	No	No	No	No	No	No	No	0
11130	19	sessile	proximal	5	TA	LGD	-32.97	1	No	No	No	No	No	No	No	0
11130	18	sessile	proximal	8	TA	LGD	-34.41	0	No	No	No	No	No	No	No	0
11177	23	sessile	proximal	7	TA	LGD	61.26	0	No	No	No	No	No	No	No	0
11177	22	sessile	proximal	7	TA	LGD	23.7	0	No	No	No	No	No	No	No	0
11177	21	pedunculated	proximal	9	TA	LGD	-27.52	0	No	No	No	No	No	No	No	0
11430	24	sessile	proximal	6	TA	LGD	-34.51	0	No	No	No	No	No	No	No	0
11430	25	pedunculated	proximal	9	TA	LGD	227.46	0	No	No	No	No	No	No	No	0
11574	64	sessile	proximal	6	TA	LGD	19	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.
11574	26	pedunculated	proximal	12	TA	LGD	33.59	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.
11574	27	pedunculated	proximal	15	TVA	LGD	88.21	0	No	No	No	No	No	No	No	0
12070	39	flat	proximal	10	TA	LGD	123.08	0	No	No	No	No	No	No	No	0
12070	36	flat	proximal	5	TA	LGD	-14.51	0	No	No	No	No	No	No	No	0
12070	37	sessile	proximal	10	TA	LGD	-0.57	6	No	Yes	Yes	No	No	Yes	No	3
12070	38	flat	distal	7	TA	LGD	93.09	1	No	Yes	No	No	No	No	No	1
12359	42	pedunculated	distal	4	TA	LGD	-47.18	0	No	No	No	No	No	No	No	0
12359	43	sessile	distal	5	TA	LGD	-31.58	0	No	No	No	No	No	No	No	0
12444	45	sessile	proximal	4	TA	LGD	-48.23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12444	66	flat	distal	3	HP	none	288.83	0	No	No	No	No	No	No	No	0
12444	46	pedunculated	distal	6	TA	LGD	17.83	2	No	Yes	Yes	No	No	No	No	2
12671	52	sessile	proximal	7	TA	LGD	137.29	0	No	No	No	No	No	No	No	0
12671	53	sessile	proximal	7	TA	LGD	174.07	0	No	No	No	No	No	No	No	0
12671	51	pedunculated	proximal	8	TA	LGD	43.23	4	Yes	Yes	No	No	No	No	No	2
12820	67	sessile	distal	5	HP	none	23.88	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12820	60	sessile	distal	8	SSP	none	64.32	0	No	No	No	No	No	No	No	0

CAE = cancer associated events, CNA = copy number alterations, excl. = exclusion, LGD = low grade dysplasia, n.d. = not determined, TA = tubular adenoma, TVA = tubulovillous adenoma.

Supplementary table 3 | Overview of 11 adenomas with at least one associated event, originating from 10 different patients

Patient	Sample ID	Sex	Age	Histopathology	Dysplasia	Growth rate	8q gain	13 gain	20q gain	8p loss	15q loss	17p loss	18q loss	Total CAEs
10052	2	Male	62	TA	LGD	108.97								1
10375	9	Male	71	TVA	LGD	97.02								2
10381	10	Male	59	TA	LGD	380.55								1
10401	12	Male	74	TVA	LGD	550.45								1
10482	15	Female	68	TA	LGD	11.92								1
11585	28	Male	73	TA	LGD	-23.30								1
12036	34	Female	59	TVA	LGD	302.40								3
12070	37	Male	61	TA	LGD	-0.57								3
12070	38	Male	61	TA	LGD	93.09								1
12444	46	Female	55	TA	LGD	17.83								2
12671	51	Male	76	TA	LGD	43.23								2
						Frequency	3	10	4	0	0	1	0	18



Supplementary figure 1 | Expression of the mismatch repair genes MLH1, MSH2, MSH6 and PMS2 for sample #47 (tubulovillous adenoma with low grade dysplasia) and sample #67 (hyperplastic polyp).

PART II.

Surveillance strategies for early colorectal cancer detection

“Success consists of going from failure to failure without loss of enthusiasm”

Winston Churchill

5.

Colorectal cancer screening by colonoscopy – putting it into perspective

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Digestive Endoscopy 2015

Abstract

Implementation of nationwide screening programs aims to decrease the disease burden of colorectal cancer (CRC) in the general population. Globally, most population screening programs for CRC are performed by either fecal occult blood test, flexible sigmoidoscopy or colonoscopy. For screening programs with colonoscopy as primary method, only circumstantial evidence from observational studies is available to prove its effectiveness, suggesting that colonoscopy effectively reduces CRC incidence and mortality. Currently, large randomized trials are being conducted to corroborate these findings. Besides the direct effect of a screening program for CRC, its protective effect is further enhanced by enrolment of patients that underwent polypectomy in surveillance programs. However, despite CRC screening and surveillance colonoscopies, interval CRCs still occur. Those are predominantly located in the right-sided colon and potential explanations, besides unfavorable tumor characteristics, are preventable operator-dependent factors relating to the quality of the colonoscopy procedure. In an effort to reduce differences in endoscopist performance and thereby the occurrence of interval CRCs, quality indicators of colonoscopy have been introduced. The meticulous inspection of the colonic mucosa not only results in the detection of advanced and relevant lesions, but also in the removal of many diminutive and small lesions leading to an increasing number of surveillance colonoscopies, known as the "high-detection paradox". More data on the cost-effectiveness of high quality colonoscopy as a primary screening method and surveillance programs with intervals based on optimal risk-stratification are eagerly awaited.

Introduction

According to the 2012 cancer statistics of the World Health Organization, CRC is the fourth most common cause of cancer death worldwide and the second in Europe.[1] Although often perceived as one disease entity, CRC is increasingly acknowledged as a heterogeneous disease with different types of precursor lesions (*polyps*).[2-5] Besides the conventional adenomatous polyps, which are believed to account for 70–80% of the CRCs,[6,7] recent literature suggests that also serrated polyps have malignant potential by progression through the serrated neoplasia pathway.[2, 8, 9] The progression of both polyp types into cancer requires the accumulation of (epi-)genetic mutations. This process covers many years, for most adenomas presumably 10 to 15 years,[7, 10, 11] thereby providing clinicians a window of opportunity to intervene prior to the development of CRC. The asymptomatic presentation of precancerous and early cancerous lesions and the relatively low-risk of endoscopic polypectomy are amongst the justifications of CRC screening.

After extensive research on cost-effectiveness, CRC screening is now increasingly implemented across Europe.[12] The major aim of a screening program is to reduce CRC mortality by detection and removal of colonic polyps and (early) cancers. Multiple countries have instigated invitational or opportunistic (self-referral or referral by general practitioner) screening programs with fecal occult blood test (FOBT), flexible sigmoidoscopy (FS) or colonoscopy as primary screening methods (table 1). One of the advantages of colonoscopy as a screening method is that, besides being the most accurate modality and its potential to reduce the incidence of CRC, it is a "one-stage screening method": it is not only possible to detect precursor lesions and early cancers in the entire colon, but also to remove them in the same procedure.

In this review, the evidence for the effect of primary colonoscopy screening on CRC incidence and mortality will be discussed. Besides, other topics with an important role in the success of CRC screening will be discussed such as risk-stratified surveillance, quality assurance of the endoscopic procedure and improvement in colonoscopy techniques.

Primary colonoscopy screening

Several countries in the Western world such as Austria, Germany, Poland and the United States, use colonoscopy as primary screening tool for CRC (table 1). The effectiveness of colonoscopy screening can be assessed by a reduction in incidence and mortality from CRC. However, to this date no randomized trial on the long-term effectiveness of colonoscopy screening compared to either no screening or another screening method, has been published and indirect evidence is used to justify its implementation. In contrast, the effectiveness of screening with FOBT or FS has been demonstrated in large randomized controlled trials (RCTs).[26-33] The disadvantages of colonoscopy as a primary screening method include its need

for a purgative bowel preparation, its invasive character, the potential complications, the high costs and the need for colonoscopy-capacity. Furthermore, participation-rates are relatively low compared to other screening modalities, ranging from 10 to 27% in population-based screening studies.[32, 34-37] The European Union taskforce has recommended to use FOBT as a primary screening method in organized programs and the majority of European countries have implemented FOBT-based programs.[38]

Table 1 | Screening programs instigated throughout the Western World†

Nation	Program	Invitations	Age (yrs)	Type	Interval
Australia [13]	Nationwide	Structured	50 – 74	FIT	Biannual
Austria‡ [14]	Nationwide	Opportunistic	>50	Colonoscopy	7-10 years
				gFOBT	Annual
Belgium [15]	Regional	Structured	50 – 74	gFOBT and FIT§	Biannual
Canada [16]	Regional	Structured	50 – 75	gFOBT and FIT§	(Bi)annual
England [17]	Nationwide	Structured	60 – 74	gFOBT	Biannual
		Pilot	55 + 60 - 74	Sigmoidoscopy	Once only
France [18]	Regional	Structured	50 – 74	FIT	Biannual
Germany‡ [19]	Nationwide	Opportunistic	50 – 54	gFOBT	Annual
			>55	Colonoscopy	10 years
				gFOBT	Biannual
Italy [20]	Regional	Structured	44 – 75	FIT	Biannual
		Pilot	58 – 60	Sigmoidoscopy	Once only
Netherlands [21]	Nationwide	Structured	55 – 74	FIT	Biannual
Poland [22]	Nationwide	Opportunistic	50 – 65	Colonoscopy	10 years
Spain [23]	Regional	Structured	60 – 69	FIT	Biannual
Switzerland [24]	Nationwide	Opportunistic	50 – 69	Colonoscopy	10 years
				gFOBT	Biannual
United States‡ [25]	Nationwide	Opportunistic	50 – 75	Colonoscopy	10 years
				Sigmoidoscopy	5 years
				gFOBT and FIT	Annual

†Table might display inconsistencies for regional programs within countries since regions sometimes use different screening tests and invite different age categories.

‡ Colonoscopy is the preferred screening option in these countries, while the other methods are an alternative for those who refuse primary colon screening.

§ Consists of either guaiac faecal occult blood test (gFOBT) or faecal immunohistochemical test (FIT) as preferred by the regional government.

Results of primary colonoscopy screening on colorectal cancer incidence and mortality

Observational studies

Among the scarce evidence, there is one landmark-study that demonstrated the protective effect of colonoscopy on long-term CRC-related mortality. The 'National Polyp Study' was

aimed to compare different surveillance strategies after removal of one or more adenomas in 2602 patients.[39, 40] Based on a comparison with US registration data of the general population (SEER-database), colonoscopy with polypectomy resulted in a reduction in CRC mortality by 53% (CI; 20–74%) after a median follow-up of nearly 16 years.[39]

There is other circumstantial evidence that provides data on the effect of primary colonoscopy screening on CRC-related mortality (table 2). Kahi et al. observed a cohort of 715 asymptomatic individuals who underwent screening colonoscopy between 1989 and 1993 at a university hospital. [41] Within 10,000 patient-years of follow-up, three patients died of CRC while the expected number was nine. [41] Although the study was not powered to detect differences in CRC-related mortality, the relative mortality reduction was estimated at 65%. [41] Comparable results were seen in a cohort study in which 22,818 individuals (aged 50–80 years) living in a precisely defined rural area in Switzerland were invited for screening colonoscopy.[42] In total 2,044 persons accepted the screening invitation, corresponding with a participation-rate of 9%.[42] After 6 years of follow-up, approximately 1 of 4 patients with CRC that died were amongst those who declined the invitation, whereas this was only 1 of 12 in the screened group, resulting in a 88% (CI; 7–99%) risk reduction in CRC-related mortality.[42] In a third study, prospective data of two large, well-characterized cohorts were collected and used to assess CRC-related mortality after having been exposed to a screening colonoscopy.[43] According to this study, screening colonoscopy reduced the risk of dying from CRC with 68% (CI; 55–75%).[43] The authors categorized the results per colonic location and interestingly, mortality from distal CRC was reduced by 82% (CI; 69–90%) compared to only 53% (CI; 24–71%) from proximal CRC.[43] A pooled analysis of the above-mentioned observational studies resulted in a reduction of CRC-related mortality of 68% (CI; 57–77%) after screening colonoscopy.[44]

Table 2 | Observational studies evaluating the protective effect of screening colonoscopy included in systematic review modified from Brenner *et al.*[44]

First author	Published	Years	Nation	Design	Mortality	Incidence
Brenner [45]	2014	2003 – 2010	Germany	Case-control	Not reported	OR 0.09 (0.07 to 0.13)
Nishihara [43]	2013	1988 – 2012	United States	Cohort	HR 0.32 (0.24 to 0.45)	Not reported
Doubeni [46]	2013	2006 – 2008	United States	Case-control	Not reported	OR 0.30 (0.15 to 0.59)
Manser [42]	2012	2001 – 2007	Switzerland	Cohort	OR 0.12 (0.01 to 0.93)	OR 0.31 (0.16 to 0.59)
Kahi [41]	2009	1989 – 2007	United States	Cohort	SMR 0.35 (0.0 to 1.06)	SIR 0.33 (0.10 to 0.62)
Cotterchio [47]	2005	1997 – 2000	Canada	Case-control	Not reported	OR 0.69 (0.44 to 1.07)

OR=adjusted odds ratio (95% confidence interval), HR=multivariate hazard ratio (95% confidence interval), SMR=standardized mortality ratio (95% confidence interval), SIR=standardized incidence ratio (95% confidence interval).

Instead of CRC mortality, other studies have focused on the effect of screening colonoscopy on the incidence of CRC (table 2). A recent German population-based case-control study selected 2,516 patients that were diagnosed with CRC between 2003 and 2010, and 2,284 randomly selected, matched controls. [45] According to registration data, only 1.7% of the cases had undergone a screening colonoscopy versus 12% of the controls.[45] The reduction in risk for left- and right-sided CRC was comparable at 92% (CI; 89–96%) and 78% (CI; 67–86%, n.s.), respectively.[45] These results were in line with a recent nested-case control study from the U.S. that was limited by a small number of screening colonoscopies; only 13 cases and 46 controls underwent a screening colonoscopy.[46] Screening colonoscopy was associated with a reduction of 70% (CI; 41–85%) in incidence of late stage CRC, defined as ≥ stage IIB, being comparable for right and left sided cancer.[46] Three additional studies have provided data on CRC incidence after screening colonoscopy.[41, 42, 47] A pooled analysis of the outcomes of these studies resulted in an incidence risk reduction of 56% for proximal CRC and 79% for distal CRC.[44]

In conclusion, screening colonoscopy seems to have the potential to achieve a substantial reduction in CRC incidence and mortality. This protective effect seems more profound for distal than for proximal CRC.[43, 44] In most recent studies this difference was less clear which might be explained by the improved quality of colonoscopy, a topic that will be discussed in more detail later in this article.[45, 46] Because most of these population studies evaluated colonoscopies that were performed many years or even decades ago, current advancements in quality of colonoscopy are not taken into account. The currently increased awareness and technical progress most probably results in an underestimation of the effectiveness of colonoscopy screening reported in these studies.[41-44, 47] The most important drawback of observational studies, however, is the lack of reliable data on participation-rates, necessary to predict the real-life screening effect. Therefore, results of RCTs comparing different methods of screening versus no screening are eagerly awaited.

Expected randomized trials

Four large RCTs are currently being conducted, all aiming to evaluate the participation-rate into a primary colonoscopy screening program and its effectiveness on the incidence and mortality of CRC (table 3).[35, 48-50] The first long-term results of these studies are expected in the next decade. Both the ColonPrev trial and the CONFIRM trial are RCTs comparing primary colonoscopy screening with (bi)annual fecal immunochemical testing (FIT).[35, 48] The Spanish ColonPrev study is designed as a non-inferiority trial,[35] whereas the CONFIRM-trial is performed in the United States and aims to prove the superiority of colonoscopy screening.[48] This is in contrast with the European NordICC trial and the Swedish SCREESCO, both RCTs designed to prove the effectiveness of CRC screening in an average risk population, compared to no screening.[49, 50] Primary colonoscopy is the intervention in the NordICC trial,[49] while the SCREESCO trial uses both biannual FIT and colonoscopy as a screening method.[50] The ColonPrev trial and the NordICC trial have both completed their first round of screening, the CONFIRM and SCREESCO trials are currently recruiting patients.

Table 3 | Summary of ongoing randomized controlled trials to investigate the effect of screening colonoscopy on CRC incidence and mortality

Name & country	Invitees	Intervention	Design	Age (yrs)	Current status	Acceptance rates
COLONPREV[35] Spain	57,000	One time colonoscopy vs. biannual FIT (1:1)	Non-inferiority trial	50 – 69	First round completed	Colonoscopy 24,6% FIT 34,2%
CONFIRM[48] United States	55,000	One time colonoscopy vs. annual FIT (1:1)	Superiority trial	50 – 75	Recruiting	Not yet published
NordICC[49] Norway, Poland, Netherlands, Sweden & Iceland	95,000	One time colonoscopy vs. usual care (1:2)	Effectiveness trial	55 – 64	One time colonoscopy completed	Not yet published
SCREESCO[50] Sweden	200,000	One time colonoscopy vs. biannual FIT vs. no screening (1:3:6)	Effectiveness trial	59 – 62	Recruiting	Not yet published

Surveillance colonoscopy after polypectomy

Besides detection of precancerous polyps and (early) CRCs, the effect of screening is optimized through the enrolment of the patients that underwent polypectomy in an endoscopic surveillance program. Currently, 20–40% of the available colonoscopy capacity is used for surveillance examinations.[51-54] To avert disproportionate growth in colonoscopy demand resulting from the implementation of population-based screening programs, post-polypectomy surveillance should be restricted to those patients that are most likely to benefit from additional colonoscopies.

Risk stratification

Patients that underwent removal of adenomatous polyps carry an increased risk to develop advanced neoplasia (adenomas ≥10 mm or with unfavorable histology or cancer) in the future.[55-58] Baseline polyp characteristics that have shown to predict future development of advanced neoplasia are size, multiplicity, a villous component (≥25%), high-grade dysplasia and proximal location.[59-61]In most guidelines, these factors are used to stratify patients into a low and a high-risk group for allocation of the appropriate surveillance interval. According to the guideline of the European Society of Gastrointestinal Endoscopy (ESGE) [62] as well as the guideline of the American Gastroenterological Association (AGA),[63] the low-risk group comprises patients with 1–2 tubular adenomas <10mm with low-grade dysplasia (table 4). Patients with adenomas with villous histology or high-grade dysplasia or size ≥10mm, and patients with ≥3 synchronous adenomas, are categorized in the high-risk group.[62, 63]

Table 4 | Current international guideline recommendations for post-polypectomy surveillance

	Most advanced lesion			
	Low-risk adenoma 1-2 tubular adenomas, < 10mm with LGD	**High-risk adenoma** villous histology, HGD, ≥10mm in size or ≥3 adenomas.	**Serrated polyp (low-risk)** <10mm without dysplasia	**Serrated polyp (high -risk)** ≥10mm or with dysplasia
ESGE guideline [62]	10 years/return to screening	3 years	10 years/return to screening	3 years
AGA guideline [63]	5†-10 years	3 years	5 years	3 years

HGD = high-grade dysplasia, LGD = low-grade dysplasia, ESGE = European Society of Gastrointestinal Endoscopy (ESGE) guideline, AGA = American Gastroenterological Association (AGA) guideline. † The AGA recommends a 5 year surveillance guideline for the low-risk group in case of inadequate bowel preparation or poor quality examination.

Low-risk versus high-risk

Several studies have reported that patients that are stratified into the low-risk group do not have an increased risk of developing CRC compared to the general population.[58, 64] Likewise, case-control studies have confirmed a low long-term risk in those patients that persists up to 10 years after detection and removal of polyps.[45, 65, 66] Furthermore, a conservative surveillance policy in a large population-based cohort study resulted in a reduction of the risk of death from CRC to a level below the risk in the general population.[67] Based on these data, the ESGE and AGA guideline advise patients in the low-risk group to undergo surveillance colonoscopy after a 10-year interval or return to a screening programme.[62, 63]

Epidemiological studies have reported that patients in the high-risk group have a standardized incidence ratio between 3.6 and 6.6 for developing CRC compared to the general population.[58, 64] By subjecting the high-risk group to colonoscopic surveillance, this excess risk can be successfully reduced.[45, 65, 66] For the high-risk group, both ESGE and AGA recommend surveillance after 3 years.[62, 63] This recommendation is mainly based on the National Polyp Study, in which the rate of advanced neoplasia between the group that received colonoscopy at both 1 and 3 years, and the group that received colonoscopy at 3 years only was not significantly different.[40] A more recent case-control study suggested that the surveillance interval for those patients could be safely prolonged from 3 to 5 years without increased risk of CRC.[66]

Serrated polyps

There is little consistency in the surveillance recommendations for the recently acknowledged serrated polyps. This entity consists of (i) hyperplastic polyps, (ii) sessile serrated adenomas/polyps and (iii) traditional serrated polyps. Even though these subtypes are believed to differ in their malignant potential, current guidelines[62, 63] do not distinguish between serrated lesions because of the high inter- and intra-observer variability among pathologists.[68-70] Whilst awaiting more reliable sub-classification of those lesions, the guidelines use size and grade of dysplasia to risk-stratify serrated polyps.[62, 63]

Expected randomized trials

Even though risk-stratified surveillance seems justified, convincing evidence on the optimal time interval is not yet available. This is due to lack of randomized studies on this subject. Besides, the rapid progression in quality assurance and techniques of colonoscopy over the past years will influence the effect of a surveillance program. The implementation of screening programs throughout the Western world, resulting in large numbers of patients diagnosed with polyps, calls for large-scale clinical surveillance trials with incidence and mortality of CRC as endpoints. In the European Polyp Surveillance trial (EPoS), that is currently instigated in several European countries, individuals are randomized to different surveillance intervals based on their presenting polyp characteristics.[71] This trial puts effort in establishing the most effective and cost-effective polyp surveillance strategy.

Interval cancers after colonoscopy

When performed in a quality-controlled setting, the effect of both screening and surveillance colonoscopy to reduce incidence and mortality of CRC seems substantial.[42, 45] However, the occurrence of interval CRCs after colonoscopy demonstrates this approach is still imperfect.[72] Colonoscopy interval CRCs are defined as carcinomas that develop after a complete colonoscopy in which all lesions have been completely removed, and before the recommended surveillance exam.[73] The reported incidence of those carcinomas in literature varies widely due to the different definitions used.[73] Colonoscopy interval CRCs make up for 3–7% of all CRCs[74-77] and are more likely to be located in the proximal colon,[72, 74, 78] which is in line with the earlier mentioned observation that screening colonoscopy seems to have a less protective effect for right-sided CRC.[44] The occurrence of colonoscopy interval CRCs can be partly attributed to the biological characteristics of fast-growing precursor lesions.[72, 74, 78] Molecular analyses of colonoscopy interval CRCs demonstrated that those lesions are more often microsatellite instable (MSI) and show DNA methylation of CpG islands (CIMP-high).[43, 74, 79] MSI tumors are considered fast growing tumors that may quickly arise from precursor lesions.[4] Furthermore, both CIMP-high and MSI are associated with serrated polyps,[4, 5] which can be difficult to detect at colonoscopy due to their morphology (often flat-elevated) and innocuous color.[80]

Another reason for interval CRCs might be poor adherence to surveillance guidelines by both patients and endoscopists as demonstrated by van Heijningen et al, who found appropriate surveillance intervals in less than 25% of cases[81] They also reported that delayed surveillance was associated with an increased rate of advanced adenomas compared with appropriately timed surveillance (8% vs. 4%, $p<0.01$) and an increased yield of CRC (1.8% vs. 0.4%, $p<0.01$).[81]

The most important causes of colonoscopy interval CRCs, however, are related to the quality of the colonoscopy procedure. Approximately half of all interval CRCs seems to result from missed lesions.[72, 82, 83] A study that reviewed back-to-back colonoscopies reported a pooled

miss rate for any adenoma of 22%.[84] Furthermore, the observation of interval CRCs occurring at the site of a previous polypectomy, suggests that incomplete resection is another cause.[72, 83] A recent study evaluating resection-margins after complete polyp removal by routinely taking biopsies after polypectomy, concluded that experienced gastroenterologists had performed incomplete resection in 10% (35 of 346) of the polyps.[85] The results of this study emphasize the importance of optimal polypectomy technique, and careful inspection and photo-documentation of the resection-margins. As procedural factors are avoidable, the introduction of quality assurance in colonoscopy programs holds promise for improvement.

Quality assurance of colonoscopy

Various studies have demonstrated a substantial variation in the performance of endoscopists and endoscopy centers,[80, 86, 87] being associated with a varying risk of interval CRCs.[77, 88] Providing structured and continuous training of endoscopists and benchmarking their quality indicators could hopefully aid to improve the variable performance of endoscopists and reduce the occurrence of interval CRCs.[89] Besides basic quality assurance, also advancements in endoscopic techniques might aid to improve colonoscopy performance.

Basic quality parameters

Screening colonoscopy is aimed to detect and remove precancerous lesions. The adenoma detection rate (ADR), which is defined as the proportion of colonoscopies in which an endoscopist detects at least one adenoma, is directly related to the risk for interval CRC in his or her patients.[90, 91] As ADRs vary widely amongst endoscopists, there is much room for improvement for the low-detectors.[91] The effort is worthwhile, as shown by Corley et al, who demonstrated that each 1% increase in ADR was associated with a 3% decrease in risk of interval CRC.[90] Therefore, nowadays minimal ADRs are being incorporated in guidelines.[92]

Other important quality parameters for colonoscopy are proper bowel preparation, cecal intubation rate and appropriate withdrawal time, all benchmarked to ensure high quality colonoscopy.[91, 93] Firstly, proper bowel preparation is vital to ensure maximum colonic visualization, and a validated scoring system should be used for structured reporting. Besides, endoscopists should reach the cecum in at least 90% of all exams and photo-document the landmarks.[92, 94] Finally, inspection of the colonic mucosa should be thorough and take at least six minutes to enhance both adenoma and serrated polyp detection.[80, 92-94]

Advanced colonoscopy techniques

Besides the implementation of basic quality parameters, several endoscopic techniques have been introduced to improve the detection of polyps during colonoscopy. Pancolonic chromoendoscopy is the only technique that has a consistent positive effect on detection of both adenomas and serrated polyps.[95, 96] However, the application of dye is time-consuming

and cumbersome and therefore not feasible for daily routine practice. Digital chromoendoscopy techniques as narrow band imaging (Olympus, Japan), Fujinon intelligent chromo endoscopy (Fujinon, Japan) and i-scan (Pentax, Japan) have not proven to increase ADR in average risk populations.[97-100]

Current standard colonoscopes generate a forward view of 170 degrees. Although adequate endoscopic skills for withdrawal as spiral-wise withdrawal and position changes of the patient are of crucial importance, polyps located on the proximal side of the colonic folds can be easily missed. Wide-angle colonoscopy, cap- and cuff-assisted colonoscopy and cecal retroflexion are all meant to facilitate visualization of polyps in difficult positions. According to the first studies, some of these techniques might increase polyp and ADR.[101-103] Nevertheless, more and larger randomized studies are needed to determine their role in colonoscopy. When assessing such studies, one should bear in mind that it is the endoscopist that determines the quality of the examination and this will not replace the importance of appropriate basic quality measures.

Reflection

CRC screening is increasingly being implemented around the world. As opposed to stool testing, its accuracy and ability to simultaneously detect and remove CRC precursor lesions and early cancers remains unrivalled. Evidence based on population studies suggests that screening colonoscopy is effective in reducing CRC incidence and mortality, even though the extent of its protection and role compared to other screening methods is still not exactly known. Risk-stratified surveillance enhances the impact of CRC screening by timely detection and removal of metachronous polyps and CRC. Colonoscopy interval CRCs typically express a biological profile that is associated with fast growing tumors, but their incidence also importantly relates to procedural shortcomings. This is the reason why training- and quality assurance programs are currently implemented in many countries. The development of emerging colonoscopy techniques might further enhance polyp detection and removal, but to this date, reliable data on cost-effectiveness of these techniques are lacking.

The trend to detect more polyps within one colonoscopic procedure, however, also poses a dilemma. Although the prevalence of polyps in adults above the age of 50 years is at least 25–30% and further increases with age,[7] the majority of these polyps will never develop into cancer.[11] High procedural quality in daily practice not only leads to the detection of clinically relevant lesions, but also promotes detection of small and diminutive lesions that are at low risk for progression.[104] According to current surveillance guidelines, this will lead to expansion of the surveillance population, whereas high quality procedures should in fact be able to reduce the necessity for frequent surveillance and the incidence of interval carcinomas. This *high-detection paradox* once again emphasizes the need for large, randomized controlled trials to evaluate the cost-effectiveness of colonoscopy in both screening and surveillance settings. This will guide us towards an evidence-based strategy for optimal reduction in mortality and morbidity from CRC.

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“You don’t fix the problem until you define it”

John Snow

6.

Molecular stool testing as an alternative for surveillance colonoscopy: a cross-sectional study*

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*published title: Molecular stool testing as an alternative for surveillance colonoscopy: a cross-sectional cohort study

BMC Cancer 2017

Abstract

Background

As in many other European countries, a nationwide screening programme for colorectal cancer (CRC) has recently been introduced in the Netherlands. As a side effect, such a screening programme will inherently yield an increase in the demand for surveillance after removal of polyps/adenomas or CRC. Although these patients are at increased risk of metachronous colorectal neoplasia, solid evidence on CRC-related mortality reduction as a result of colonoscopy-based surveillance programmes is lacking. Furthermore, colonoscopy-based surveillance leads to high patient burden, high logistic demands and high costs. Therefore, new surveillance strategies are needed. The aim of the present study, named Molecular stool testing for Colorectal CAncer Surveillance (MOCCAS), is to determine the performance characteristics of two established non-invasive tests, *i.e.* the multitarget stool DNA test Cologuard and the faecal immunochemical test (FIT) in the detection of CRC and advanced adenomas as an alternative for colonoscopy surveillance.

Methods

In this observational cross-sectional study, subjects aged 50 to 75 years will be approached to collect (whole-) stool samples for molecular testing and a FIT prior to their scheduled surveillance colonoscopy. The results of the tests will allow calculation of test sensitivities and specificities in the context of surveillance. This will provide the required input for the Dutch ASCCA model (Adenoma and Serrated pathway to Colorectal CAncer) to simulate surveillance strategies differing in frequency and duration. The model will allow predictions of lifetime health effects and costs. Multiple centres in the Netherlands will participate in the study that aims to include 4,000 individuals.

Discussion

The outcome of this study will inform on the (cost-) effectiveness of stool based molecular testing as an alternative for colonoscopy in the rapidly expanding surveillance population.

Registered on 17th February 2016 at ClinicalTrials.gov: NCT02715141

Background

Colorectal cancer (CRC) is a major health concern worldwide, ranking third in males and second in females, with over 1.2 million new cancer cases and an estimated 608,700 deaths in 2008.[1] Survival of colorectal cancer is inversely related to the stage at diagnosis. Five-year survival rates range from more than 90% for stage I to less than 10 % for stage IV CRC.[2] Therefore, detection and removal of the tumour in an early, or preferably, a premalignant stage is vital.[3] Since CRC often only becomes symptomatic when progressed to an advanced state, secondary prevention through screening is an important instrument for reducing death from CRC.[4]

CRC as a disease lends itself well for screening as it has a high prevalence and a well-defined precursor lesion (*i.e.* adenoma) with a long dwell-time, providing a window of opportunity for detection and resection of a lesion before becoming symptomatic. Indeed, incidence and mortality rates of CRC have declined in countries where screening has been introduced.[5–7] Recently a nationwide screening programme was also implemented in the Netherlands, using a faecal immunochemical test (FIT). Men and women aged 55 to 75 years are invited every 2 years, which amounts to a total number of 2.2 million individuals per year in the Netherlands being invited to participate in the programme. Of the estimated 7% FIT-positive screenees, approximately half will have advanced adenomas or carcinomas at colonoscopy, equal to over 45.000 individuals annually.[8] As these patients carry an increased risk to develop metachronous advanced lesions in the future[9–12] it is standard practice to enrol these individuals in a colonoscopy-based surveillance programme.[13–15]

However, there are several downsides to the current approach for managing the cancer risk in the surveillance population. Firstly, evidence of the impact of current surveillance strategies on the ultimate endpoint CRC-related mortality is very limited. The effect of surveillance has primarily been evaluated for intermediate endpoints, *i.e.* the yield of (advanced) adenomas upon surveillance colonoscopy.[12,16] However, adenomas are very common with a prevalence of 18–35% reported in screening series,[17,18] whilst only up to 5% of these adenomas will eventually progress to malignancy.[19] This suggests that focussing on adenoma yield as a primary endpoint represents actual overdiagnosis. Second, the technical advancements in colonoscopy-equipment and the recent emphasis on quality assurance have resulted in increasingly more and often smaller adenomas being detected. These small lesions are more likely to remain stable over time.[20,21] As a result, the surveillance population will expand even further, putting the colonoscopy capacity and health care budgets under pressure.[22,23] Finally, it will expose post-polypectomy patients to a burdensome and risky procedure that has not proven to be effective for this population.

For these reasons there is a demand for a surveillance tool that is easy to apply, well-tolerated and accurate in identifying high risk adenomas, as to reserve colonoscopy only for those individuals that are most likely to benefit.

Today, despite its limitations, colonoscopy is still the only test used for surveillance of patients after removal of polyps and CRC. The FIT, which detects small amounts of human haemoglobin

in the faeces, has been proposed as a method for surveillance.[24] While simple and increasing the likelihood of neoplasia being present when positive, its sensitivity is relatively low due to the fact that not all CRCs, and especially not all advanced adenomas bleed.[25,26] Yet, it was shown that the diagnosis of CRC and advanced adenomas was made 25 and 24 months (median) earlier, respectively, when offering a yearly FIT in the interval between colonoscopies and shortening the interval in case of a positive test. This indicates that FIT could be used to detect missed or rapidly developing lesions in surveillance programmes.[24]

In contrast to tests detecting blood, tests based on molecular markers derived from the neoplastic cells in the colon have the potential to be more accurate. CRCs are known to acquire discriminating epigenetic and genetic changes as they develop and progress, which form the basis of stool based DNA testing. Recently, a multitarget stool DNA test (Cologuard, Exact Sciences, Madison, WI, USA) combining a molecular assay for hypermethylated promoter CpG islands (*NDRG4* and *BMP3*) and mutant *KRAS* with an immunoassay for human haemoglobin has been reported.[27] Sensitivities for the detection of CRC were 92.3% with Cologuard and 73.8% with FIT (p =0.015). Also, Cologuard detected significantly more advanced adenomas than the FIT (69.2% versus 46.2%, p = 0.004) and significantly more sessile serrated polyps measuring 1 cm or more (42.4% versus 5.1%). Specificities of Cologuard and FIT were 86.6 and 94.9%, respectively. The results of this study have led to FDA approval of Cologuard in 2014.

We hypothesise that Cologuard- or FIT-based surveillance is a cost-effective first-line surveillance strategy to select individuals that need colonoscopy for confirmation of the diagnosis and therapeutic removal.

Objective

Primary objective

The primary goal of this study named Molecular stool testing for Colorectal CAncer Surveillance (MOCCAS), is to evaluate the performance of the molecular stool test Cologuard in post-polypectomy, CRC and FCC surveillance. To this end, we will determine the performance characteristics of Cologuard in the detection of CRC and advanced adenomas in a surveillance setting. The performance of Cologuard will be compared to two commercially available FITs (OC Sensor, Eiken Chemical Co., Tokyo, Japan and FOB Gold, Sentinel, Milan, Italy) and the reference standard colonoscopy. The test performance data will be used as input in the ASCCA (Adenoma and Serrated pathway to Colorectal CAncer) model that was developed using Dutch data.[28] The model will allow predictions of lifetime health effects and costs for a number of surveillance strategies differing in frequency and duration (Figure 1).

Secondary objectives

As a secondary objective, we aim to analyse whether the diagnostic markers included in Cologuard are present in the tissue samples of lesions identified and removed during the

surveillance colonoscopy procedure. These data will then be correlated to the Cologuard results. In addition, these same tissue samples will be analysed for the presence of particular genomic alterations known to be indicative of progression to cancer[29] and the performance of Cologuard will be determined for detecting such high risk precursor lesions. This will allow refined analysis of the diagnostic performance of these assays. Through modelling, the impact of using a molecular-based definition of high risk adenoma on predicted health effect and burden will be assessed for alternative surveillance strategies. Moreover, we will incorporate previously identified risk factors for the development of advanced neoplasia in the model.[30] To this end participants will be asked to complete a validated online questionnaire including these risk factors.[31]

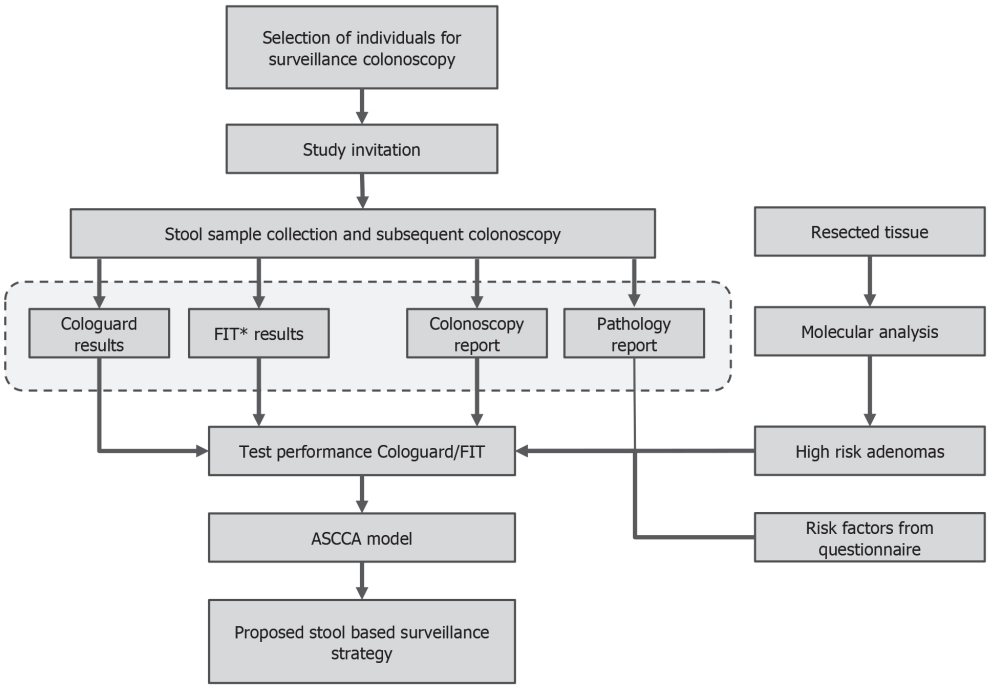

Figure 1 | Overview of the MOCCAS study.

* FIT = faecal immunochemical test, consisting of the OC-sensor (Eiken Chemical Co., Tokyo, Japan) and FOB Gold (Sentinel, Milan, Italy). ASCCA = Adenoma and Serrated pathway to Colorectal Cancer.

Individuals with an indication for surveillance colonoscopy are selected and invited to participate. After oral consent the home stool collection kit is sent to the individuals home address. The test results of Cologuard (Exact Sciences, Madison, WI, USA) and FIT will be compared to the findings described in the colonoscopy and pathology report in order to yield the test performances. This will then feed the ASCCA model for the simulation of different surveillance strategies.

The tissue of lesions removed during the surveillance colonoscopy will be used for molecular analysis of progression biomarkers to define high risk adenomas. This alternative, molecular-based intermediate endpoint will impact the test performances and thus the ASCCA modelling.

By adding identified risk factors to the obtained test performance data, new sensitivity data will be acquired and used to repeat the model simulations of surveillance strategies.

Methods

Study design

This is a multi-centre, cross-sectional observational study in the Netherlands that aims to include 4,000 patients. The study has been approved by the Medical Ethical Committee of the Academic Medical Center (AMC), Amsterdam and has been registered in ClinicalTrials.gov (NCT02715141).

Study population

All individuals that have an indication for a surveillance colonoscopy according to the previous (2001)[15] or current (2013)[15] Dutch guidelines are eligible for this study. Those guidelines include subjects with a history of polypectomy or CRC, as well as subjects under surveillance for familial colorectal cancer (FCC). In order to complete the risk questionnaire and give informed consent, subjects must have sufficient understanding of the Dutch language. Subjects with inflammatory bowel disease (IBD) or genetic cancer syndromes such as Lynch syndrome, familial adenomatous polyposis (FAP), attenuated FAP (AFAP), MUTYH associated polyposis (MAP), serrated polyposis syndrome (SPS) and other polyposis syndromes are excluded from participation. Also, a colonoscopy in the previous 6 months, having undergone a proctocolectomy or a life expectancy of less than three years are exclusion criteria.

Study algorithm

Subject recruitment and sample collection

Subjects will be invited to participate one to two weeks prior to their routine surveillance colonoscopy by a member of the research team. When oral consent is given, a package containing the study information and -instructions, informed consent form, FITs and stool collection kit will be sent to their home address. Simultaneously, an email containing the link to the online questionnaire will be sent, or a hardcopy version of the questionnaire will be added to the package in case email is not available. This validated questionnaire evaluates the risk factors: age, body mass index (BMI), family history for CRC (first degree relatives), regular aspirin or non-steroid anti-inflammatory drug (NSAID) use, current smoking, history of smoking, alcohol intake, total calcium intake, physical activity and postmenopausal hormone replacement therapy.[31] Regular NSAID intake is defined as the use of NSAIDs three or more times a week during the last month. Calcium intake is estimated by questions about food and supplement intake.[31]

For stool collection as part of Cologuard, a dedicated kit is used as provided by Exact Sciences. This kit comprises materials to collect at home stool from one full bowel movement (whole stool sample), in an easy and hygienic way. Subjects are instructed to collect the stool sample, perform FIT-sampling by swiping the test on the faecal surface, and afterwards add stabilisation buffer to the remaining stool sample for DNA preservation. The sealed samples may be stored at room temperature until the colonoscopy appointment. The allowed time frame between collection of the sample and processing in the laboratory is restricted to 72 hours.

On arrival at the endoscopy department subjects will hand in the signed informed consent form and the sealed package containing the whole stool- and FIT-samples. Samples from individuals of whom written informed consent is lacking will be excluded from further processing or analysis. The sealed package will be stored at the endoscopy department until transfer to the laboratory by a courier service.

Laboratory procedures

On arrival in the laboratory the FITs and the whole stool sample will be handled separately. The FITs used are automated tests (OC Sensor, Eiken Chemical Co., Tokyo, Japan and FOB Gold, Sentinel, Milan, Italy) with a quantitative outcome. The FITs will be analysed on the OC –sensor DIANA (Eiken Chemical Co.) and SENTiFIT 270 (Sentinel) according to the manufacturer's instructions by an experienced technician, who is unaware of the colonoscopy outcomes. The stool sample will be homogenised, aliquoted and stored at -80°C. The homogenised samples will be shipped in batches under strict conditions to Exact Science Corporation for analysis. As Cologuard is composed of a molecular assay plus an immunochemical test for haemoglobin detection, an algorithm derived from these two assays will determine the test result. Researchers performing the analyses will be blinded for the colonoscopy results.

First, the diagnostic results of the FITs and Cologuard will be compared to the yield of colonoscopy. Colonoscopy is the reference standard, and neither participants nor doctors will be informed about the test results. Second, tissue samples of lesions removed during the surveillance colonoscopy procedure will be collected from the pathology archives and subjected to further molecular characterisation. Expression of the diagnostic Cologuard markers will be tested through methylation-specific PCR and mutation analysis in order to determine which polyps are likely to have contributed to the test result. Lastly, in a separate analysis, DNA copy number changes that are associated with adenoma to carcinoma progression will be assessed for the identification of high risk adenomas.[29] These changes include losses in 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21, and gains in 8q23-qter, 13q14-31, and 20q13. The presence of two or more of the seven aforementioned chromosomal changes defines a high risk adenoma.

Clinical procedures

Colonoscopies will be performed or supervised by experienced gastroenterologists. A complete colonoscopy will be defined as intubation of the caecum with identification of the ileocaecal valve or appendiceal orifice. Quality parameters for colonoscopy will be reported.[32,33] Patients with an incomplete colonoscopy and/or insufficient bowel preparation will be rescheduled for colonoscopy. Patients that undergo the re-colonoscopy at more than 26 weeks (6 months) after the initial surveillance colonoscopy, and thus collection of the whole stool sample, will be excluded for analyses. Only in case of detection of colonic lesions, an incomplete colonoscopy is no reason for exclusion.

Lesions that are resected during surveillance colonoscopy, will be evaluated by pathologists at the participating centres. Adenomas ≥ 10mm, with high-grade dysplasia and/or villous characteristics (*i.e.* tubulovillous or villous adenoma) will be classified as advanced adenomas.[13–15] CRC and/or advanced adenomas are considered advanced neoplasia. CRC will be staged according to the AJCC cancer and TNM staging manual.[34]
Formalin-fixed paraffin-embedded (FFPE) tissue samples from all the lesions removed during colonoscopy will be stored in the respective pathology departments of the participating centres. These FFPE blocks will be retrieved for molecular analysis through the Dutch national pathology registry (PALGA)[35] and the Dutch National Tissuebank Portal (DNTP)[36], in the context of the Biobanking and BioMolecular resources Research Infrastructure the Netherlands (BBMRI-NL).

Data collection

Clinical data will be collected in a database, which is validated to global regulatory standards. Variables that will be assessed include subject age, sex, indication and date of current surveillance colonoscopy, recommended surveillance interval and findings of the previous colonoscopies. For our main study endpoint, *i.e.* the accuracy of Cologuard and FIT in detecting advanced neoplasia, endoscopic- and pathologic characteristics of the lesions found during the subsequent surveillance colonoscopy will be collected. A dedicated system for registering laboratory- and pathology processes will be used to gather information on the test characteristics and results of Cologuard and FITs.

Data analysis

Accuracy of Cologuard and FIT

This study will yield estimates for relative sensitivity and specificity of Cologuard and FITs versus colonoscopy. The analyses will be based on data from all participants who had valid results on Cologuard and/or FIT and colonoscopy. In case of missing values on outcome variables, the patient will be excluded. Exact binomial confidence intervals will be calculated around relative sensitivity, relative specificity, positive and negative predictive values.

Estimation of lesion specific positivity rates required for model-based analyses

For the cost-effectiveness analyses comparing multiple surveillance strategies, the ASCCA model (Adenoma and Serrated pathway to Colorectal Cancer) will be used. The ASCCA model describes the development of colorectal cancer from adenomatous and serrated precursor lesions. As input, the ASCCA model requires test positivity rates per lesion in each of the model categories of no/small/medium/large adenomas and serrated lesions. These cannot be directly taken from the cross-sectional diagnostic study; the study yields estimates for *relative* sensitivity and specificity of Cologuard and FITs versus colonoscopy, because colonoscopy is treated as the reference standard. Therefore, we apply a process called calibration. In essence, test-specific positivity rates per lesion are drawn randomly from a wide range of probable

values. Subsequently, the model is used to simulate the present cross-sectional surveillance study using these randomly drawn positivity rates. Predictions for the number of positive test results within individuals with small/medium/large adenomas and serrated lesions on colonoscopy are compared to the observed data for Cologuard and FITs. Sets of positivity rates that reproduce the observed data are kept, whereas estimates that produce predictions that diverge from the data are discarded. For this calibration process, we will run ~10K+ simulations each involving ~100K+ individuals (the actual number will depend on stopping criteria for achieving less than a pre-specified level of statistical uncertainty around the predictions). Statistical methods to assess goodness-of-fit will be used to identify the best-fitting sets of positivity rates.

Modelling alternative surveillance strategies

The surveillance schedule from the Dutch guidelines 'Colonoscopy Surveillance' (2013)[15] will be implemented in the ASCCA model. Subsequently, alternative surveillance strategies will be implemented using the estimates for lesion-specific positivity rates as described in the paragraph above. Different frequencies of molecular testing with Cologuard and FIT (every 1, 1.5 or 2 years) and rules for referral back to the screening population (after 3, 4, or 5 consecutive negative tests) will be evaluated.

Each model evaluation will involve simulation of 1,000,000 individuals, which represents a 'virtual' sample of the Dutch surveillance population. Predicted outcomes will include cancer incidence and mortality, resource utilisation (including number of surveillance tests and demand for colonoscopies) and cost-effectiveness. The evaluation will be accompanied by extensive sensitivity analyses in which, among others, the impact of changes in adenoma and serrated lesion incidence in the surveillance population, test positivity rates in each of the size categories and costs of molecular testing will be evaluated.

Modelling the impact of molecularly defined high risk adenomas

In the current version of the model, only advanced adenomas have the possibility to progress to CRC. Advanced adenomas in the model are defined on the basis size and histology. To evaluate the impact of an alternative, molecular-based intermediate endpoint, we will extend the ASCCA model such that it includes the most relevant progression biomarkers.[29] That is, on the basis of these progression biomarkers, the category 'advanced adenoma' will be replaced by 'molecularly high risk adenoma'.

Transition probabilities from the different adenoma health states to a high risk adenoma will be derived by the automatic calibration procedure as described above, such that the molecular version of the ASCCA model also correctly reproduces Dutch age- and sex-specific adenoma prevalence and serrated polyp prevalence, the observed proportion of molecularly high risk adenomas within the subgroups of small/medium/large adenomas, and Dutch CRC incidence and mortality. The result will be the first CRC surveillance model that includes the molecular biology of CRC development.

The model will then be used to repeat the described simulation analyses of alternative surveillance strategies. The hypothesis is that the predictions for health benefits and cost-effectiveness of molecular stool test-based surveillance are underestimated, because the current classification ignores the ability of Cologuard to detect specifically those adenomas at high risk of progression.

Modelling the impact of a risk-based questionnaire

We will build a multivariable logistic regression model by adding all risk factors from the questionnaire and the obtained accuracy data from Cologuard and FITs, using advanced neoplasia as the dependent variable. Missing data in the questionnaires will be handled by multiple imputations. The newly obtained sensitivity data will be used to repeat the model simulations of surveillance strategies.

Sample size calculation

Based on previous reports,[27,39] we assume for the power analysis that Cologuard has a sensitivity of 50% for advanced adenomas in individuals under surveillance. To obtain an accuracy of 5% around the sensitivity estimate (SE 2.5%, 95% confidence interval of width 10%), a total of 400 individuals with advanced neoplasia are needed. In a recent Dutch surveillance study an advanced adenoma was found in one per ten individuals.[22] Based on this ratio, we will include 4,000 individuals in the current study. This will allow a highly accurate estimate of the specificity of Cologuard and FIT in this population (estimated width of 95% confidence interval = 2%).

Discussion

In this cross-sectional observational MOCCAS study, the performance of a multitarget stool DNA test (Cologuard) and FITs will be assessed as an alternative for surveillance colonoscopy. To this end, the results of the stool based molecular tests and FITs will be compared to the findings of the (routine) colonoscopy in a surveillance population. These data will then be used as input for model-based analyses. By simulating different surveillance strategies varying in testing frequency and rules for referral to the screening population, the model will predict outcomes such as cancer incidence and mortality, resource utilisation and cost-effectiveness. Multiple modalities are available in the prevention of CRC. Colonoscopy is a one-staged method in which lesions are detected and removed simultaneously. Other methods, such as CT-colonography, faecal immunochemical tests and multitarget stool DNA tests, are two-staged and are used as triage for colonoscopy. The two-staged methods are associated with lower sensitivities, but are generally advantageous in terms of participation rates, risks and costs.[27,28,40] When choosing an optimal strategy it should be emphasised that the weight of these factors is different for surveillance compared to screening. Where screening targets the

"healthy" population, surveillance is aimed at a narrow high risk group with a higher positivity rate for colonoscopy. Sensitivity of the method is therefore most important. The accuracy of molecular tests might approach colonoscopy in the detection of (high risk) colorectal polyps and –carcinomas when the test is performed more frequently than colonoscopy. Participation in these high risk groups is generally high, as a result of a patient's awareness of his/her risk. The optimal model-predicted surveillance strategy, as identified in the current study, will be evaluated in clinical practice through a randomised study. Besides investigating a potential alternative to surveillance colonoscopy, this study will generate new insights in the molecular profiles of precancerous lesions by relating the expression of both diagnostic biomarkers and progression biomarkers to the results of Cologuard. Moreover, because whole stool samples, as well as FIT samples are collected, a large biobank is established that provides extensive opportunities for future research.

Ethics approval and consent

Ethical approval was obtained from the Medical Ethical Committee, Academic Medical Center, Amsterdam (reference number 2015_070). Participants will give written informed consent.

Acknowledgements

The authors thank Exact Sciences (Madison, WI, USA) for assisting in the logistics and analysis concerning their Cologuard.

We thank Sentinel (Milan, Italy) for providing the FOB Gold infrastructure necessary to perform this study.

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“Nothing new that is really interesting comes without collaboration”

James Watson

7.

Multitarget stool DNA testing and faecal immunochemical testing (FIT) as alternative for colonoscopy-based surveillance: an interim analysis

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To be submitted after completion of the MOCCAS study

Abstract

Introduction

The current colonoscopy-based surveillance programme for early detection of colorectal cancer (CRC) is a major burden for the healthcare system and likely not cost-effective. In the MOCCAS (MOlecular stool testing for Colorectal CAncer Surveillance) study, we aimed to assess whether the multitarget stool DNA test (Cologuard) could be used to preselect patients with advanced neoplasia (AN) for surveillance colonoscopy. Cologuard performance was compared to the faecal immunochemical test (FIT). Here we present an interim analysis.

Methods

In this cross-sectional study, whole stool samples for Cologuard testing and two FITs (OC Sensor and FOB Gold) were collected prior to their scheduled colonoscopy in post-CRC, post-polypectomy and familial risk surveillance patients. Sensitivity and specificity of Cologuard were calculated, applying the screening-validated threshold, and compared to FIT with a 15µg Hb/g faeces threshold for test positivity. Test performances were also compared at equal positivity rate of 50%, translating into a reduction of surveillance colonoscopy volume by 50%. Primary endpoint was AN detection as most advanced lesion. AN included CRC, advanced adenomas (AA) and advanced serrated polyps (ASP).

Results

Cologuard results were available for 1,551 patients, of which the majority (61%) underwent post-polypectomy surveillance. AN was detected in 180 (11.6%) patients, including 13 (0.8%) CRC, 112 (7.2%) AA and 55 (3.6%) ASP. Cologuard detected AN with a sensitivity of 59% (95% CI 52% to 67%) and specificity of 76% (95% CI 74% to 79%). Sensitivity for CRC was 77% (95% CI 46% to 95%), for AA 51% (95% CI 42% to 61%) and for ASP 71% (95% CI 57% to 82%). Both FIT OC Sensor and FOB Gold had lower sensitivity for AN (27% (95% CI 20% to 34%) and 34% (95% CI 27% to 41%), respectively, $p<0.001$) and higher specificity (89% (95% CI 97% to 91%) and 86% (95% CI 84% to 88%), respectively, $p<0.001$) than Cologuard. At 50% positivity rate, Cologuard sensitivity for AN was 76% (95% CI 69% to 82%) and specificity 56% (95% CI 53% to 58%). At this threshold, Cologuard test sensitivity for AN remained significantly higher than both FITs (OC Sensor 63% (95% CI 55% to 70%) and FOB Gold 57% (95% CI 49% to 65%), $p<0.01$), while specificities were comparable.

Conclusion

The multitarget stool DNA test (Cologuard) has higher sensitivity than FIT for detection of AN in the surveillance population. Applying a low test cut-off to reach 50% positivity rate would further increase Cologuard sensitivity and still reduce the number of unnecessary colonoscopies by half. Mathematical modelling approaches will be applied to the final study data in order to determine an optimal stool-based strategy for surveillance.

ClinicalTrials.gov: NCT02715141

Introduction

Screening programmes have been implemented in many countries to reduce colorectal cancer (CRC) incidence and mortality. Independently of the screening method used, this leads to more patients being diagnosed with CRC and polyps. Observational studies have shown that patients having undergone curative CRC resection or polypectomy are at increased risk for CRC compared to the general population.[1–3] Therefore, this population is recommended to undergo regular surveillance colonoscopies, reason why screening programmes indirectly also lead to an increase in surveillance.

Similar to screening, the goal of a surveillance programme is to prevent CRC and death from CRC by early detection of (pre)malignant lesions. However, the benefit of the current colonoscopy-based surveillance strategies remains unclear.[4] Recommendations are based on older cohorts,[5,6] but since then substantial improvements in colonoscopy quality have been made. This has reduced the number of missed or incompletely removed lesions.[7] Only few studies have investigated the effect of surveillance on long-term CRC incidence, instead of the effect on incidence of advanced adenomas (AA).[6,8] A recent study showed that a proportion of patients that are advised surveillance colonoscopy every three years might not benefit from this.[9]

Because of limited long-term follow-up data, a microsimulation model was used to examine the added value of colonoscopy surveillance in a screening setting.[4] They found that colonoscopy based surveillance on top of a FIT based screening programme reduced CRC mortality by an extra 1.7% (from 50.4% to 52.1%), whilst increasing the lifetime colonoscopy demand by 62%. Thus, in this modelling study, adding colonoscopy surveillance to FIT screening was not cost-effective.[4]

Taken together, the current colonoscopy-based surveillance strategy likely leads to overdiagnosis. Patients are exposed to unnecessary harm and the limited colonoscopy capacity is used inefficiently. In addition, colonoscopy is expensive and associated with a low risk of bleeding and perforation.[10] Currently, 20–25% of colonoscopies are performed for post-polypectomy surveillance.[11,12] Without changing policies, this number will gradually increase further due to the implementation of population screening programmes and the performance of modern, high-quality endoscopy. Colonoscopy has been the primary method for surveillance since the early 1980s[13], but comparative studies with other modalities are scarce. With the availability of new stool tests, the question has become relevant to study whether these tests could be used for surveillance and potentially postpone colonoscopy safely.

The faecal immunochemical test (FIT), produced by multiple manufacturers, detects human haemoglobin (Hb) in faeces. FIT has proven to be an effective method for early CRC detection and is widely used for screening.[14] A recent surveillance study with three FIT rounds showed that annual testing was a cost-saving strategy in the intermediate risk population.[15] Yet, sensitivity for AN of a single FIT round at low threshold in the surveillance setting was 20–34% at specificity of 88–92%.[15] The multitarget stool DNA test (Cologuard, Exact Sciences, Madison, WI, USA) combines the detection of human Hb with several DNA markers and has only be tested in the

screening setting. In the screening population, Cologuard detected advanced neoplasia (AN) with a sensitivity of 46% at a specificity of 87%.[16]

In the MOCCAS (MOlecular stool testing for Colorectal CAncer Surveillance) study, we hypothesized that stool testing could be used to preselect patients for surveillance colonoscopies. The primary aim of this study was to assess whether the multitarget stool DNA test (Cologuard) could accurately patients with advanced neoplasia (AN) for surveillance colonoscopy. Secondly, we compared the performance of Cologuard to FIT. Ultimately, these data will be used as input for cost-effectiveness modelling to find the optimal stool-based surveillance strategy. Here, we present an interim analysis of the MOCCAS study.

Methods

Study design

This interim analysis is part of the MOlecular stool testing for Colorectal CAncer Surveillance (MOCCAS) study, a prospective, multicentre, cross-sectional study ongoing in eleven endoscopy centres throughout the Netherlands. For the interim analysis, patients were enrolled between November 2015 and April 2018. The study was approved by the Medical Ethical Committee of the Amsterdam UMC, the Netherlands and registered in ClinicalTrials.gov (NCT02715141). Details on the study protocol can be found elsewhere.[17]

Study population

Patients between 50–75 years old, with an indication for surveillance colonoscopy according to the Dutch surveillance guidelines (*i.e.* post-polypectomy, history of CRC or familial risk) visiting any of the participating centres were eligible.[18] In case of inflammatory bowel disease (IBD), genetic cancer syndromes (*e.g.* Lynch syndrome and familial adenomatous polyposis), colonoscopy in the previous 6 months, proctocolectomy or life expectancy less than three years, were excluded. Patients had to have sufficient understanding of the Dutch language in order to provide written informed consent.

Enrolment procedures

Patients with a scheduled routine surveillance colonoscopy were invited by a member of the research team to participate two weeks prior to their colonoscopy. After the patient's consent, the test kit containing instructions and stool tests (Cologuard and two different FITs) were shipped to the patients' home address. Patients performed a whole stool specimen and FIT collection from the same bowel movement at home before starting bowel preparation. Before colonoscopy examination, patients returned the kit at the endoscopy department. Within 72 hours after collection, stool samples were transferred from the endoscopy department to the laboratory site and processed.

Colonoscopy examination

Colonoscopy was performed according to standard practice. The endoscopists documented caecal intubation status and bowel preparation score as colonoscopy quality parameters and also location, size and morphology of any lesions detected. Bowel preparation of ≥6 on the Boston Bowel Preparation Scale was considered good and <6 poor.[19] Lesions removed were collected for histological evaluation. Endoscopists and pathologists were blinded for the results of the stool tests. Patient data were extracted from endoscopy data systems, the Dutch national pathology archive PALGA and hospital electronic health records.

Patients were not informed about the faeces study test results, as they already underwent a colonoscopy.

The collection of clinical data was performed according to the Dutch Personal Data Protection Act, using a predefined CRF with a dedicated electronic data capture system validated to global regulatory standards (*i.e.* OpenClinica, Waltham, MA, USA).

Laboratory procedures

Stool specimens were shipped to one of the two central laboratories in the Netherlands. For Cologuard, buffered stool samples were homogenised, aliquoted and frozen at -80°C in compliance standard operating procedures. Stool aliquots were shipped in batches to Exact Sciences, where analyses were performed. Cologuard consists of an immunochemical assay for human Hb and molecular assays for mutations in codon 12 of the *KRAS* gene (referred to as KRAS1) and in codon 13 of the *KRAS* gene (referred to as KRAS2), and hypermethylation of *NDRG4* and *BMP3* promoter regions. Also, β-*actin* is part of this molecular assay, acting as reference gene for human DNA quantity. Quantitative measurements for each marker separately were obtained, as well as a single result for the multitarget stool DNA test. For Hb, the maximum value was 60 μg Hb/g faeces (*i.e.* 600 ng Hb/ml buffer). Calling of an individual test as positive or negative was done using the screening-validated threshold of 182.[16]

The two FITs used in this study were OC Sensor (Eiken Chemical Co, Tokyo, Japan) and FOB Gold (Sentinel, Milano, Italy). FIT samples were stored at 4°C upon arrival and measured according to the manufacturer's instruction by a trained technician, yielding quantitative measures of Hb concentration. Pre-defined thresholds were applied, which were used in previous screening trials in the Netherlands.[20] For both the OC Sensor and FOB Gold, this threshold was 15μg Hb/g faeces (*i.e.* 75 ng Hb/ml buffer and 88 ng Hb/ml buffer, respectively).[21] Measurements of all tests were performed blinded for the colonoscopy results.

Outcome measures

Primary outcome measures were sensitivity and specificity of Cologuard for AN. AN included CRC, AA (adenoma ≥ 10mm and/or with high grade dysplasia and/or ≥25% villous component), and advanced serrated polyps (ASP; hyperplastic polyp/sessile serrated lesion/traditional serrated adenoma ≥ 10mm or with dysplasia) as most advanced lesion. Secondary outcomes were sensitivity

and specificity of the two FITs. In addition, Hb concentrations and DNA marker values were used for comparison between different lesion types.

Sample size calculation

The test characteristics of Cologuard were used to calculate the required sample size. A similar test sensitivity of 50% for AN, as was previously reported for a large screening population, was assumed for the surveillance population.[16] In order to obtain an accuracy of 5% around the sensitivity estimate, a total of 400 individuals with AN was needed. In a recent Dutch surveillance study AA were detected in one per ten individuals.[22] Based on this ratio, a sample size of 4,000 individuals was needed to allow an accurate estimate of the specificity of Cologuard in this population (2% estimated width of 95% confidence interval (CI)). This interim analysis presents the results of a subset of 1,756 cases.

Statistical analysis

For Cologuard as well as the respective FITs, sensitivities were calculated using the pre-specified screening thresholds for test positivity. In addition, sensitivities were compared using equal positivity rate of 50%, which would translate into a reduction of surveillance colonoscopy volume by 50%. Colonoscopy results combined with histopathology results were considered the reference standard. Calculation of the specificity was done considering all patients with non-advanced adenomas, non-advanced serrated lesions, non-neoplastic findings or a negative colonoscopy as controls. In addition, specificity was calculated considering only patients with a complete negative colonoscopy as controls. For determining the 95% CI around the test characteristics, the exact binomial test was applied using the R package epiR. The McNemar test was used to compare differences in lesion detection between the respective diagnostic tests.

Receiver Operating Characteristic (ROC) curves were constructed in order to calculate the ability of the tests to discriminate between patients with and without the disease (*i.e.* CRC or AN versus non-advanced neoplasia or lesser findings) for different thresholds. The discriminatory ability was summarised in the area under the curve (AUC) and DeLong's method was used to test the differences in AUC between tests. For these calculations, the R package pROC was used.

To compare the Hb concentrations of Cologuard and the FITs, a maximum value of 60 µg Hb/g faeces was applied for the FITs in the same way as for Cologuard. The Wilcoxon rank sum test was used to test the differences in concentration of Hb and the various Cologuard markers for patients with CRC, AA and ASP as most advanced lesion, because of the non-normal distribution of these values.

Subgroup analyses were performed based on the most severe surveillance indication. Assuming patients with a history of CRC have the highest risk for metachronous CRC, followed by patients with a polypectomy and then by patients with a familial risk, three groups were distinguished: 1) post-CRC surveillance population, 2) post-polypectomy surveillance

population, without history of CRC, 3) familial risk surveillance population, without history of CRC or post-polypectomy indication. To calculate differences in lesion distribution among surveillance indication subgroups, the Fisher's Exact test was used.
All analyses were conducted in R studio, version 1.1.383 (R studio, Boston, MA, USA).

Results

Study population

A total of 1,756 patients were enrolled in the study at the time of data-extraction on April 12th 2018. Of these, 1,551 patients had Cologuard results completed. Reasons for drop-out are listed in the flowchart in Figure 1.
Of the 1,551 patients that were included in the primary analysis, 54% were male and mean age was 66 years (sd 6.4) (Table 1). The most common indication for colonoscopy was post-polypectomy surveillance (61%), followed by post-CRC surveillance (33%) and familial risk for CRC (6%). The caecum was reached in 96% and bowel preparation was good in 85%. At the surveillance colonoscopy, AN was detected in 11.6%, concerning 0.8% CRC, 7.2% AA and 3.6% ASP.

Table 1 | Baseline characteristics

Total cohort	**n=1,551**
PATIENT DEMOGRAPHICS	
Male, n (%)	839 (54)
Age, years (sd)	66 (6.4)
Surveillance indication, n (%)	
Post-CRC	507 (33)
Post-polypectomy	942 (61)
Familial risk	102 (6)
QUALITY OF COLONOSCOPY	
Caecum reached, n (%)	
Yes	1,486 (95.8)
No	4 (0.3)
Not reported	61 (3.9)
Bowel preparation, n (%)	
Good	1,314 (84.7)
Poor	2 (0.1)
Not reported	235(15.2)
FINDINGS AT SURVEILLANCE most advanced lesion	
Advanced neoplasia, n (%)	
Colorectal cancers	13 (0.8)
Advanced adenomas	112 (7.2)
Advanced serrated polyps	55 (3.6)
Total	**180 (11.6)**

Cologuard performance

Cologuard detected 106 of 180 patients with AN, corresponding to a sensitivity of Cologuard of 59% (95% CI 51% to 66%) (Table 2). Out of the thirteen patients with CRC, Cologuard detected ten (77%, 95% CI 46% to 95.0%). Sensitivity for detecting AA was 51% (57 of 112, 95% CI 41% to 61%) % and for ASP 71% (39 of 55, 95% CI 57% to 82%).

Among 1,371 patients who had findings other than AN or negative colonoscopy, the Cologuard identified 1,045 patients correctly as negative (specificity 76%, 95% CI 74% to 79%). For those patients with a totally negative colonoscopy, specificity was 81% (483 of 600, 95% CI 77% to 84%).

Table 2 | Test characteristics of Cologuard at screening threshold

Most advanced lesion (n=1,551)	Colonoscopy	Cologuard (score 182)	
		+ result (n=432)	Sensitivity (%)
Advanced neoplasia	180	106	59 (51 to 66)
Colorectal cancers	13	10	77 (46 to 95.0)
Advanced adenomas	112	57	51 (41 to 61)
Advanced serrated polyps	55	39	71 (57 to 82)
Non-advanced neoplasia	600	172	29 (25 to 33)
		- result	Specificity (%)
Non-advanced lesions, other lesions and negative colonoscopy	1,371	1,045	76 (74 to 79)
Negative colonoscopy	600	483	81 (77 to 84)

Comparison of Cologuard to FIT

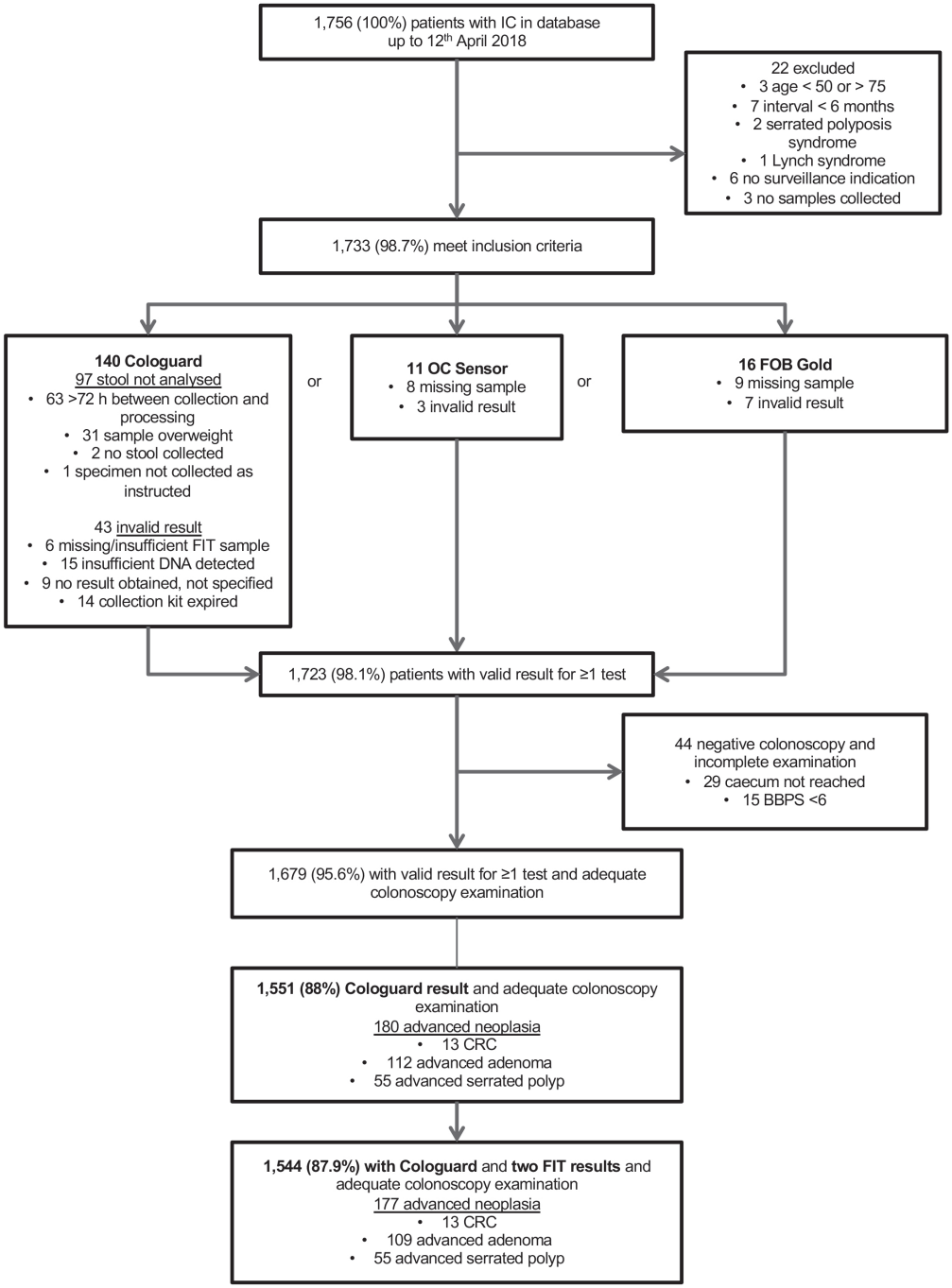
Test performance at screening threshold

For the comparison of Cologuard to FIT, participants with test results for Cologuard as well as the two FITs were used, yielding 1,544 patients. The performance of Cologuard in these individuals (Table 3) was similar to that of the entire group of study participants (Table 2).

OC Sensor had a sensitivity for AN of 27% (95% CI 20% to 34%) (Table 3). Sensitivity for CRC was 62% (95% CI 32% to 86%), for AA 30% (95% CI 22% to 40%) and for ASP 11% (95% CI 4.1% to 22%). Compared to Cologuard, the sensitivity for detection of AN, AA and ASP was significantly lower for OC Sensor ($p<0.001$). Sensitivity for CRC did not differ significantly between Cologuard and OC Sensor.

Specificity of OC Sensor for AN was 89% (95% CI 87% to 90.8%). Specificity was 92.5% (95% CI 90.1%–94.5%) considering patients with fully negative colonoscopy as controls. This was significantly higher than the specificity of Cologuard for these two groups ($p<0.001$).

FOB Gold had a sensitivity for AN of 34% (95% CI 27% to 41%). The sensitivity for CRC was 54% (95% CI 25% to 81%), for AA 39% (95% CI 30% to 49%) and for ASP 18% (95% CI 9.1% to 30%). Sensitivity for detection of AN, AA and ASP was significantly lower for FOB Gold than for Cologuard ($p<0.05$). Also here, sensitivity for CRC did not differ significantly between Cologuard and FOB Gold.



BBPS = Boston Bowel Preparation Score. CRC = colorectal cancer

Figure 1 | Flowchart of interim study population

Specificity of FOB Gold for AN was 86% (95% CI 84% to 88%). Specificity was 90.5% (95% CI 88% to 92.7%) considering patients with totally negative colonoscopy as controls. In both cases specificity was significantly higher compared to Cologuard (p<0.001).

Discriminatory ability

For discriminating between patients with and without AN, the AUC was 0.73 for Cologuard. This was significantly higher than the AUC for OC Sensor of 0.64 (p<0.001) and the AUC for FOB Gold of 0.61 (p<0.001) (Figure 2A). The AUC for CRC was 0.80 for Cologuard, compared to 0.87 and 0.86 for FIT (Figure 2B). These differences in AUC were not significant. In Supplementary figures 1B and 1D, ROC curves are shown considering patients with negative colonoscopy only as the control group.

Test performance at 50% positivity rate

Cologuard was compared to FIT at equal positivity rate of 50%. When FOB Gold threshold was lowered to 0 µg Hb/g faeces (*i.e.* all patients with FOB Gold values > 0 µg Hb/g faeces were considered positive and all patients with values equal to zero were considered negative), test positivity rate was 48%. Because the FOB Gold threshold could not be lowered beyond this value, all tests were compared at positivity rate of 48%, equal to 736 referrals for colonoscopy. At this positivity rate, Cologuard sensitivity for AN detection was 76% (95% CI 69% to 82%) (Table 4). Cologuard sensitivity for CRC was 77% (95% CI 46% to 95.0%), for AA 73% (95% CI 63% to 81%) and for ASP 84% (95% CI 71% to 92.2%). Cologuard specificity for AN was 56% (95% CI 53% to 59%).

Sensitivity for AN was significantly higher for Cologuard than for OC Sensor (63%, 95% CI 55% to 70%) and FOB Gold (57%, 95% CI 49% to 65%) (p<0.01) (Table 4). Cologuard detected ASP with significantly higher sensitivity compared to OC Sensor (44%, 95% CI 30% to 58%) and FOB Gold (36%, 95% CI 24% to 50%) (p<0.001). The sensitivity of the three tests did not differ significantly for CRC and AA detection. Also specificities were similar for all three tests.

Test markers in relation to lesion type

In Figure 3A Hb values of the three tests are shown in relation to the most advanced lesion, *i.e.* CRC, AA and ASP. For all three tests, median Hb values were significantly lower in patients with AA compared to patients with CRC (p<0.05). Hb values were also lower in patients with ASP compared to those with CRCs (p<0.0001) and AA (p<0.01) as most advanced lesion.

In Figure 3B the molecular DNA markers KRAS1, KRAS2, BMP3 and NDRG4 of Cologuard are shown in relation to the most advanced lesion. The median values of the hypermethylation markers BMP3 and NDRG4 differed significantly across lesion types and was higher in patients with ASP than in those AA (p<0.001).

Table 3 | Test characteristics of Cologuard and FIT at screening thresholds

Most advanced lesion (n=1,544)	Colonoscopy	Cologuard (score 182)		OC Sensor (15 µg Hb/g faeces)*			FOB Gold (15 µg Hb/g faeces)*		
		+ result (n=430)	Sensitivity (%)	+ result (n=194)	Sensitivity (%)	p-value	+ result (n=249)	Sensitivity (%)	p-value
Advanced neoplasia	**177**	**105**	**59 (52 to 67)**	**47**	**27 (20 to 34)**	<0.001	**60**	34 (27 to 41)	**<0.001**
CRC	13	10	77 (46 to 95.0)	8	62 (32 to 86)	0.62	7	54 (25 to 81)	0.25
AA	109	56	51 (42 to 61)	33	30 (22 to 40)	<0.001	**43**	39 (30 to 49)	0.04
ASP	55	39	71 (57 to 82)	6	11 (4.1 to 22)	<0.001	10	18 (9.1 to 30)	<0.001
Non-advanced neoplasia	**598**	**171**	**29 (25 to 32)**	**74**	**13 (10 to 15)**	<0.001	**102**	17 (14 to 20)	**<0.001**
		- result (1114)	Specificity (%)	- result (n=1350)	Specificity (%)		- result (1295)	Specificity (%)	p-value
Non advanced lesions, other lesions and negative colonoscopy	1,367	1042	76 (74 to 79)	1,220	89 (87 to 90.8)	<0.001	**1,178**	86 (84 to 88)	**<0.001**
Negative colonoscopy	**598**	**481**	**80 (77 to 84)**	**553**	**92.5 (90.1 to 94.5)**	<0.001	**541**	90.5 (88 to 92.7)	<0.001

*threshold used in all the screening pilot studies in preparation for the Dutch screening program. P-values represent statistical testing between Cologuard and the other test.

Lesion distribution by surveillance-indication

Most CRC (9/13) were found in patients undergoing post-CRC surveillance (Figure 4). This was reflected by a significantly higher risk of CRC at colonoscopy in the post-CRC surveillance population compared to the post-polypectomy surveillance population (OR 4.2 (95% CI 1.2 to 19), p<0.05). In patients undergoing post-polypectomy surveillance, AA was more frequently detected compared to the post-CRC population (OR 2.2 (95% CI 1.4 to 3.8), p<0.001), as well as the familial risk surveillance population (OR 3.4 (95% CI 1.1 to 17), p<0.05). The incidence of ASP did not differ across the surveillance indication populations.

Discussion

In this study, we assessed the performance of the multitarget stool DNA test Cologuard in the surveillance setting. When applying relatively high thresholds for test positivity, as used for screening, sensitivity of Cologuard for the detection of AN was 59% and exceeded that of the two FITs (OC Sensor 27% and FOB Gold 34%). In particular, Cologuard was more sensitive for detection of advanced precursor lesions. This can be attributed to the DNA marker component of the test, as reflected by the relative low Hb concentration in the stool sample of patients with AA, and especially with ASP as most advanced lesion. ASP was mostly picked up through the hypermethylation markers of the Cologuard. The FITs, on the other hand, were more

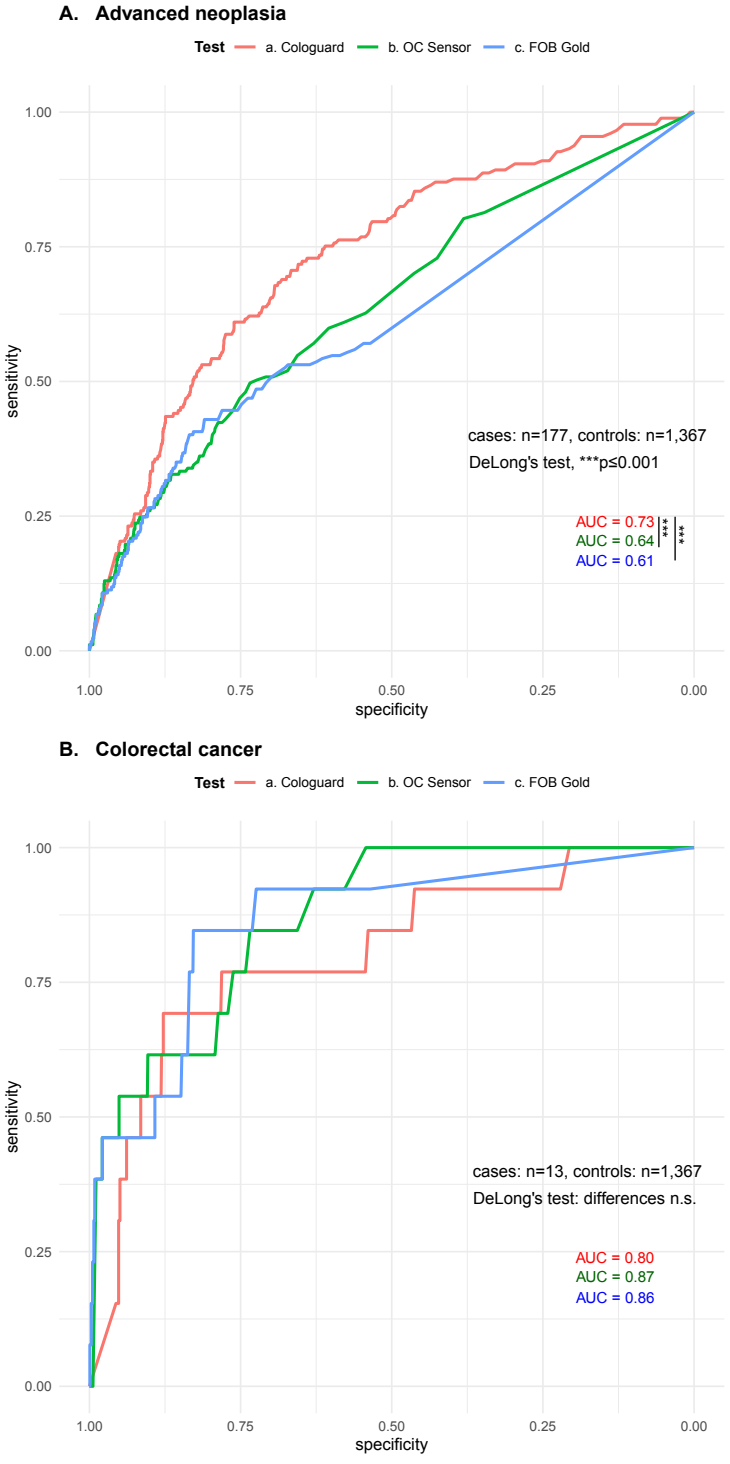
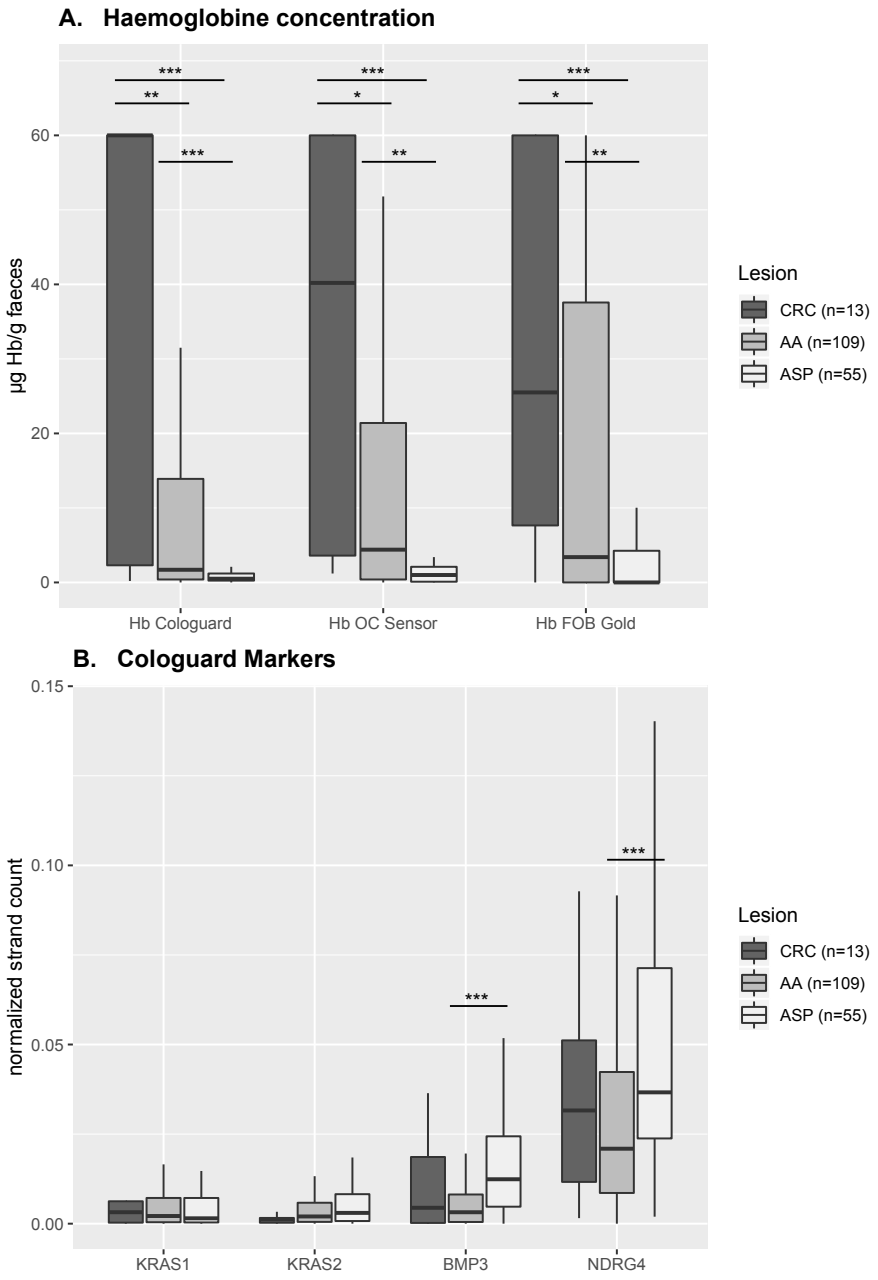


Figure 2 | ROC curve comparing Cologuard with FIT for advanced neoplasia and colorectal cancer detection



CRC = colorectal cancer, AA = advanced adenoma, ASP = advanced serrated polyp
Wilcoxon rank sum test, *p<0.05, **p<0.01, ***p<0.001

Figure 3 | Markers values versus lesion type A. Haemoglobin (Hb) concentration for Cologuard, OC Sensor and FOB Gold per lesion type B. Values of Cologuard DNA markers per lesion type

specific for non-advanced or negative findings (OC Sensor 89% and FOB Gold 86% versus Cologuard 76%).

The accuracy of the Cologuard was evaluated previously in a large cross-sectional study with average risk individuals.[16] Even though the same cut-off was used, lower sensitivity for advanced precursor lesions of 42% was reported (compared to 58% in the current study), against higher specificity of 87%. Possibly, biological differences in the detected lesions in the surveillance population play a role, such as the relative high contribution of ASP. In contrast, the sensitivity for CRC was higher in the previous study (92.3%) compared to the current study (77%). Even though the difference was not significant, a plausible explanation is the early stage of the majority of cancers detected in this surveillance cohort. Nine out of 13 CRC concerned stage I cancers (Supplementary table S1), which may have less shedding of neoplastic cells compared to more advanced cancers. Also the lower FIT sensitivity for CRC compared to screening studies may be explained by the early stage of the CRC.[23] However, even though FIT sensitivity is known to improve with cancer stage,[16,24] this has not been described for Cologuard.[16] Other explanations are different morphological (smaller size or non-polypoid shape)[25,26] and molecular features[27] of the tumours encountered during surveillance compared to screening-detected tumours.

The surveillance population consists of patients with increased risk for CRC. These patients are monitored by colonoscopy. Thus, when evaluating alternative stool-based surveillance strategies, especially false negative test results are undesirable, while higher rates of false positivity can be accepted. Lowering the screening cut-offs to reach 50% positivity rate, increased the sensitivity of Cologuard and FITs considerably, thereby reducing the risk of missing AN. In this scenario, a single round of Cologuard would miss 24% of AN, while FIT would miss a significantly higher proportion of 40%. In practice, the possibility to lower the threshold is constrained by the limit of quantification of the tests.[28]

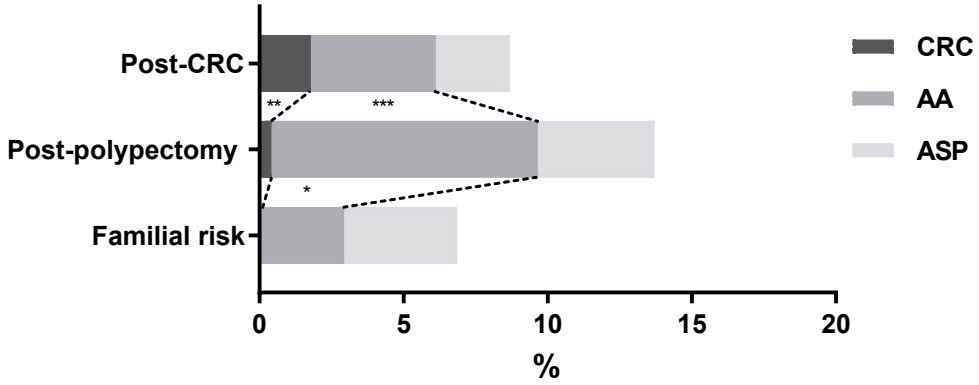
Alternatively, also repeated testing can increase the likelihood of detecting AN within a given timeframe. Even though delayed detection may be acceptable for AA and ASP considering the slow rate of malignant transformation, for CRC this is debatable. There, diagnostic delay may result in more progressed disease and reduced survival. Because Cologuard is more expensive than FIT (~€600 versus ~€20, respectively),[4,29] efficiency of repeated testing also needs to be evaluated against test costs. More frequent testing would lead to a larger number of patients having a positive test ánd subsequent colonoscopy; therefore it is unlikely that repeat mt-sDNA testing would be cost-effective. Upon full study completion, ASCCA (Adenoma and Serrated pathway to Colorectal CAncer) modelling will be performed to identify the optimal stool-based surveillance strategy. Besides different intervals between the stool tests, other factors to consider are logistic issues and ease to perform the test. This can influence the number of analytical test drop-outs and participation rates. As seen in Figure 1, the number of drop-outs due to analytical problems is almost tenfold higher with Cologuard testing compared to FIT.

In the current study, patients with different surveillance indications, and therefore different risk profiles, were included. According to our subgroup analysis, the *a priori* risk for CRC is highest

Table 4 | Test characteristics of Cologuard and FIT at equal positivity rate of 50%*

Most advanced lesion (n=1,544)	Colonoscopy	Cologuard (score 79)		OC Sensor (1 µg Hb/g faeces)†			FOB Gold (0 µg Hb/g faeces)		
		+ result (n=736)	Sensitivity (%)	+ result (n=736)	Sensitivity (%)	p-value	+ result (n=736)	Sensitivity (%)	p-value
Advanced neoplasia	177	135	76 (69 to 82)	111	63 (55 to 70)	0.007	101	57 (49 to 65)	<0.001
CRC	13	10	77 (46 to 95.0)	13	100 (75 to 100)	0.25	12	92.3 (64 to 99.8)	0.62
AA	109	79	73 (63 to 81)	74	68 (58 to 77)	0.51	69	63 (54 to 72)	0.14
ASP	55	46	84 (71 to 92.2)	24	44 (30 to 58)	<0.001	20	36 (24 to 50)	<0.001
Non-advanced neoplasia	598	311	52 (48 to 56)	304	51 (47 to 55)	0.72	315	53 (49 to 57)	0.86
		- result (n=808)	Specificity (%)	- result (n=808)	Specificity (%)	p-value	- result (n=808)	Specificity (%)	p-value
Non advanced lesions, other lesions and negative colonoscopy	1,367	764	56 (53 to 59)	741	54 (52 to 57)	0.39	732	54 (51 to 56)	0.20
Negative colonoscopy	598	372	62 (58 to 66)	360	60 (56 to 64)	0.49	361	60 (56 to 64)	0.52

*The threshold of FOB Gold could not be lowered beyond 0 µg Hb/ml. At this level, the positivity rate was 48%. Therefore, all tests were compared at positivity rate of 48%, equal to 736 colonoscopy referrals. †For this specificity the threshold of OC Sensor is below the limit of quantification. P-values represent statistical McNemar testing comparing Cologuard and the other test.



CRC = colorectal cancer, AA = advanced adenoma, ASP = advanced serrated polyp
Fisher's Exact test, *p<0.05, **p<0.01, ***p<0.001

Figure 4 | Lesion types detected during the surveillance colonoscopy stratified by surveillance indication (*i.e.* post-CRC, post-polypectomy and familial risk)

in the post-CRC population. Therefore it may be warranted for this subgroup to maintain colonoscopy-based surveillance, especially during the first years after curative resection when CRC risk is most pronounced.[30–32] Indeed, also in the present study most surveillance-detected CRC (6/9; 67%) occurred within two years after initial CRC diagnosis (Supplementary table S1). The lower prevalence of AA in the post-CRC population may be explained by the reduced

length of the colon still *in situ* after CRC resection. The post-polypectomy and familial risk subgroups, accounting for 67% of the population, harboured mostly advanced precursor lesions or non-advanced findings. Considering the relatively high sensitivity of Cologuard for advanced precursor lesions, surveillance based on stool testing may prove a suitable alternative for the colonoscopy-based strategy in the final analysis.

This study has several limitations. Firstly, we present an interim analysis including 1,551 of the 4,000 scheduled patients, and therefore the study is still underpowered in determining the accuracy of the stool tests. Secondly, the surveillance population in this study was a mixed group, comprising post-CRC, post-polypectomy and familial risk patients, potentially requiring different surveillance approaches. The study was not powered to perform subgroups analysis on test accuracy. Lastly, we did not evaluate the performance of stool tests over multiple rounds.

Nevertheless, this study is the first to our knowledge to assess multitarget stool DNA testing in the surveillance population and compare it to FIT. Considering the low evidence for the colonoscopy-based strategy and the high colonoscopy demand it generates, alternative strategies are highly warranted. In addition, endoscopists and pathologists were blinded for the test results, as were the laboratory workers for the clinical findings. Because Cologuard and FITs were performed on the same stool sample, direct comparison between the tests was possible. Furthermore, the inclusion of the entire range of surveillance patients can provide insights in test performance in different subgroups.

In conclusion, the multitarget stool DNA test (Cologuard) has higher sensitivity than FIT for detection of advanced neoplasia in the surveillance population. In the surveillance setting, lower specificity can be accepted to increase test sensitivity, while still reducing the number of unnecessary colonoscopies. Mathematical modelling approaches will be applied to the final study data in order to determine an optimal stool-based strategy for surveillance.

Acknowledgements

We thank Health RI (Utrecht, the Netherlands) for providing tools for the integration of clinical and test data and for facilitating long-term data storage. We thank the technicians for their invaluable work in assisting with the processing of the stool samples, the research nurses for their efforts to include patients and Pauline van Mulligen for the project management. We also wish to thank the involved personnel at the participating centres, as well as all participants.

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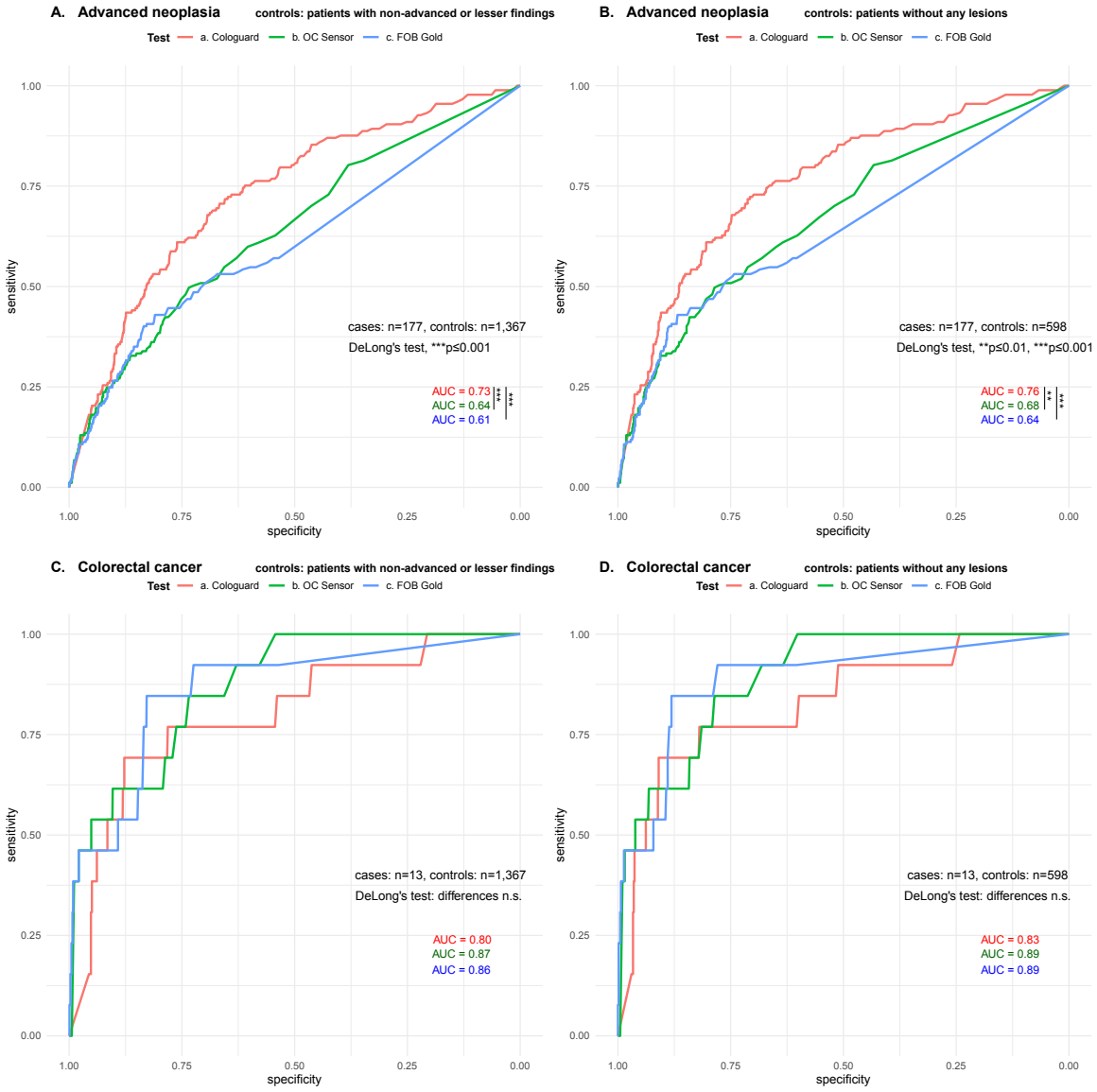
Supplementary Material



Supplementary figure 1 | ROC curve comparing Cologuard with FIT for advanced neoplasia and colorectal cancer detection, using different control groups

Supplementary table 1 | Details of patients with colorectal cancer at the current surveillance colonoscopy

	Indication	Previous CRC			Current CRC						Cologuard	OC Sensor	FOB Gold
		Year	Stage	Diff.	Year	Stage	TNM	Diff	Type	Location	µg Hb/g faeces	µg Hb/g faeces	µg Hb/g faeces
MOC_A_0062	CRC	2015	III	g/m	2016	I	T2N0M0	g/m	recurrence	rectum	60	40.2	25.5
MOC_B_0010	CRC	2015	I	g/m	2016	I		g/m	recurrence	desc. colon	1.6	3.6	10
MOC_B_0014	CRC	2015	III	g/m	2016	I		g/m	recurrence	rectum	7.3	16.6	7.7
MOC_B_0144	Polypectomy				2017	I		g/m		sigmoid	2.5	3	18
MOC_E_0114	Polypectomy				2016	I	T1N0M0	g/m		desc. colon	2	1.2	0
MOC_E_0145	CRC	2015	III	signet ring	2016	IV	T4N2M1	signet ring	recurrence	neocaecum	60	200	1,420
MOC_E_2010	CRC	2004	II	p/n	2016	I	T1N0M0	g/m	2nd primary	caecum	60	200	1,036
MOC_G_0164	CRC	2015	I	g/m	2017	III	T3N2M0	p/n	recurrence	sigmoid	2.3	1.8	7.8
MOC_H_0201	CRC	2012	I	p/n	2017	I	T1N0M0	p/n	2nd primary	asc. colon	1.4	4.4	6.8
MOC_K_0017	Polypectomy				2016	I	T2N0M0	g/m		sigmoid	60	200	1,240
MOC_K_0050	CRC	2014	I	g/m	2016	III	T4N2M0	g/m	recurrence	rectum	60	117	400
MOC_L_0024	Polypectomy				2017	I	TxN0M0	g/m		asc. colon	60	200	1,855
MOC_L_0008	CRC	2009	III	p/n	2017	IV	T4N0M1	g/m	2nd primary	desc. colon	60	200	827

The sequence number represents the number of surveillance colonoscopy the patient has undergone, including the current colonoscopy. Green coloured cells reflects overall test positivity and grey coloured cells test negativity, applying the screening-validated cut-off for Cologuard and 15µg Hb/ml cut-off for OC Sensor and FOB Gold. For all three tests. the number within the cells depicts the haemoglobin value. g/m = good/moderate, n.a. = not applicable, p/n = poor/none.

“Have no fear for perfection, you’ll never reach it”

Marie Curie

8.

Improving preselection for surveillance colonoscopy by complementing FIT with clinicopathological risk factors

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To be submitted after completion of the MOCCAS study

Abstract

Introduction

Preselection by FIT for surveillance colonoscopy could reduce the number of unnecessary colonoscopies. However, FIT sensitivity for advanced neoplasia (AN) is suboptimal. Based on a model that was previously developed to predict AN risk in the screening setting (COCOS model), we examined whether the performance of FIT in detecting AN could be improved by adding clinicopathological risk factors.

Methods

In the cross-sectional MOlecular stool testing for Colorectal CAncer Surveillance (MOCCAS) study, post-CRC (>5 years), post-polypectomy and familial risk surveillance patients performed FIT sampling and filled in a questionnaire prior to surveillance colonoscopy. The FIT result and questionnaire variables included in the COCOS model were revised and additional variables, reflecting historical colonoscopy findings, were considered for model extension. Model performance was evaluated with calibration statistics ($p>0.05$ corresponding to good calibration) and the area under the receiver operating characteristic curve (AUC). Model performance was compared to FIT with the generalised likelihood ratio test (LR).

Results

AN was detected in 147 of the 1,138 (13%) analysed surveillance patients with a FIT result and questionnaire response. The updated model included FIT result, age, calcium intake, smoking habits, (tubulo)villous adenoma in previous colonoscopy and large lesions in previous colonoscopy. The AUC of the updated model was 0.71 (95% CI 0.66 to 0.76) (calibration test: $p=0.23$) compared to an AUC of 0.66 (95% CI 0.60 to 0.70) for FIT only (LR, $p <0.001$). At a positivity rate of 50%, sensitivity increased from 68% (95% CI 58% to 76%) with FIT only to 75% (95% CI 66% to 82%) with the updated model at similar specificity of 51% (95% CI 47% to 54%) and 52% (95% CI 48% to 55%). At a positivity rate of 25%, sensitivity increased from 47% (95% CI 38% to 56%) with FIT only to 49% (95% CI 40% to 58) with the updated model at equal specificity of 78%.

Conclusion

Preselection for surveillance colonoscopy could be improved by complementing FIT with clinicopathological risk factors in a prediction model.

ClinicalTrials.gov: NCT02715141

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death worldwide and has a high incidence of 1.7 million annually.[1] Endoscopic removal of precursor lesions, *i.e.* adenomas and serrated polyps, can reduce CRC incidence and mortality.[2,3] An important part of early detection is colonoscopy surveillance, which aims to monitor those patients at increased risk of CRC, *e.g.* patients with prior curative CRC resection, prior polypectomy or a familial risk.[4] Due to recent implementation of screening programmes, the population with an indication for surveillance colonoscopy is growing. Currently, already 20-25% of colonoscopy capacity (in the UK and USA) is for surveillance.[5,6] The expected increase will put more pressure on the colonoscopy capacity.

The yield of relevant pathology, *i.e.* advanced neoplasia, at surveillance colonoscopy is around 10%.[7] Therefore, efforts that try to minimise the number of unnecessary colonoscopies and ensure availability for patients at the highest risk are warranted. In general, the effectiveness of current surveillance strategies has not been convincingly demonstrated for all patient categories.[8–10] Furthermore, colonoscopy as surveillance method is associated with several issues. It is an invasive procedure, associated with a risk of bleeding and perforation in a small proportion of cases.[11] In addition, the procedure is costly and has limited capacity.

In short, there is a need for alternative and less invasive surveillance strategies. Stool-based testing is a non-invasive method that could be used to select patients at increased risk for AN for referral to surveillance colonoscopy. The faecal immunochemical test (FIT) detects human haemoglobin (Hb) in faeces and has proven to be an effective method for early CRC detection in screening.[12] A recent surveillance study with three FIT rounds showed that annual testing was a cost-saving surveillance strategy in the intermediate risk group, defined as patients with one-to-two large (≥10 mm) adenomas, or three-to-four small adenomas.[13] However, also 15-30% CRCs and 40-70% of advanced adenomas were missed, depending on the FIT cut-off. A multitarget stool DNA (mt-sDNA) test combining the detection of human Hb with several DNA markers detects AN with significantly higher sensitivity than FIT.[14,15] Yet, the mt-sDNA test is also more expensive, more complicated to perform and comes with logistical challenges as it requires whole-bowel movement stool samples. This means that other strategies to increase the efficiency of surveillance are worth considering.

Previous studies in a screening setting have shown that combining FIT results with established risk factors for AN increases the accuracy of FIT.[16] These risk factors were easily obtained using a questionnaire and included total calcium intake, family history, smoking habits and age. It is unknown whether this prediction model (from here on referred to as the COCOS model) would also provide additional value in the surveillance setting. Studies have shown that factors predicting the index event do not necessarily predict recurrence, a phenomenon known as 'index-event bias'.[17] Moreover, in the surveillance population historical colonoscopy data are also available to predict AN, information that is by definition unavailable in a (first-round) stool-based screening setting.

The aim of the present study was to assess whether the performance of FIT in preselecting patients for surveillance colonoscopy can be improved by applying a diagnostic prediction model that includes additional risk factors. For this purpose, we first evaluated the performance of the previously developed COCOS model. We then updated the model by performing variable revision and adding clinicopathological variables associated with metachronous AN.[18–20] The performance of the updated model was compared to the performance of FIT only.

Methods

Study design

This research is part of the MOlecular stool testing for Colorectal CAncer Surveillance (MOCCAS) study, a cross-sectional study ongoing in eleven centres throughout the Netherlands. The MOCCAS study aims to assess whether stool-based tests, including FIT (OC Sensor, Eiken Chemical Co, Tokyo, Japan) and the mt-sDNA test (Cologuard, Exact Sciences, Madison, WI, USA), can accurately identify patients with AN in a surveillance population. In addition to submitting a stool sample, patients filled in a questionnaire regarding risk factors for CRC.

The study was approved by the Medical Ethical Committee of the Amsterdam UMC, the Netherlands and registered in ClinicalTrials.gov (NCT02715141). Details on the study protocol can be found elsewhere. [21]

Study population

In the MOCCAS study, patients between 50-75 years old with an indication for surveillance colonoscopy according to the Dutch guidelines (*i.e.* post-polypectomy, history of CRC and/or familial risk) were eligible. Patients were excluded in case of inflammatory bowel disease (IBD), genetic cancer syndromes (*e.g.* Lynch syndrome and familial adenomatous polyposis), colonoscopy in the last 6 months, proctocolectomy or life expectancy less than three years. In addition, patients with CRC diagnosed less than 5 years prior to a surveillance colonoscopy were excluded because it is known that these patients have a pronounced CRC risk in the first few years after resection. For this reason, these patients likely keep an indication for colonoscopy.[22,23] In order to give written informed consent and fill in the questionnaire, patients also had to have sufficient understanding of the Dutch language.

Stool testing

Patients with a scheduled routine surveillance colonoscopy were invited to participate two weeks prior to the colonoscopy by a member of the MOCCAS research team. Consenting patients performed mt-sDNA whole stool sampling and FIT sampling on stool from the same bowel movement at home before starting the bowel preparation. The collected stool specimen of the mt-sDNA test and FIT were analysed as described elsewhere.[21] This resulted

in quantitative results which, in case of the mt-sDNA test, was a final test score combining the Hb and DNA marker concentrations and, in case of FIT, was the Hb concentration. All measurements were performed blinded for the colonoscopy results.

Questionnaire

In addition to performing stool sampling, patients were asked to fill in an online questionnaire. Patients needed to answer all questions in order to proceed. Study participants could also request a hardcopy version of the questionnaire. In case of missing answers in a hardcopy questionnaire, patients were approached by phone to collect the missing information.
The questionnaire had previously been validated and was identical to the one used in the study in which the COCOS model had been developed.[16] The questionnaire contained 28 questions concerning the risk factors age, first degree family members with CRC history, alcohol intake, smoking habits, BMI, regular aspirin or non-steroid anti-inflammatory drug (NSAID) use, total calcium intake, physical activity and oestrogen supplementation.[24]

Colonoscopy

Colonoscopy was performed according to standard practice. The endoscopists described cecal intubation, quality of bowel preparation and location, size and morphology of the detected lesions. Resected lesions were sent to pathology for assessment of histological features according to protocol.[25] The endoscopists and pathologists were blinded for the results of the stool tests. Patients were routinely informed about the colonoscopy findings, the reference standard for colorectal polyp detection, but not about the stool-based test results.

Clinical data collection

Data on the surveillance indication and findings at the previous and current colonoscopy, *i.e.* number and characteristics of detected lesions, were collected. The collection of clinical data was performed according to the Dutch Personal Data Protection Act, using a predefined database validated to global regulatory standards (*i.e.* OpenClinica, Waltham, MA, USA).

Outcome measures

The primary outcome of this study was the detection of AN at colonoscopy. The definition of AN included CRC, advanced adenomas (AAs, *i.e.* adenoma ≥ 10mm and/or with high grade dysplasia and/or ≥25% villous component), and advanced serrated polyps (ASPs, *i.e.* hyperplastic polyps, sessile serrated lesions, or traditional serrated lesions ≥ 10mm or with dysplasia).

Statistical analysis

External validation and update of COCOS model

The COCOS model was originally developed in a screening setting, using logistic regression analysis to predict AN at colonoscopy. In addition to the FIT result (in ng Hb/ml), the model

included a square root transformation of the FIT result (from here on referred to as FIT sqrt) to better approach linearity of the relation between the logit of the risk and FIT result. and the following risk factors:

- Age (years).
- Relatives with CRC: number of first-degree family members with CRC.
- Total calcium intake (mg/day): calculated from the amount of calcium in dairy products, vegetables and bread consumed daily.[26]
- Smoking habits: dichotomised into smokers versus former and non-smoker.

First, we evaluated the performance of the original COCOS model by applying it to the MOCCAS dataset. All variable definitions were the same as in the development study.
Thereafter, the model was updated to fit the surveillance population by following the methods described by Steyerberg.[27] This was done by recalibrating the intercept and slope and by subsequently revising the regression coefficients of the original variables and extending the model with additional variables. The variables that were considered for addition to the model reflected historic colonoscopy findings associated with increased risk of metachronous AN in previous studies.[15,18–20] These variables could not be included in the COCOS model as individuals in a FIT screening setting did not previously undergo colonoscopy. The variables considered for addition to the model were:

- Post-CRC as surveillance indication: history of CRC >5 years ago (yes/no).
- Post-polypectomy as surveillance indication (yes/no).
- Familial risk as surveillance indication (yes/no).
- Number of adenomas: total number of adenomas found in the previous colonoscopy.
- Proximal adenomas: presence of at least one proximal adenoma in the previous colonoscopy (yes/no).
- (Tubulo)villous adenomas (T)VAs: presence of at least one adenoma with villous histology at the previous colonoscopy (yes/no).
- Dysplastic lesion: presence of at least one adenoma with HGD or dysplastic serrated polyp in the previous colonoscopy (yes/no).
- Large lesion: presence of at least one large (≥10mm) adenoma or serrated polyp in the previous colonoscopy (yes/no).

A stepwise selection procedure was used to update the model. For each of the variables already present in the original model we evaluated whether the regression coefficient was significantly different in the MOCCAS dataset, in a model including the linear predictor as offset variable. In case of significance, the coefficient was revised. Next, when the revised coefficient no longer added predictive value to the model (*i.e.* was no longer statistically significant), it was removed. For each of the new variables that were considered, we evaluated whether it contributed significantly to the model. A p-value of 0.157 was used as significance level, based on Akaike's Information Criterion and according to the recommendations for developing prediction models.[28]

Internal validation of new diagnostic model

Generally, prediction models perform best in the dataset they were developed in. This may lead to overfitting and too optimistic performance.[28] Internal validation of the extended model was done to estimate the optimism, using bootstrapping with 500 repetitions.

Model performance

The performance of the original model and updated model were examined with measures of calibration and discrimination. Calibration reflects the agreement between the predicted risk of AN and the actually observed risks in the dataset. Calibration belts were used to assess calibration.[29] These calibration belts show confidence bands (with 80% and 95% confidence levels) for the calibration curve. This allowed for assessing the ranges of risk for which there would be a significant deviation from the ideal calibration, visualised as the 45-degree line, and the direction of the deviation. The calibration test associated with the calibration belt is based on a series of likelihood ratio tests.[30] Non-significance of the calibration test indicates adequate calibration.

Discrimination reflects the model's ability to distinguish between patients with and without AN. Discrimination was expressed by calculating the area under the receiver operating characteristic (ROC) curve (AUC). Improvement in performance was tested with the generalised likelihood ratio test (LR).[31]

The sensitivity and specificity of FIT only and the updated diagnostic model were compared at equal positivity rates of 50% and 25%. The positivity rate is defined as the number of patients with a positive test, divided by the total number of patients. Thus, at equal positivity rates, the number of patients referred for colonoscopies would be the same, while the number of patients detected with AN at those colonoscopies is dependent on the selection method used (*i.e.* FIT or the updated diagnostic model). In the scenario of a 50% positivity rate, 50% of colonoscopies would be saved compared to the current colonoscopy surveillance; in the scenario of 25% positivity rate, 75% of colonoscopies would be saved. Reclassification tables were made to calculate the net reclassification improvement (NRI) for both scenarios.

In addition, the sensitivity and specificity of the mt-sDNA test were calculated at a positivity rate of 50% and 25% and compared with FIT only and the updated model.

Software

Statistical analyses were performed using the packages givitiR, Hmisc, rms and PredictABEL in R studio version 1.1.453 (R studio, Boston, MA, USA).

Results

Study population

A total of 1,756 patients were enrolled in the study at the time of data extraction on April 12th 2018 (Figure 1). The questionnaire was filled in by 1,505 of the 1,678 patients with a valid FIT result and adequate colonoscopy examination (response rate 90%). After excluding patients diagnosed with CRC less than 5 years ago, 1,138 patients remained.

The majority of the 1,138 respondents was male (53%) and the mean age was 66 years (sd 6.6) (Table 1). Post-polypectomy surveillance was the most common indication for surveillance (85%).

At surveillance colonoscopy, AN was detected in 147 (12.9%) patients, including 6 (0.5%) CRCs, 92 (8.1%) AAs and 49 (4.3%) ASPs.

As shown in Table 1, data were missing on severable variables concerning previous colonoscopy findings. For comparability of model performance, all analyses were performed on patients with complete cases, *i.e.* 1,026 patients of which 134 with AN. The characteristics of these patients are summarised in Supplementary table 1.

Evaluation of COCOS model

The COCOS model was applied to the MOCCAS dataset to evaluate its performance in an external dataset. Its discriminatory ability, indicated by the AUC, was 0.63 (95% CI 0.58 to 0.68), which was significantly lower than the AUC of 0.76 reported in the development study.[16]

The calibration belt showed an overestimation of the calculated probabilities compared to the observed proportions in the range 0.17-0.92 at 95% CI ($p<0.001$) (Figure 2A). Calculated probabilities were distributed around the prevalence of AN (12.9%) (Figure 2B). The median calculated probability was of 0.12 (IQR 0.07 to 0.95).

Updating the COCOS model

We then evaluated whether revising and updating the model would improve the performance in the MOCCAS data. First, the intercept and slope were recalibrated. This resulted in adequate calibration of the model over the entire range of risks ($p=0.08$). Since the ranking of the calculated probabilities is not affected by the recalibrated intercept and slope, discrimination did not change.

Subsequently, we performed model revision. The regression coefficients of the variables smoking and FIT sqrt were revised (Table 2). In addition, having relatives with CRC was removed from the model: this variable was associated with an increased risk of AN in the COCOS model (OR 1.6, 95% CI 1.1 to 2.4), but appeared to be a non-significant factor in the MOCCAS dataset (OR 0.90, 95% CI 0.70 to 1.2).

Two variables were added to the model: (T)VAs in previous colonoscopy (OR: 1.6, 95% CI 1.0 to 2.5) and large lesions in previous colonoscopy (2.1, 95% CI 1.3 to 3.3)(Table 2).

Table 1 | Baseline characteristics

	Total cohort (n=1,138)	Patients with AN (n=147)
PATIENT DEMOGRAPHICS		
Male, n (%)	602 (53)	79 (54)
Age in years, mean (sd)	66 (6.6)	66 (5.8)
Surveillance indication*		
Post-CRC	122 (11)	10 (7)
Post-polypectomy	968 (85)	130 (88)
Familial risk	275 (24)	7 (5)
STOOL TEST		
FIT result, n (%)†, median (IQR)		
<15 ng Hb/ml	831 (73)	76 (52)
≥15 ng Hb/ml	307 (27)	71 (48)
QUESTIONNAIRE		
Calcium intake in mg/day, median (IQR)	690 (398)	753 (448)
Relatives with CRC, n (%)†		
0	618 (54)	94 (64)
1	352 (31)	34 (23)
≥2	168 (15)	19 (13)
Smoking status, n (%)		
Current	178 (16)	33 (22)
No/former	960 (84)	114 (78)
PREVIOUS COLONOSCOPY FINDINGS		
Total number of adenomas, n (%)†		
0	422 (37)	43 (29)
1	388 (34)	49 (33)
2	134 (12)	21 (14)
3	79 (7)	14 (10)
4	37 (3)	5 (4)
≥5	48 (4)	12 (8)
Missing	29 (3)	3 (2)
≥1 proximal adenomas, n (%)		
Yes	376 (33)	53 (36)
No	715 (63)	91 (62)
Missing	47 (4)	3 (2)
≥1 (T)VA, n (%)		
Yes	233 (21)	48 (33)
No	856 (75)	94 (64)
Missing	49 (4)	5 (3)
≥1 adenoma with HGD or SP with dysplasia, n (%)		
Yes	77 (7)	20 (14)
No	987 (87)	119 (81.0)
Missing	74 (6)	8 (5)
≥1 large adenoma or SP, n (%)		
Yes	222 (20)	54 (37)
No	845 (74)	85 (58)
Missing	71 (6)	8 (5)

Table 1 | Continued

FINDINGS AT SURVEILLANCE		
Advanced neoplasia, n (%)		
Colorectal cancers	6 (0.5)	6 (4)
Advanced adenomas	92 (8.1)	92 (63)
Advanced serrated polyps	49 (4.3)	49 (33)
Total	147 (12.9)	147 (100)

*Patients could have multiple surveillance indications. †Variable categorised in table, but used as continuous variable in the model. AN = advanced neoplasia, CRC = colorectal cancer, FIT = faecal immunochemical test, Hb = haemoglobin, HGD = high grade dysplasia, SP = serrated polyp, (T)VA = (tubulo)villous adenoma.

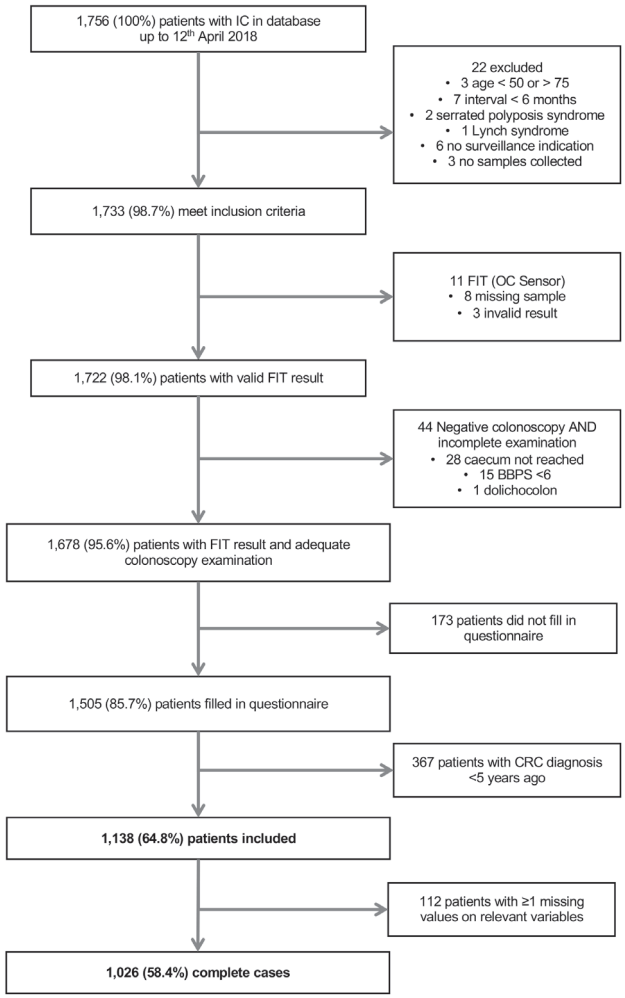

Figure 1 | Flowchart of study population

Taken together, the updated model included the variables age, FIT, FIT sqrt, calcium intake, smoking, (T)VAs in previous colonoscopy and large lesions in previous colonoscopy. The calibration of the updated model remained adequate over the entire range of risks (p=0.36) (Figure 2C). Again, the calculated probabilities were distributed around the prevalence of AN (Figure 2D), with a median of 0.09 (IQR 0.07 to 0.15). As examples, risk profiles of patients at the extremes of the distribution are provided in Supplementary table 2. The AUC of the updated model improved to 0.71 (95% CI 0.66 to 0.76)(Table 2 and Figure 3).

Table 2 | Model parameters and model performance in the MOCCAS dataset of original model, updated model and final model after internal validation

n=1,026		COCOS model applied in MOCCAS dataset	Updated model	Updated model after internal validation
Model parameters	Intercept	-5.243	-3.036	-2.958
	Age (years)	0.0446	0.00685	0.00643
	FIT (ng Hb/ml)	-0.0031	-0.000474	-0.000445
	FIT sqrt (ng Hb/ml)	0.2145	0.0838 (se 0.017 p<0.001)	0.0787
	Calcium intake (mg/day)	-0.0009	-0.000133	-0.000125
	Relatives with CRC	0.5065	removed	-
	Smoking	0.6054	0.519 (se 0.238, p=0.043)	0.488
	≥1 (T)VA	-	0.462 (se 0.238, p=0.091)	0.434
	≥1 Large adenoma or SP	-	0.744 (se 0.231, p=0.001)	0.699
	Calibration slope for COCOS model		0.153	-
	Calibration slope for internal validation	-	-	0.939
Calibration	Calibration test	<0.001	0.357	0.357
Discrimination	AUC (95% CI)	0.63 (0.58 to 0.68)	0.71 (0.66 to 0.76)	0.70 (0.65 to 0.75)

AUC = area under the curve, CRC = colorectal cancer, FIT = faecal immunochemical test, FIT sqrt = square root transformation of FIT result, Hb = haemoglobin, SP = serrated polyp, (T)VA = (tubulo)villous adenoma.

Comparison of FIT only and updated model

The AUC of FIT only was 0.66 (95% CI 0.60 to 0.70), compared to the mentioned AUC of 0.71 for the updated model (LR, p<0.001) (Figure 3). Because it was not possible to reach exactly 50% and 25% FIT positivity rates, cut-offs were selected resulting in rates closest to these numbers. This lead to FIT positivity rates of 52% and 25%, respectively. The other tests were compared at the exact same positivity rates.

At a positivity rate of ~50%, sensitivity of FIT only was 68% (95% CI 58% to 76%) at a specificity of 51% (95% CI 47% to 54%) (Table 3). Using the updated model while maintaining the same positivity rate, sensitivity improved to 75% (95% CI 66% to 82%) and specificity to 52% (95% CI 48% to 55%). This translated into 9 more patients with AN being detected with

the updated model, in 502 colonoscopies. The updated model detected 7 more AAs and 3 more ASPs but missed 1 CRC that was detected with FIT only. Classification improved with the updated model, but this was not significant; NRI was 0.084 (p=0.12) (Supplementary table 3).

At a positivity rate of ~25%, sensitivity of FIT only was 47% (95% CI 38% to 56%) compared to 50% (95% CI 40% to 59%) for the updated model (Table 3). The specificity of both FIT only and the updated model was 78% (95% CI 75% to 81%). In 246 colonoscopies, 4 more cases with AN were detected using the model instead of FIT only. One more CRC and 6 more ASPs were detected with the model, but 2 more AAs were missed. The improvement in classification was not significant (NRI 0.028, p=0.54) (Supplementary table 3).

Table 3 | Performance of different surveillance methods in the detection of advanced neoplasia

n=974*		**FIT**	**Updated model**	**Mt-sDNA test**
50% Positivity rate†		**Cut-off: 4.5 ng Hb/ml**	**Risk threshold: 0.09**	**Cut-off: 71**
Sensitivity (95% CI)		68% (58% to 76%)	75% (66% to 82%)	82% (74% to 88%)
Specificity (95% CI)		51% (47% to 54%)	52% (48% to 55%)	53% (50% to 56%)
Number of colonoscopies:		502	502	502
With AN		83	92	101
Without AN		419	410	401
Positive tests among	n=			
patients with: AN	123	83	92	101
CRC	6	6	5	5
AA	78	56	63	62
ASP	39	21	24	34
25% Positivity rate†		**Cut-off: 13.5 ng Hb/ml**	**Risk threshold: 0.15**	**Cut-off: 202**
Sensitivity		47% (38% to 56%)	50% (40% to 59%)	55% (45% to 64%)
Specificity		78% (75% to 81%)	78% (75% to 81%)	79% (76% to 82%)
Number of colonoscopies:		246	246	246
With AN		58	62	67
Without AN		188	187	179
Positive tests among	n=			
patients with: AN	123	58	61	67
CRC	6	4	4	5
AA	78	45	43	37
ASP	39	9	14	25

*In these analyses, patients with mt-sDNA test results were included. Therefore, this subgroup is smaller than the total study group. †Because it was not possible to reach exactly 50% and 25% FIT positivity rates, cut-offs were selected resulting in rates closest to these numbers. This resulted FIT positivity rates of 52.2% and 25.4%, respectively. The other tests were compared at equal positivity rates.

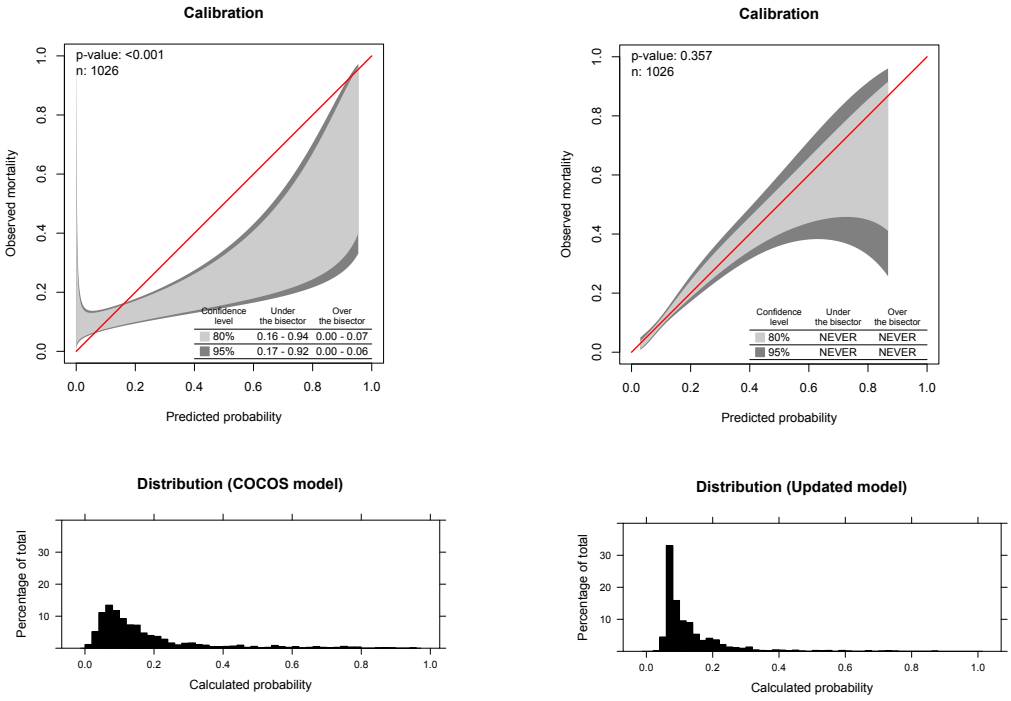

Figure 2 | Calibration belts and histograms of COCOS model (panel A and B) and updated model (panel C and D) for prediction of advanced neoplasia

Calibration: Red line indicates perfect calibration. Confidence levels γ = 80% and q = 95% (light and dark gray, respectively), with ranges depicting significant disagreement between calculated probabilities and observed frequencies. Histogram: Applying 40 breaks. In our patient cohort 13% had advanced neoplasia.

Comparison with mt-sDNA test

The AUC of the mt-sDNA test was 0.74 (95% CI 0.70 to 0.79) (Figure 3). The AUC point estimate and 95% CI of the mt-sDNA test were not overlapping with the 95% CI of the FIT (CI 0.60 to 0.70), but were contained within the 95% CI of the updated model (95% CI 0.66 to 0.76). At a positivity rate of ~50%, sensitivity for the mt-sDNA test was 82% (95% CI 74% to 88%) and specificity 53% (95% CI 50% to 56%) (Table 3). In 502 colonoscopies, 18 more patients with AN were detected compared to FIT only and 9 more patients compared to the updated model. While detecting more AAs and ASPs, the mt-sDNA test missed 1 CRC that was detected with FIT only.

At a positivity rate of ~25%, the sensitivity of the mt-sDNA test was 55% (95% CI 45% to 64%) and specificity 79% (95% CI 76% to 82%)(Table 3). This resulted in 9 more patients with AN being detected with the mt-sDNA test compared to FIT only and 5 more patients compared to the model. The mt-sDNA test detected 1 more CRC and more ASPs, but less AAs compared to FIT only and the updated model.

Internal validation

After internal validation, the calculated AUC was 0.70 (95% CI 0.65 to 0.75) (Table 2). Model calibration remained adequate (p=0.36). The regression equation is summarised in Box 1.

Box 1 | Calculating the probability of advanced neoplasia (AN) with the updated model after internal validation

Regression equation of updated model:

Ln(odds AN) = -3.036 + 0.00643*age - 0.000445*FIT + 0.0787*FIT sqrt - 0.000125*calcium intake + 0.488*smoking + 0.434*≥1(T)VA + 0.699*≥1 large lesion

Probability of advanced neoplasia:

$$P(AN) = \frac{\exp(\text{odds AN})}{1+\exp(\text{odds AN})}$$

Example

For a 65 years old patient with FIT result 68 ng Hb/ml, calcium intake 180 mg/day, who is a non-smoker and had 1 TVA and no large lesions at previous colonoscopy, the probability of advanced neoplasia is calculated as follows:

Ln(odds AN) = -3.036 + 0.00643*65 - 0.000445*68 + 0.0787*8.25 - 0.000125*180 + 0.488*0 + 0.434*1 + 0.699*0 = -1.59

$$P(AN) = \frac{\exp(-1.644)}{1+\exp(-1.644)} = 0.17$$

Using the risk threshold of 0.09 or 0.15, corresponding with positivity rates of 50% and 25% (table 3), respectively, this patient would be classified as risk positive, *i.e.* as having advanced neoplasia.

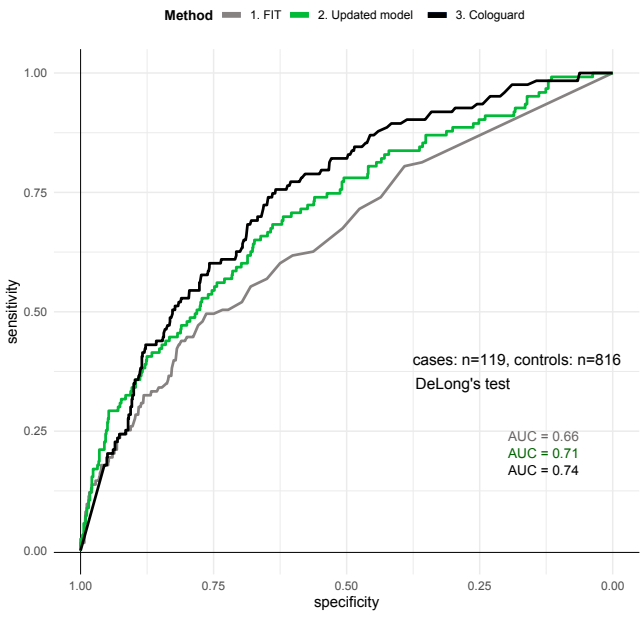

Figure 3 | ROC curve comparing FIT and the updated model

Discussion

Against the background of the need to increase the efficiency and effectiveness of colonoscopy surveillance, in the present study we evaluated whether the performance of FIT for preselecting patients for colonoscopy could be improved by complementation with clinicopathological risk factors. We found that the original COCOS model, developed in a screening population, did not improve the performance of FIT in the surveillance setting. After revising and updating, the new model included the FIT result, age, calcium intake, smoking, (tubulo)villous adenomas in previous colonoscopy and large lesions (adenomas or serrated polyps) in previous colonoscopy. This approach improved the discriminatory ability of the model and resulted in more patients with AN being detected with an equal number of colonoscopies. The performance of this updated FIT plus risk factors model was comparable to the mt-sDNA test in detecting patients with AN.

Having relatives with CRC, which was identified as a risk factor for AN in a screening population, turned out not to be a predictive factor in the surveillance population. This risk apparently fades against other risk factors that are present in this population. On the other hand, presence of adenomas with villous histology and large adenomas or serrated polyps in the previous colonoscopy were added as risk factors to the model. Both factors are currently used in the Dutch surveillance risk-stratification guideline.[4] Because these findings at baseline are associated with detection of AN during the surveillance period,[32] patients with these lesion characteristics are recommended to undergo colonoscopy at a shorter surveillance interval. Indeed, in our cohort patients with (T)VAs or large lesions had shorter colonoscopy intervals compared to patients without these lesions. Nevertheless, villous histology and large lesions apparently remain predictive for AN detection at this earlier surveillance colonoscopy.

Another risk factor in the Dutch surveillance guideline is proximal adenoma location. A large registry study found that the OR associated with AN in patients with proximal adenomas was 1.6.[32] In the current study, however, proximal location of a previously detected adenoma came forward as a significant protective factor. An explanation for this contradictory finding may be that detection of a proximal adenoma reflects a complete and meticulous colon examination, with cecal intubation and detection of less obvious non-polypoid lesions, which are more abundant in the proximal colon.[33] To avoid overfitting and subsequent poor external applicability of the updated model, proximal adenoma location was not included as a predictor in the final model.

In the present study, sensitivity and specificity of different surveillance methods were compared at 50% and 25% positivity rate. Although such high positivity rates would not be realistic in a screening setting, in the surveillance setting they may, as in the current situation all these patients already qualify for colonoscopy.

A recent report demonstrated that a surveillance strategy consisting of annual FIT with a low cut-off during a three year period had a high cumulative sensitivity of 85% in detecting CRC and would be cost-saving compared with colonoscopy once every three years.[13] The programme

sensitivity after three FIT rounds for AAs, however, was only 57%. The FIT sensitivity for ASPs was not taken into account, but is known to be considerably lower than for AAs.[14] The current study shows that by adding clinicopathological risk factors to the FIT result, more patients with AAs and ASPs can be detected, depending on the positivity rate. This could increase the efficiency of the surveillance programme, likely without substantially increasing programme costs. Nevertheless, also one more CRC was missed by the updated model compared to FIT only at 50% positivity rate, which was also missed by the mt-sDNA test. Because the current dataset included few cancers, it should be tested on a larger cohort whether the model does not result in more missed cancers. Evidently, diagnostic delay of cancers is more harmful than of precursor lesions, as it may result in more progressed disease and reduced survival.

The performance of the updated model was comparable to that of the mt-sDNA test. The user-friendliness and logistic organisation is, however, more favourable with FIT, which could translate into a reduced number of non-participants and analytical drop-outs in a FIT-based programme compared to a mt-sDNA-based programme.[15] In addition, the costs of the FIT(~€20) are substantially less than that of the mt-sDNA test (~€600). A formal health technology and patient preference analysis, to further investigate these aspects, was beyond the scope of the current study.

It should be kept in mind that the current model can only be applied in those patients that had colonoscopy as their last surveillance examination. If the surveillance programme would become FIT-based, the model could not be applied to patients that received FIT without colonoscopy as previous examination. For this patient category, that is yet inexistent, a new model should be developed.

A limitation of this study was that data on several variables concerning previous colonoscopy findings were missing. This was due to a lack of previous colonoscopy and pathology reports, for example when patients were referred from a different hospital. We tested whether these data were missing at random and did not find selective missingness. The number of cancers in our cohort was very low so CRC sensitivity could not reliably be compared between the different surveillance methods. In addition, small difference in performance between the updated model and mt-sDNA test cannot be excluded based on our data and should be investigated in a larger study.

Strengths of this study are that the questionnaire was exactly the same as in the original COCOS study. This means that comparability of the current study, performed in a surveillance setting, and the development study, performed in a screening setting, was maximised. The response rate was high and completeness of questionnaire data was ensured by using an online application.

In conclusion, this study showed that preselection for surveillance colonoscopy could be improved by complementing FIT with clinicopathological risk factors in a prediction model. The magnitude of the improvement was dependent on the positivity rate. The multivariable model could be an alternative for the more logistically challenging and expensive multitarget stool DNA test.

Acknowledgements

We thank Health RI (Utrecht, the Netherlands) for providing tools for the integration of clinical and test data and for facilitating long-term data storage. We thank the technicians for their invaluable work in assisting with the processing of the stool samples, the research nurses for their efforts to include patients and Pauline van Mulligen for the project management. We also wish to thank the involved personnel at the participating centres, as well as all participants.

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Supplementary Material

Supplementary table 1 | Baseline characteristics

	Total cohort (n=1,026)	Patients with AN (n=134)
PATIENT DEMOGRAPHICS		
Male, n (%)	549 (48)	71 (53)
Age in years, mean (sd)	65.6 (6.6)	66.3 (5.8)
Surveillance indication*		
Post-CRC	115 (11)	9 (7)
Post-polypectomy	812 (79)	118 (88)
Familial risk	99 (10)	7 (5)
STOOL TEST		
FIT result, n (%)†, median (IQR)		
<15 ng Hb/ml	748 (73)	76 (52)
≥15 ng Hb/ml	278 (27)	71 (48)
QUESTIONNAIRE		
Calcium intake in mg/day, median (IQR)	690 (408)	650 (451)
Relatives with CRC, n (%)†		
0	558 (54)	86 (64)
1	317 (31)	30 (22)
≥2	151 (15)	18 (13)
Smoking status, n (%)		
Current	160 (14)	32 (22)
No/former	866 (86)	102 (78)
PREVIOUS COLONOSCOPY FINDINGS		
Total number of adenomas, n (%)†		
0	411 (40)	42 (31)
1	338 (33)	43 (32)
2	121 (12)	20 (15)
3	75 (7)	13 (10)
4	36 (4)	4 (4)
≥5	45 (4)	11 (8)
≥1 proximal adenomas, n (%)		
Yes	335 (35)	49 (37)
No	671 (65)	85 (63)
≥1 (T)VA, n (%)		
Yes	206 (20)	44 (33)
No	820 (80)	90 (67)
≥1 adenoma with HGD or SP with dysplasia, n (%)		
Yes	74 (7)	20 (14.9)
No	952 (93)	114 (85.1)
≥1 large adenoma or SP, n (%)		
Yes	210 (20)	51 (38)
No	816 (80)	83 (62)

Supplementary table 1 | Continued

FINDINGS AT SURVEILLANCE		
Advanced neoplasia, n (%)		
Colorectal cancers	5 (0.5)	5 (4)
Advanced adenomas	83 (8.1)	83 (62)
Advanced serrated polyps	46 (4.5)	46 (34)
Total	134 (13.1)	147 (100)

*Patients could have multiple surveillance indications. †Variable categorised in table, but used as continuous variable in the model. AN = advanced neoplasia, CRC = colorectal cancer, FIT = faecal immunochemical test, HGD = high grade dysplasia, SP = serrated polyp, (T)VA = (tubulo)villous adenoma.

Supplementary table 2 | Risk profiles of the three patients with the lowest calculated probability and three patients with the highest calculated probability of advanced neoplasia according to the updated model

Subject ID	Calculated probability	Age (years)	FIT (ng Hb/ml)	Calcium intake (mg)	Smoking	≥1 (T)VA	≥1 Large adenoma or SP	Event
Lowest risk patients								
MOC_H_2038	0.0300	65	0	6788	no	no	no	no
MOC_E_0216	0.0358	67	0	5490	no	no	no	no
MOC_C_0093	0.0523	49	0	1491	no	no	no	no
Highest risk patients								
MOC_K_0164	0.725	60	809	998	yes	yes	yes	yes (ASP)
MOC_H_2060	0.748	70	9504	428	no	no	no	yes (AA)
MOC_G_0290	0.869	66	3659	560	no	yes	yes	yes (AA)

AA = advanced adenomas, ASP = advanced serrated polyp, FIT = faecal immunochemical test, SP = serrated polyp, (T)VA = (tubulo) villous adenoma.

Supplementary table 3 | Reclassification in patients with and without advanced neoplasia (AN) according to FIT result and risk as predicted by the updated model

50% Positivity rate

FIT cut-off: 1 ug/g, risk cut-off: 0.09

n=974*	Risk positive	Risk negative	Total
With AN			
FIT positive	69	14	83
FIT negative	23	17	40
Total	92	31	123
Without AN			
FIT positive	254	165	419
FIT negative	156	276	432
Total	410	441	851

NRI = 0.084 (p=0.12), NRI for events = 0.073 (p=0.14), NRI for non-events = 0.011 (p=0.62)

25% Positivity rate

FIT cut-off: 3 ug/g, risk cut-off: 0.15

n=974*	Risk positive	Risk negative	Total
With AN			
FIT positive	46	12	58
FIT negative	15	50	65
Total	61	62	123
Without AN			
FIT positive	85	103	188
FIT negative	100	563	663
Total	185	666	851

NRI = 0.028 (p=0.54), NRI for events = 0.024 (p=0.56), NRI for non-events = 0.0035 (p=0.83)

*For comparability the same number of patients were used as in table 3. FIT = faecal immunochemical test.

“Kleine dingen goed doen is een stap
om grote dingen beter te doen”

Vincent van Gogh

9.

Yield of surveillance colonoscopies 1 year after curative surgical colorectal cancer resections

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Clinical Gastroenterology and Hepatology 2019

Abstract

Background & Aims

Endoscopic surveillance after curative colorectal cancer (CRC) resection is routine. However, there is controversy whether the 1-year interval between pre- and postoperative colonoscopy is justified, due to improved colonoscopy standards. We aimed to assess the yield of surveillance colonoscopies 1 year after CRC surgery.

Methods

We performed a retrospective cohort study of 572 patients (54.9% male, mean age 66.2 (±9.9) years), who underwent curative surgical resection of a first CRC from June 2013 through April 2016 in the Northwest region of the Netherlands. Patients were included if a complete clearing colonoscopy was performed before surgery and the interval between the pre- and postoperative colonoscopy was 12 months (range 6-20 months), conforming to Dutch guidelines. The primary outcome of the study was the yield of CRC at the surveillance colonoscopy performed 1 year after curative resection. A secondary outcome was the yield of advanced neoplasia.

Results

After a mean surveillance interval of 13.7 (±2.8) months, 10/572 patients (1.7%; 95% CI, 0.7% to 2.8%) received a diagnosis of CRC. Of these, 5 CRCs were apparently metachronous cancers (3 were stage stage III or IV) and 5 were recurrences at the anastomosis (1 was stage IV). In 11.4% of patients (95% CI, 8.9% to 13.8%), advanced neoplasia was detected at the 1-year follow-up colonoscopy. Synchronous advanced neoplasia at baseline colonoscopy was a risk factor for detection of advanced neoplasia at the follow-up colonoscopy (odds ratio 2.2; 95% CI, 1.3 to 3.8, $P \leq .01$).

Conclusion

Despite high colonoscopy quality, the yield of CRC at surveillance colonoscopy 1 year after CRC resection was 1.7%. These were metachronous CRCs and recurrences, often of advanced stage. The high yield justifies the recommendation of a 1-year surveillance interval after surgical CRC resection.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, with 1.7 million new cases diagnosed in 2015.[1] At diagnosis, approximately 80% of the CRCs is confined to the colon wall with or without regional lymph nodes, for which surgery is the optimal curative treatment option.[1] Ideally, all patients should undergo endoscopic examination of the entire colon prior to surgery in order to confirm the diagnosis of cancer by histopathology and detect synchronous neoplasia.

After curative resection, up to 13% of patients will develop a metachronous CRC or local recurrence.[2–4] Postoperative endoscopic surveillance is aimed at reducing disease-specific mortality by early detection of these cancers. Even though surveillance has become routine part of clinical practice, controversy exists on the optimal timing of the first postoperative colonoscopy, especially considering the improvement in colonoscopy quality that has been made over recent years. Because randomized trials are lacking, the surveillance intervals recommended by guidelines vary from 1 to 5 years after resection.[5–7] In the Netherlands, previously a surveillance colonoscopy interval of three years was recommended. Because of evidence suggesting that the risk of CRC is highest during the first three years after initial diagnosis, the guideline was adapted in 2013 to endorse a 1-year surveillance interval.[5,8,9] In a recent review of 31 studies (1990-2014) this increased post-surgery risk of CRC was confirmed, finding that approximately 30% of the CRCs was detected within two years after resection.[6]

The implementation of the more stringent 1-year surveillance interval inevitably leads to a higher rate of surveillance colonoscopies. This is not only burdensome for the patients, but also puts pressure on the available colonoscopy capacity. In order to judge whether this patient and healthcare burden is justified, we deemed it important to evaluate the outcome of the new guideline in a recent cohort with current colonoscopy technology and quality indicators. The aim of this study was to assess the yield of metachronous CRCs and recurrent CRCs at 1-year surveillance colonoscopy.

Methods

Study population

In this retrospective cohort study, records of patients having undergone a curative surgical resection of a first CRC between June 2013 (implementation date of the new Dutch guideline) and April 2016 were checked for eligibility in four hospitals in the Northwest region of the Netherlands. Two of these hospitals represented tertiary referral centers and 2 were large secondary referral centers. Patients with an indication for 1-year surveillance colonoscopy according to the Dutch guideline were included and comprised patients who 1) underwent a complete preoperative colonoscopy with caecum intubation and clearance of all synchronous

lesions, 2) underwent an incomplete preoperative colonoscopy followed by a CT colonography without synchronous lesions, or 3) underwent an incomplete preoperative colonoscopy followed by surgical resection of the non-inspected colonic segment.[5] The interval between the preoperative colonoscopy and postoperative colonoscopy was confined to 6-20 months; the lower limit being based on the definition of metachronous CRC[10] and the upper limit chosen to allow inclusion of patients with neoadjuvant treatment. Patients were excluded when they underwent proctocolectomy, or were diagnosed with hereditary CRC syndrome (such as Lynch syndrome and familial adenomatous polyposis), inflammatory bowel disease or colorectal tumors of non-adenocarcinoma histology (*i.e.* neuroendocrine tumors, squamous cell carcinomas or soft connective tissue sarcomas).

The study was evaluated by the ethical board of North-Holland. The Medical Research Involving Human Subjects Act (WMO) was not applicable to this study, since data for this study were collected retrospectively and no additional patient interventions were undertaken.

Data collection

From the hospital files, data were collected on patient demographics and characteristics of the baseline CRC. All tumors located in or proximal to the splenic flexure were considered proximal, in the descending colon, sigmoid or rectosigmoid distal and in the rectum rectal.[8] The exact location of the rectal tumor was determined based on endoscopy and (MRI) imaging. Stage I and II tumors were classified as 'early' and stage III and IV tumors as 'advanced'. The medullary variant of colorectal adenocarcinoma was classified under the well/moderately differentiated subclass and the signet ring cell variant under the poorly differentiated subclass.

Of the preoperative colonoscopy, quality parameters such as bowel preparation and caecum intubation were recorded, together with the endoscopic and histologic characteristics of the synchronous lesions. For preoperative colonoscopies that were performed in one of the participating centers, the endoscopists' adenoma detection rates (ADRs) were not available for colonoscopies they performed within the regular program. Most endoscopists performing the baseline colonoscopies were, however, qualified to perform screening colonoscopies. Within the screening program, their ADRs were available. Therefore, the screening-derived ADR was used as general measure for the quality of endoscopists' performance for all colonoscopies the specific endoscopist performed. Measures of bowel preparation used in the different hospitals were dichotomized into 'good bowel preparation' when Boston Bowel Preparation Score (BBPS) ≥ 6 or Ottawa Score ≤7 and 'poor bowel preparation' when BBPS <6 or Ottawa Score >7. Regarding baseline surgery, all patients had an R0 resection. All hospitals fulfilled the standards of surgical care, including volume requirements and participation in the national audit that monitors surgical outcome.[11,12] The surveillance interval was calculated from the dates between the pre- and postoperative colonoscopy.

For the postoperative colonoscopy, the same quality parameters and lesion characteristics were recorded as described for the preoperative colonoscopy. In case CRC was detected, detailed available information on the tumor, subsequent treatment and molecular profile was collected.

The cancers were classified as metachronous CRC or recurrent CRC. Metachronous CRC was defined according to the criteria of Moertel *et al*. as 1) a pathologically proven adenocarcinoma, 2) distinctly separated from the previous line of anastomosis, and 3) diagnosed at a minimal interval of 6 months after the baseline carcinoma.[10] Because it is likely that these cancers, detected only 1 year after the baseline colonoscopy, also include synchronous missed lesions, they are referred to in the present study as 'apparently metachronous CRC'. Recurrent CRC was defined as a histologically proven adenocarcinoma occurring at the site of the anastomosis. Even though recurrences are generally not restaged, for this study also these cancers were classified according to the TNM-classification.
Data collection was performed according to the Dutch Personal Data Protection Act, using a database validated to global regulatory standards (*i.e.* OpenClinica, Waltham, MA, USA).

Outcome measures

The primary outcome of the study was the yield of apparently metachronous and recurrent CRCs at the 1-year surveillance colonoscopy after curative resection.
Secondary outcome was the yield of advanced neoplasia. Advanced neoplasia included (apparently metachronous and recurrent) CRCs, advanced adenomas (adenoma ≥10mm or with high grade dysplasia or a villous component) and advanced serrated polyps (hyperplastic polyp, sessile serrated lesion, or traditional serrated lesion ≥10mm or with dysplasia).

Study size

The primary aim of this study was to evaluate the yield of CRCs at the 1-year surveillance colonoscopy after surgical resection of a first CRC. In Europe, the CRC yield of primary colonoscopy screening is 0.5%, which was used as benchmark for the current number needed to scope to detect 1 CRC.[13] The alternative hypothesis was that CRC yield would be at least as high as 0.5%. The expected CRC yield in the increased risk surveillance population was 1.5%.[8,9,14] Based on these numbers, we needed a sample size of 571 individuals to achieve 80% power to reject this null hypothesis. The power analysis was done using R-software version 2.14 (R studio, Boston, MA, USA) with the pwr library, assuming α=.05 (type I error) and β=.20 (type II error) for the 1 sample binomial test (1 sided).

Statistical analyses

The proportion of CRC and advanced neoplasia detected at the 1-year surveillance colonoscopy in the entire population was determined. Based on clinical knowledge and literature, potential baseline risk factors associated with the detection of advanced neoplasia at follow-up were selected. The factors age and surveillance interval were categorized into ordinal (<60, 60-69, and ≥70 years) and binary variables (≤12 months and >12 months), respectively. For each risk factor, the univariate association with advanced neoplasia at follow-up was calculated and presented as an odds ratio (OR) with 95% confidence intervals (CI). Multivariable logistic regression was used to assess the adjusted associations between these risk factors

and advanced neoplasia at follow-up. Those risk-factors with a *P*-value <.2 in the univariate analysis were included in the multivariable analysis. Missing data were assumed to be missing at random. Multiple stochastic imputation using a full multivariable model was carried out to adjust for missing data. Analyses were performed using ten imputed datasets with library mice, R-software version 3.4.3.

Results

Patient characteristics

From the 2074 identified candidates that underwent a curative CRC resection, 574 patients were enrolled in the database (Figure 1). During analysis, an additional 2 patients were excluded, because of local resection by transanal endoscopic microsurgery (TEM) as treatment for the baseline CRC. In the final cohort of 572 patients were included, 54.9% being male, with a mean age of 66.2 (±9.9) years and mean surveillance interval of 13.7 (±2.8) months (Table 1).

From 467/572 (81.6%) patients, the baseline colonoscopy was performed in 1 of the 4 participating centers. Approximately a quarter of the patients was referred for colonoscopy through the national screening program using fecal immunochemical testing (FIT) (Table 1). Most of these colonoscopies (410/467 (87.8%)) were carried out by endoscopists that were qualified for screening. The ADR for these FIT positive colonoscopies was 65.1% (range 58.2-71.7) and used as general measure of the endoscopists' colonoscopy performance.

The quality of the bowel preparation was scored as good in 88.8% of endoscopy reports. For those cases in which caecum intubation could not be performed (13.8%), either the non-inspected part was resected during surgery (64.6%) or the colonoscopy was complemented by means of a CT colonography (35.4%). The CRCs detected at baseline colonoscopy were mostly early staged cancers (57.5%), located in the distal colon or rectum (61.4%). Besides CRC, the most advanced synchronous lesion during baseline colonoscopy was advanced adenoma in 25.7% and advanced serrated polyps in 1.2% of patients (Table 2).

Baseline findings

Of the 5 patients with apparently metachronous CRC at 1-year surveillance colonoscopy, 3 patients had stage I or II CRC at baseline (Table 3). These cancers were located proximal to the splenic flexure in 4/5 patients and treated with right-sided hemicolectomy. The resection margins of all tumors were clear.

During the preoperative colonoscopy, caecum intubation was performed in 3/5 patients. In the other 2 patients the colonoscopy was followed by right-sided hemicolectomy including the non-inspected part. Bowel preparation was poor in 1 patient.

In 1/5 patients a synchronous advanced adenoma was resected *in toto* at the preoperative colonoscopy in the same segment as the apparently metachronous cancer was detected 1 year later. Clear resection margin of this adenoma was explicitly stated in the pathology report.

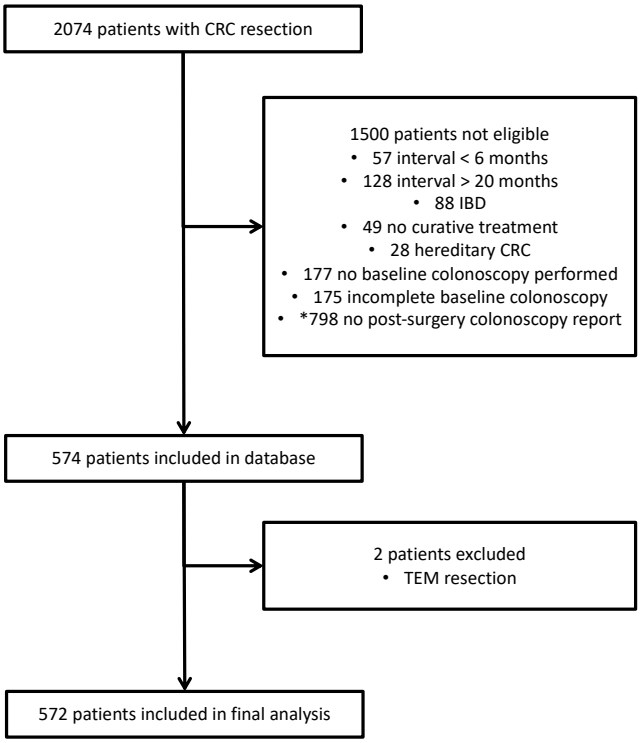

Figure 1 | Flowchart of study population

*Including patients that had not yet undergone surveillance colonoscopy, were referred to different hospital for surveillance, were not amenable for curative treatment or underwent resection for other reasons than CRC. CRC = colorectal cancer, IBD = inflammatory bowel disease, TEM = transanal endoscopic microsurgery

Table 1 | Baseline characteristics

PATIENT DEMOGRAPHICS	
Male, n (%)	314 (54.9)
Age at diagnosis [years], mean (s.d.)	66.2 (9.9)
Surveillance interval [months], mean (s.d.)	13.7 (2.8)
FIT screening*, n (%)	138 (24.1)
Hospital of surgery‡, n (%)	
Hospital 1	25 (4.4)
Hospital 2	110 (19.2)
Hospital 3	295 (51.6)
Hospital 4	142 (24.8)
BASELINE CRC CHARACTERISTICS	
Diameter [mm], mean (s.d.)	36.8 (±21.8)
Location, n (%)	
Proximal	221 (38.6)
Distal	179 (31.3)
Rectal	172 (30.1)

Table 1 | Continued

Differentiation, n (%)	
Good/moderate	462 (80.8)
Poor	48 (8.4)
Mucinous	41 (7.1)
ypT0/not evaluable	20 (3.5)/1 (0.2)
T stage , n (%)	
(yp)T0	20 (3.5)
T1	77 (13.5)
T2	117 (20.4)
T3	315 (55.1)
T4	43 (7.5)
N Stage, n (%)	
N0	332 (58.0)
N1	151 (26.4)
N2	89 (15.6)
M Stage, n (%)	
M0	557 (97.4)
M1	15 (2.6)
Overall Stage, n (%)	
Early (stage I/II)	329 (57.5)
Advanced (stage III/IV)	243 (42.5)
Type of resection, n (%)	
Ileocecal resection	1 (0.2)
Extended colectomy	1 (0.2)
Right hemicolectomy	194 (33.9)
Transverse colon resection	8 (1.4)
Left Hemicolectomy	43 (7.5)
Sigmoid resection	115 (20.1)
Total mesorectal excision	210 (36.7)
Synchronous CRC, n (%)	21 (3.7)
BASELINE COLONOSCOPY	
Bowel preparation, n (%)	
Good	406 (88.8)
Poor	51 (11.2)
Missing	115
Cecal intubation, n (%)	
Yes	493 (86.2)
No	79 (13.8)

*Patients undergoing baseline colonoscopy after a positive fecal immunochemical test (FIT) as part of the national screening program for colorectal cancer.

‡Hospital 1 and 2 represent tertiary referral centers and hospital 3 and 4 large secondary referral centers.

Table 2 | Lesions detected at baseline colonoscopy and at follow-up (n=572)

	BASELINE COLONOSCOPY	SURVEILLANCE COLONOSCOPY
	Most advanced lesion besides baseline CRC, n (%)	Most advanced lesion, n (%)
CRC	n.a.	10 (1.7)
Advanced neoplasia		
Advanced adenomas	147 (25.7)	37 (6.5)
Advanced serrated polyps	7 (1.2)	18 (3.2)
Total	154 (26.9)	55 (11.4)
Non-advanced neoplasia		
Non-advanced adenomas	57 (10.0)	94 (16.4)
Non-advanced serrated polyps	26 (4.6)	43 (7.5)
Total	83 (14.6)	137 (23.9)
No neoplasia		
Non-neoplastic or no lesions	335 (58.5)	370 (64.7)

Yield of colorectal carcinomas at the 1-year surveillance colonoscopy

In 10/572 patients (1.7%; 95% CI, 0.7% to 2.8%, Table 2) CRC was detected. These included 5 apparently metachronous cancers and 5 recurrences at the site of the anastomosis. The mean age at baseline of these 10 patients was 73.1 years (±7.5), which was significantly higher than that of the total study-population (independent t-test, *P*=.03).

Apparently metachronous colorectal carcinomas

Surveillance findings

Three of the 5 apparently metachronous cancers were stage III and IV cancers (Table 3). Two of these qualified for palliative treatment only due to peritoneal metastases. One tumor was a T1 cancer that was treated with TEM. One of the 5 apparently metachronous CRCs occurred in the proximal colon, 3 in the distal colon and 1 in the rectum.

Molecular analysis was performed on tumor tissue of 4/5 cancers. The baseline tumor of 1 patient was MSI-high due to hypermethylation of the MLH1 promotor region, while the tumor of the apparently metachronous cancer of this patient was MMR proficient. In none of the other cancers microsatellite instability (MSI) or loss of expression of the mismatch-repair (MMR) proteins was found.

Recurrent colorectal carcinomas

Baseline findings

Of the 5 patients with recurrent CRC at the surveillance colonoscopy, 3 had stage I or II CRC at baseline (Table 3). One right-sided cancer was treated with right-sided hemicolectomy, 1 cancer in the splenic flexure with left-sided hemicolectomy, 1 sigmoid cancer with sigmoid resection and 1 rectosigmoid cancer and 1 rectal cancer with neoadjuvant (chemo)radiotherapy

Table 3 | Characteristics of patients with colorectal carcinomas detected at 1-year surveillance colonoscopy

	GENERAL				BASELINE FINDINGS								SURVEILLANCE FINDINGS					
					CRC characteristics					Colonoscopy			CRC characteristics					Colonoscopy
	Number	Age*	Family history CRC	Surveillance interval (months)	Location	Differen-tiation	TNM stage	Treatment	Resection margin / Molecular analysis	Lesions	Cecum intubation	Bowel preparation	Location	Differentiation	TNM stage	Treatment	Molecular analysis	Lesions
Apparently metachronous CRCs	1	82	negative	16	ascending colon	g/m	pT4N1M0	right hemicolectomy + adjuvant CT	clear / n.p.	none	no	good	sigmoid	g/m	cT3N0M1	palliative CT	-	none
	2	77	negative	13	cecum	g/m	pT3N0M0	right hemicolectomy	clear / MSI-H, MLH-1 methylation	none	yes	poor	rectum	g/m	pT1N-M0	TEM	MMR proficient	2x HP, 3mm, sigmoid
	3	66	negative	8	sigmoid	g/m	pT3N0M0	sigmoid resection	clear / n.p.	TA, 3mm, LGD, sigmoid	yes	n.i.	transverse colon	g/m	pT4N1M0	right hemicolectomy	MSS	none
	4	79	negative	13	ascending colon	g/m	pT2N0M0	right hemicolectomy	clear / n.p.	- TA, 6mm, LGD, transverse colon - HP, 8mm, sigmoid	yes	good	sigmoid	g /m	pT3N0M0	sigmoid resection	MSS	HP, 5mm, sigmoid
	5	80	n.i.	12	ascending colon	g/m	pT3N1M0	right hemicolectomy	clear/ MMR proficient	- TVA, 25mm, LGD, descending colon - TVA, 25mm, LGD, sigmoid	no	n.i.	descending colon	g/m	pT4N1M1	palliative subtotal colectomy	BRAF mutation, no other mutations	none
Recurrent CRCs	6	81	n.i.	15	rectosig-moid	g/m	ypT3N0M0	neoadjuvant RT + TME	clear / n.p.	none	yes	good	rectum	g/m	pT2N0M0‡	APR	-	none
	7	66	FD age 74	6	sigmoid	g/m	pT2N0M0	sigmoid resection	clear/ n.p.	TVA, 9mm, LGD, cecum	yes	good	rectum	g/m	ypT3N0M0‡	neoadjuvant CRT + TME	-	none
	8	70	FD age 65	18	rectum	g/m	ypT3N1M0	neoadjvant CRT + TME	clear / n.p.	none	yes	good	rectosig-moid	g/m	pT4N0M0‡	sigmoid resection	-	none
	9	69	negative	11	ascending colon	signet ring cell	pT4N2M0	right hemicolectomy	clear (retroperitoneal n.e.) / MSS	none	no	good	transverse colon	signet ring cell	pT4N2M1‡	palliative resection with ileotransverse colostomy + CT	-	none
	10	61	n.i.	13	splenic flexure	mucinous	pT4N0M0	left hemicolectomy + adjuvant CT	clear / MMR proficient	none	no (+CTC)	good	transverse colon	mucinous	pT4N0M0‡	multivisceral resection + proximal colon resection	-	none

*Age at diagnosis of the first CRC. ‡Although restaging is not performed for recurrent cancer, TNM stage is provided to indicate the degree of tumor advancement. APR = abdominoperineal resection, FD = first degree family member, CT = chemotherapy, CTC = computed tomography colonography, CRT = chemoradiotherapy, g/m = good/moderate, HP = hyperplastic polyp, LGD = low grade dysplasia, MSI = microsatellite instable, MSI-H= MSI-high, MSS= microsatellite stable, MMR = mismatch repair, n.i. = no information, n.p. = not performed, RT = radiotherapy, TA = tubular adenoma, TEM = transanal endoscopic microsurgery, TME = total mesorectal excision, TVA = tubulovillous adenoma.

During the preoperative colonoscopy, the caecum was reached in 3/5 patients. In 1 of the 2 remaining patients, colonoscopy was followed by a right-sided hemicolectomy and in the other patient a CT-colonography was performed. The quality of the bowel preparation at baseline colonoscopy was good in all 5 patients.

Surveillance findings

One of the 5 CRCs recurring at the site of the anastomosis was a stage IV signet ring cell carcinoma in the transverse colon with lymph node and peritoneal metastases for which palliative chemotherapy was started (Table 3). The remaining 4 patients underwent curative resection. Two of these recurrent cancers were located in the rectum and 1 in the rectosigmoid. The other recurrence was located in the transverse colon and of mucinous subtype, in concordance with subtype of the baseline tumor.

Upon detection of the recurrent CRC, molecular analysis was performed retrospectively on tumor tissue of 2/5 index CRCs. Both primary tumors were microsatellite stable (MSS) / MMR proficient.

Yield of advanced neoplasia at surveillance

Advanced neoplasia was detected at 1-year surveillance colonoscopy in 65/572 patients (11.4%; 95% CI, 8.9% to 13.8%) (Table 2). In addition to the 10 (1.7%) patients with CRC described above, these consisted of 37 (6.5%) patients with advanced adenomas and 18 (3.2%) patients with advanced serrated polyps as the most advanced lesion. Additionally, 137 (23.9%) patients had non-advanced adenomas or non-advanced serrated polyps at follow-up.

Risk-factors for advanced neoplasia at surveillance

Risk factors for the detection of advanced neoplasia at 1-year surveillance colonoscopy are presented in Table 4. Univariate analysis showed a significant association with advanced neoplasia at follow up in patients who had synchronous advanced lesions next to the CRC at baseline colonoscopy (OR 2.5; 95% CI, 1.5 to 4.2, $P \leq .001$).

Based on P-value <.2 the risk factors sex, age, stage, bowel preparation (good-poor) and synchronous advanced lesions at baseline colonoscopy, were included in the multivariable logistic regression analysis. Only the detection of synchronous advanced lesions at baseline colonoscopy was independently associated with an increased risk of having advanced neoplasia at follow up (OR 2.2; 95% CI, 1.3 to 3.8, $P \leq .01$). Although not significant, poor bowel preparation during the baseline colonoscopy also tended to increase the risk for advanced neoplasia at follow up (OR 1.9; 95% CI, 0.9 to 3.7, $P = .08$).

Table 4 | Baseline risk factors for detection of advanced neoplasia at 1-year surveillance colonoscopy

	Patients with AN at follow-up	Patients without AN at follow-up	Univariate OR (95% CI)	p-value	Mutivariable OR (95% CI)	p-value
PATIENT DEMOGRAPHICS	**n=65**	**n= 507**				
Male (%)	41 (63.1)	273 (53.8)	1.5 (0.9 to 2.5)	0.19	1.4 (0.8 to 2.4)	0.24
Population screening (%)	17 (26.2)	121 (23.9)	0.9 (0.5 to 1.6)	0.65		
Age (%) <60 years	7 (10.8)	109 (21.5)	reference	0.12	reference	0.13
60-69 years	33 (50.8)	228 (45.0)	2.3 (1.0 to 5.9)		2.0 (0.9 to 5.2)	
≥70 years	25 (38.5)	170 (33.5)	2.3 (1.0 to 5.7)		2.0 (0.9 to 5.0)	
Surveillance interval >12 months (%)	43 (66.2)	336 (66.3)	1.0 (0.6 to 1.7)	1.00		
BASELINE CRC CHARACTERISTICS						
Diameter in mm; mean (s.d.)	39.1 (±18.7)	36.5 (±22.2)	1.0 (0.9 to 1.0)	0.37		
Location (%) Proximal	26 (40.0)	195 (38.5)	reference	0.20		
Distal	25 (38.5)	153 (30.2)	1.2 (0.7 to 2.2)			
Rectal	14 (21.5)	159 (31.4)	0.7 (0.3 to 1.3)			
Advanced Stage (%)	22 (33.8)	221 (43.6)	0.7 (0.4 to 1.1)	0.14	0.7 (0.4 to 1.2)	0.19
Synchronous CRC (%)	1 (1.5)	20 (3.9)	0.4 (0.1 to 2.9)	0.50		
BASELINE COLONOSCOPY						
Poor bowel preparation (%)	10 (15.4)	41 (8.1)	2.0 (0.9 to 4.2)	0.11	1.9 (0.9 to 3.7)	0.08
CT colonography	5 (9.2)	23 (5.1)	1.8 (0.6 to 4.8)	0.23		
Synchronous advanced lesions (%)	29 (44.6)	125 (24.7)	2.5 (1.5 to 4.2)	≤0.001	2.2 (1.3 to 3.8)	≤0.01

AN = advanced neoplasia, *i.e.* colorectal cancer, advanced adenomas and advanced serrated polyps, CI = confidence interval, CRC = colorectal cancer, CT = computed tomography, OR = odds ratio.

Discussion

In this retrospective cohort study, the detection rate of CRC 1 year after curative CRC resection was 1.7%, corresponding to a number of 59 colonoscopies needed to detect 1 CRC. Half of the cancers was apparently metachronous and the other half recurrent, of which in total 40% was of advanced stage. Advanced neoplasia was detected in 11.4%. Synchronous advanced lesions at baseline colonoscopy was identified as risk factor for having advanced neoplasia at follow up.

The results of this study show that CRC risk after curative resection remains high. Earlier colonoscopy surveillance studies reported CRC incidence rates of 1.3-1.4%[15,16] 1 year after surgery. The slightly higher rate in the present study might be due to the larger sample size of this study, thereby providing a more accurate estimation, or the larger proportion of patients with stage III and IV baseline tumors in our cohort.

The baseline colonoscopies were performed recently (2013-2016), in the era of heightened awareness about the importance of high colonoscopy quality. All patients had complete clearance of the entire colon still *in situ* after CRC resection and the majority (88.8%) had good fecal cleaning at baseline colonoscopy. The mean screening-derived ADR after positive FIT, that was used as general measure for the quality of the endoscopists' performance for colonoscopies in- and outside the screening setting, was high (65.1%, range 58.2-71.7). Recently, the United States Multi-Society Task Force recommended an ADR benchmark of >45% (males) and >35% (females) for FIT positive colonoscopies.[17] The ADRs of all endoscopists in the current cohort were well above these thresholds.

Above factors point towards generally high colonoscopy quality in the studied cohort. For this reason, the 11.4% yield of advanced neoplasia detected only 1 year after examination is striking. Even though colonoscopy is the gold standard for the detection and removal of colonic lesions, it is known that lesions are missed.[18] As a consequence, missed lesions account for a great proportion of the post-colonoscopy colorectal cancers.[19] In the current study, 5 metachronous CRCs were detected. Even though aggressive tumor biology resulting in quick tumor progression from a previously benign lesion cannot be ruled out, more likely these cancers represent missed CRCs at baseline colonoscopy. Thus, the CRC miss rate could be calculated as the number of missed CRCs (n=5) proportionate to the total number of CRCs present at baseline (*i.e.* 572 CRCs + 21 synchronous CRCs + 5 apparently metachronous CRCs), equal to 0.8%. Compared to the 2.1% miss rate of large adenomas, this rate is relatively high.[18] Explanations include fecal contamination at baseline due to tumor obstruction, or endoscopist-related factors, like distraction or a quick withdrawal once a CRC is detected. These points are supported by the observation that most of the apparently metachronous CRCs were found distal from the index cancer.

For all 5 cases with recurrent CRC, resection margins showed complete resection of the baseline tumor. Two patients had poorly differentiated tumors (*i.e.* 1 mucinous adenocarcinoma and 1 signet-ring cell carcinoma), associated with a poorer prognosis.[20] In 2 other patients the tumor was located in the rectum with a known higher local recurrence.[6] Four out of 5 recurrences were found primarily at surveillance colonoscopy. For the remaining case, MRI of the rectum was performed for suspicion of a rectovaginal fistula and revealed a rectal mass, which was followed by colonoscopy confirming recurrent cancer. From this we may conclude that colonoscopy is an important modality in the surveillance after CRC resection.

For the 6.5% patients with advanced adenomas and 3.2% with advanced serrated polyps at follow up, the surveillance interval was timely, since early detection and removal prevented them from potential malignant progression. The finding that synchronous advanced neoplasia at baseline colonoscopy was a risk factor for advanced neoplasia at follow up is in agreement with previous studies.[8,14] This may be related to patient-specific factors, such as genetic make-up or life-style. However, also here lesions may have been missed, as is illustrated by the finding that patients with advanced neoplasia at surveillance almost twice as often had poor bowel preparation at the pre-operative colonoscopy, compared to those without advanced

neoplasia. Previous studies show a miss rate of 22% for polyps.[18] This again emphasizes the need for meticulous inspection of the entire colon at baseline.

Besides quality of the colonoscopy procedure, also guideline adherence contributes to the success of surveillance. Previously, it has been shown that adherence to surveillance guidelines is low.[21] Indeed, the 128 patients that underwent colonoscopy with a surveillance interval of more than 20 months and a proportion of the 798 patients without post-surgery report (Figure 1), did not adhere to the surveillance protocol. This may have led to a missed opportunity to detect CRC and therefore to underestimation of the CRC yield in the current study. The recent introduction of the surveillance guideline, recommending a 1-year instead of 3-year surveillance interval, may be partly responsible for the lack of adherence.

This study has several limitations. Because of the retrospective design, confounding by indication can have caused high-risk patients to be referred for earlier surveillance colonoscopy. Furthermore, our definition to classify a surveillance cancer as metachronous or recurrent was based on the tumor location in relation to that of the first primary cancer, but not on molecular profiling.

Nevertheless, we believe this study provides a valid estimation of the CRC risk 1 year after curative resection. Before start of the study a power calculation was performed and a database was designed for collection of relevant variables. In addition, it was ascertained that all patients complied to the 1-year surveillance recommendation. Furthermore, all patients were recruited in a recent and short time period, giving an up-to-date estimation of the CRC risk, minimally distorted by changes in endoscopic and surgical techniques or oncological treatment. Lastly, a predefined distinction was made between apparently metachronous and recurrent cancers, which is important for interpreting the possible etiologies.

In conclusion, the CRC risk 1 year after curative CRC resection remains high, despite improvement in colonoscopy standards over the last decade. The high yield of 1.7% CRCs at justifies the recommendation of a 1-year surveillance interval. Future research should further investigate the procedural and biological factors responsible for this finding, as to optimize the effectiveness of the post-CRC surveillance program.

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EPILOGUE

“It always seems impossible until it’s done”

Nelson Mandela

10.

Thesis summary and future perspectives

Thesis summary

Colorectal cancer (CRC) is a large health problem worldwide. Early detection through screening and surveillance aims to reduce CRC burden by diagnosing cancers in a curable stage and removing precancerous lesions. While screening is aimed at the general risk population, surveillance is aimed at the increased-risk population.
In the current surveillance strategy, individuals at increased risk are monitored with regular colonoscopies. There are however several problems assiociated with this program. Firstly, discussion exists on the added value of colonoscopy surveillance. On the one hand, multiple epidemiological studies have shown that the risk of CRC is lower in patients who are in surveillance programs compared to patients without surveillance. On the other hand, these data mostly stem from the pre-screening era. The question has arisen whether the value of surveillance is maintained on top of screening. Secondly, the colonoscopies are a burden on patients. Patients typically experience bowel preparation as unpleasant and also the procedure itself can be uncomfortable or painful. Besides, colonoscopy is an invasive procedure and leads to complications in a small proportion of cases. Thirdly, the program is a burden on society. With the implementation of screening programs around the world, more individuals with polyps are being diagnosed. As a consequence surveillance consumes an increasing proportion of endoscopy and health care resources. Considering advanced neoplasia is only found in around 10% of surveillance colonoscopies, many colonoscopies are performed without clear benefits. Lastly, during colonoscopy generally all precursor lesions are detected and removed, while only an estimated 5% of these lesions would have eventually progressed to malignancy. This can be regarded as overtreatment.
In this thesis I addressed the issues outlined above, aiming to improve current surveillance strategies for early detection of CRC. I have sought to achieve this by improving our understanding of the molecular changes that define the different stages of CRC progression and by validating a clinically applicable tool for the detection of these changes (**part I**). In addition, I have clinically evaluated different surveillance strategies, focussing especially on the potential of stool tests to select patients for colonoscopy (**part II**).

Part I

Chapter 2 is a review presenting the genomic changes occurring during colorectal adenoma-to-carcinoma progression. Understanding biology has led to the discovery of molecular markers that can be used for early detection of CRC in stool and blood specimens. Efforts are ongoing to develop new tests that are both accurate and clinically applicable with respect to costs, user-friendliness and logistics. The perceived effectiveness of new tests also depends on the accuracy of the intermediate endpoint used. Progression biomarkers, such as somatic DNA copy number alterations (SCNAs), may provide a more precise measure for the identification

of high-risk adenomas than the currently used phenotypical features size, grade of dysplasia and villous histology that define advanced adenomas. Also in other cancer types, insights in the genomic basis of progression have enhanced early detection strategies.

In **chapter 3** a novel method for the detection of SCNAs was validated. By amplifying specific genomic loci using a single primer pair according to the FAST-SeqS method and analysing the data with the associated tool 'conliga', we were able to generate SCNA profiles that maintained high-resolution SCNA information. With the FAS-SeqS approach, the complicated and expensive library preparation steps associated with whole genome sequencing (WGS) were replaced by a two round polymerase chain reaction (PCR). This resulted in reduced preparation time and lower costs compared to WGS techniques. FAST-SeqS was able to detect SCNAs also in samples with low tumour purity. FAST-SeqS could have potential to be applied in the clinic, for example in patient screening and surveillance for cancer.

In **chapter 4** the natural behaviour of colorectal lesions, including adenomas and serrated polyps, was studied. For this, we had access to a unique set of small polyps with longitudinal follow up data over a period of approximately 3 years. On these polyps a comprehensive analysis of DNA alterations was performed. We found that higher polyp growth rates were related to presence of non-random SCNAs associated with adenoma-to-carcinoma progression (cancer associated events or CAEs), as well as to increased mutation burden. Regressed lesions did not have CAEs, but did harbour some mutations, which concerned mostly APC mutations, an early event in adenoma genesis. Altogether this longitudinal study provides *in vivo* support in the human setting for the functional role of these molecular alterations, that mostly have been identified by cross sectional observations in tissue samples of colorectal adenomas and cancers.

Part II

Chapter 5 provided an overview of the effectiveness of primary screening colonoscopy in reducing CRC incidence and mortality. Studies have shown that the effect of colonoscopy screening is more pronounced for distal cancers than proximal cancers. Indeed, interval cancers are predominantly located in the proximal colon. This may be due to unfavourable tumour characteristics, but also to potentially avoidable factors relating to colonoscopy quality. Surveillance programs are in place to enhance the protective effect of screening. The so named 'high-detection paradox' refers to the detection of more diminutive and small polyps due to improved colonoscopy quality and imaging techniques, leading to an increasing number of surveillance colonoscopies. This trend emphasises that the optimal interval between surveillance examinations for different patient categories requires re-examination.

In **chapter 6** we outlined a large cross-sectional study which started in 2015 and is still ongoing in multiple centres throughout the Netherlands. The aim of the MOCCAS (Molecular stool testing for Colorectal CAncer Surveillance) study is to evaluate whether stool testing

could be used in the surveillance setting as a triage method to select patients with advanced neoplasia for therapeutic colonoscopy. For this, surveillance patients are asked to collect stool specimens for the multitarget stool DNA (mt-sDNA) test and two FITs (OC Sensor and FOB Gold), prior to their scheduled colonoscopy.

The interim results of the MOCCAS study were presented in **chapter 7**. We found that mt-sDNA had a higher sensitivity for the detection of advanced neoplasia than FIT. Lowering the screening cut-offs to reach 50% positivity rate, increased the sensitivity of the mt-sDNA test and FITs considerably, thereby reducing the risk of missing advanced neoplasia. In this scenario, a single round of mt-sDNA testing missed 24% of advanced neoplasia, while FIT missed a significantly higher proportion of 40%. Mathematical modelling approaches will be applied to the final study data in order to determine an optimal stool-based strategy for surveillance.

Despite the higher sensitivity of the mt-sDNA test, the test is also more expensive, more complicated to perform and comes with logistic challenges as it requires whole stool samples. Therefore, in chapter 8 we tested whether the performance of FIT could be improved by complementation with clinicopathological risk factors. In addition to performing stool collection, all patients included in the MOCCAS study filled in a questionnaire with risk factors for CRC. The questionnaire data were combined with the FIT result and historical colonoscopy findings to update a model that was previously developed in the screening setting to predict the risk of advanced neoplasia. The updated model included FIT result, age, calcium intake, smoking habits, (tubulo)villous adenoma in previous colonoscopy and large lesions in previous colonoscopy. Application of the updated model improved the performance of the FIT in the detection of advanced neoplasia significantly. At equal positivity rate of 50%, the sensitivity of FIT only was 68% compared to 75% when applying the model.

In **chapter 9** the patient category undergoing surveillance after curative surgical CRC resection was studied more closely. These patients are currently recommended to have their first surveillance colonoscopy already one year after CRC resection. However, there is controversy whether the one-year interval between pre- and postoperative colonoscopy is indeed justified, due to improved colonoscopy quality standards. Despite confirmed high quality colonoscopies, we found that the yield of CRC was 1.7% one year after resection of the first CRC. The detected cancers included metachronous, as well as recurrent tumours. Considering the late stage of apparently metachronous cancers, these might actually represent CRCs that were missed during the previous colonoscopy. The high yield justifies the recommendation of a one-year surveillance interval after surgical CRC resection.

Future perspectives

Once metastasised to distant organs, cancer survival rates decrease dramatically and treatment burden and costs increase.[1] When thinking about reducing CRC mortality, the focus

should therefore not be on curing advanced cases, but rather on early disease detection. As CRC develops over a course of 10-15 years,[2] a wide window of opportunity exists for doing so. The aim of surveillance for CRC is to reduce disease incidence and mortality and to do so with a sustainable amount of medical and economical resources.[3] At present, approximately 25% of the colonoscopy capacity is consumed by surveillance. Due to screening, the burden of surveillance is likely to further increase in the near future. This results in high costs and may lead to longer waiting times for colonoscopies for this as well as other indications. This situation is problematic, especially because strong evidence for the effectiveness of surveillance for all different subgroups is lacking.[4,5] Therefore, the focus of this thesis was to improve surveillance strategies.

Surveillance starts with the performance of a high quality baseline colonoscopy with complete removal of all detected lesions. Only if quality parameters are met, patients are entered into the surveillance program. To avoid overuse of colonoscopy but still prevent CRC, the timing of the surveillance examination is essential. Currently, colonoscopy is used as a diagnostic as well as a therapeutic intervention for polyps. Stool tests have the potential to replace colonoscopy as diagnostic procedure and rather select those patients with relevant lesions for subsequent treatment with colonoscopy. Ideally, surveillance would not detect tumours when they have already become invasive, but just before, in a premalignant stage. Molecular alterations could be used to more precisely pinpoint premalignant lesions that are at high risk of progression. Below, all of these issues are considered in more detail.

Quality of baseline colonoscopy

The effectiveness of colonoscopy for the prevention of CRC depends, amongst others, on the quality of the performance.[6,7] There is no evidence that an initial poor examination can be compensated by overuse of endoscopic surveillance. For this reason, surveillance guidelines only apply to patients that have had a high quality baseline colonoscopy.[3,8]

Multiple studies have shown that missed or incompletely removed lesions contribute to the development of post-colonoscopy colorectal cancers (PCCRCs),[9,10] defined as cancers diagnosed after a colonoscopy during which no cancer was found, but before the next due surveillance colonoscopy.[11] Despite colonoscopy being the reference standard for polyp detection and removal, it misses an estimated 20% of polyps[12] and 0.8% of cancers (in the setting of synchronous CRC), as demonstrated in **chapter 9**. Reasons for missed lesions include inadequate bowel preparation and endoscopist-related factors, such as completeness of colonoscopy,[6] withdrawal time[13] and adenoma detection rate (ADR).[14] Therefore, these parameters are incorporated in colonoscopy quality standards. Because serrated polyps are notorious for being easily missed,[15,16], also the proximal serrated polyp detection rate has been proposed as autonomous colonoscopy quality parameter. More research is needed to determine the association between endoscopists' proximal serrated polyp detection rates and the risk of interval cancer. Another proposed performance indicator of colonoscopy is the Performance Indicator of Colonic Intubation (PICI). This measure combines cecal intubation

rate, comfort and use of sedation during colonoscopy. Since less skilled endoscopists might more forcefully intubate the colon and cause more pain, which especially is remembered by those patients who are less sedated, the PICI may give a good reflection of the skills of the endoscopist. PICI could be used to identify and support low-performers and for benchmarking. An estimated 10% of PCCRCs is caused by incompletely resected polyps.[10] Especially large polyps resected in a piecemeal fashion are associated with inadequate polypectomy and relatively low rate of radical resection.[17] For this reason, the Dutch and European surveillance guidelines advice endoscopic follow-up within 4-6 months after a piecemeal resection. Endoscopic submucosal dissection (ESD) enables en-bloc resection also in large polyps. Compared to piecemeal endoscopic mucosal resections (EMR), ESD however is more difficult, resulting in a longer procedure time and more complications.[18] Future cost-effectiveness studies comparing piecemeal EMR with ESD should evaluate whether the reduced recurrence rate and higher number of radical resection after ESD outweigh these drawbacks.[19]

Timing of colonoscopy

Timely colonoscopy should be offered to those patients with a substantial residual risk. So far, no randomised controlled trials have examined the effect of different surveillance intervals per risk group on long-term outcomes. It has been hypothesised that due to the improved colonoscopy quality over the last decade, current intervals recommended in the guidelines are too strict. A large-scale ongoing European trial, the EpoS-study, randomises patients from different risk categories between shorter and longer surveillance intervals.[20] After a follow-up period of 10 years, the incidence of CRC in the various randomisation arms will be compared to identify the optimal interval between colonoscopies.

Methods of surveillance

To reduce the number of patients referred for surveillance colonoscopies, stool testing could be used as a diagnostic tool to select patients at high risk for advanced neoplasia for subsequent treatment with colonoscopy. Such a triage strategy is currently applied in the Dutch FIT screening program. In surveillance it is important that test sensitivity is high, while a lower specificity might be acceptable because all these patients currently get colonoscopy. The mt-sDNA test was previously shown to have a higher sensitivity in the screening setting than FIT.[21] Therefore we hypothesised that the mt-sDNA test could be an appropriate triage test for surveillance.

Indeed, in **chapter 7** we described that the mt-sDNA test detected more advanced neoplasia than FIT in a surveillance setting. Yet, the mt-sDNA test missed 3/10 CRCs even after lowering the test cut-off, compared to colonoscopy. For perspective, the negative predictive value (NPV) of the mt-sDNA test for CRC was 98.8% and the positive predictive value (PPV) 1.4%. A previous study conducted in the surveillance setting reported that after three rounds of annual FIT, using the low cut-off of 10 µg haemoglobin (Hb)/g faeces, 28% of CRCs were missed.[22] In that study, the NPV of the FIT for CRC after three rounds was 99.8% and PPV

1.4%. While triage with stool tests could potentially reduce the number of colonoscopies, the clinical acceptability of such miss rates remains to be resolved.

Repeated testing could reduce the number of missed lesions within a certain period. This comes however with several caveats. First, missed cancers could be detected with later tests, but then the disease then may be more progressed. Second, stool test return could drop following a negative test and lead to false reassurance. Third, the program costs would rise. Because a larger number of patients would have a positive test ánd subsequent colonoscopy, the test costs need to be low to allow the strategy to be cost-effective. At the moment, costs of the mt-sDNA test are around 600 euro's, while FIT is around 20 euro. Therefore it is unlikely that repeat mt-sDNA testing would be a cost-effective strategy. Fourth, the relieve on the colonoscopy capacity would be less pronounced. To quantify these considerations, modelling studies are required to assess the long term effect of stool-based surveillance on CRC mortality and colonoscopy burden.

Instead of using stool tests with one cut-off for all, also a more tailored approach could be considered. One option is to use clinicopathological risk factors, such as age, life-style factors or previous colonoscopy findings, in addition to the FIT value, as described in **chapter 8**. The diagnostic prediction model we created included FIT result, age, calcium intake, smoking habits, (tubulo) villous adenoma in previous colonoscopy and large lesions in previous colonoscopy and was able to improve advanced neoplasia detection compared to FIT only. The model should be externally validated in a cohort of surveillance patients, before implementing this strategy in the clinic. In addition, similar efforts could be made to improve mt-sDNA test performance by developing a new prediction model. A second approach is to use the quantitative FIT value to personalise the frequency of surveillance colonoscopy.[23] It has been demonstrated that FIT values below the cut-off value are predictive for advanced neoplasia detection at follow up, in screening as well as surveillance cohorts.[23–25] This suggests that colonoscopy intervals could be lengthened relative to FIT Hb concentrations. Further modelling studies are needed to assess the risk of advanced neoplasia based on Hb and optimal time intervals. Yet another approach comes from fruits of the genomic era. Based on our increasing knowledge of DNA variants associated with CRC risk and the ease with which these now can be determined, the concept of polygenic risk scores (PRS) has been developed.[26] The potential of these PRS for personalising screening, as well as strategies require further clinical validation.

Successful implementation of stool testing in surveillance also depends on several practical matters. The test has to be user-friendly and easy to apply at large scale. Therefore, participation rates and number of analytical drop-outs should be incorporated in modelling studies in order to identify the optimal stool-based surveillance strategy.

In general, the probability of the disease not only depends on the result of the test, but also on the probability of the disease before the test was performed. For proper implementation of the stool testing in surveillance, it is essential to take the *a priori* chance of advanced neoplasia, and especially CRC, into account. In **chapter 7** we found that patients with a previous diagnosis of CRC, had a higher risk of subsequent CRC (1.7%) compared to post-polypectomy (0.4%) and familial

risk patients (zero). As outlined in **chapter 9**, this may be explained by CRCs being missed due to quicker withdrawal once a CRC is detected or maybe suboptimal bowel preparation due to a stenosing cancer. Also the background risk of the patients (high-risk genetic make-up/ life-style) may play a role. Because of the pronounced risk of the post-CRC surveillance patients, colonoscopy likely remains warranted for this population especially in the first few years after resection.[27,28] For the post-polypectomy and familial risk population with a low *a priori* chance of CRC, stool testing seems more appropriate.

Lastly patient attitude towards stool tests replacing routine colonoscopy should be considered. Surveillance-experienced patients have previously expressed concerns about the sensitivity of FIT and did therefore not endorse the idea of FIT as alternative for colonoscopic surveillance.[22,23] Whether patient preference would be different for the mt-sDNA test or is dependent on historical colonoscopy findings, needs to be evaluated.

Target for surveillance

Ideally, surveillance would target those lesions that are not yet malignant, but would have likely progressed to cancer when left *in situ*. Yet, leaving colorectal polyps in place an following them over time until they progress, is considered unethical. The standard endoscopic removal of polyps disrupts the natural behaviour of polyps and hinders the identification of the exact characteristics of these high-risk lesions. Most of the knowledge on the adenoma-to-carcinoma progression is based on cross-sectional data, comparing molecular profiles of premalignant with malignant tissue.[29–32] From these studies it can be concluded that in adenomas, chromosomal instability (CIN) occurs at a late stage and this is a critical step in progression to cancer. The role of CIN in malignant transformation has also been confirmed in mouse and human intestinal organoid models.[33–35] Instead of randomly, these chromosomal alterations arise in specific patterns.[29,31] When leading to amplification of oncogenes or deletion of tumour suppression genes, this may confer a growth advantage, as has been shown for *CDK8* on the 13q ampicon[36] and *AURKA* on the 20q amplicon.[37] Altogether, these findings suggest that SCNAs could be used to distinguish adenomas that are likely to progress from the ones that are not, i.e. the low-risk ones.

A previous study has attempted to more specifically define the regions of copy number gains and losses that could be used as adenoma progression biomarkers. In that study, the presence of two or more out of seven frequently occurring DNA copy number alterations (CAEs) distinguished adenomas with and without a malignant component with high accuracy (78% sensitivity and 78% specificity).[29] In **chapter 4** we found that these CAEs were present in adenomas that grew over time, as well as those that remained stable, but were absent in polyps that had regressed. This provides evidence for the functional role of these alterations in a clinical setting. Furthermore, when comparing to the traditionally used advanced adenoma criterion, a much smaller proportion of polyps was classified as molecular high-risk adenomas, suggesting an overestimation of the number of high-risk lesions with the current definition. Therefore, molecular high-risk adenomas could potentially be a useful measure in clinical

practice. The implementation of the concept of molecular high-risk adenomas is envisioned in several ways.

First, instead of measuring sensitivity and specificity of novel diagnostic stool tests against advanced adenomas, molecular high-risk adenomas could be applied as a more precise intermediate endpoint for CRC. Clinical use of tests that are in particular sensitive for molecular high-risk adenomas, could then reduce the number of patients being referred for colonoscopy, while still effectively reducing CRC mortality. In such a scenario, the polyp tissue resected during colonoscopy would undergo copy number profiling to identify high-risk and low-risk adenomas. The FAST-SeqS method described in **chapter 3** could provide an assay for realising copy number profiling of tissues in large cohorts of patients, as it is a simple and low-cost technique which can easily automated in a high throughput platform.

Second, molecular high-risk adenomas could be used to predict future CRC risk. A research collaboration between the Netherlands and Norway called IntEnd has recently been initiated for this purpose. In this study, DNA copy number profiling will be performed on a large retrospective series of advanced and non-advanced adenomas. Molecular high- and low-risk lesions will be related to the risk of metachronous CRC during 10 year follow-up. If indeed molecularly-defined intermediate endpoints appear to be more accurate in predicting future risk than the currently used concept of advanced adenomas, this could eventually lead to revision of surveillance guidelines.

Lastly, the changes that define molecular high-risk adenomas, *i.e.* the seven CAEs, could be used as diagnostic markers to design new diagnostic stool tests. For example, the mt-sDNA test is currently based on the detection of methylation and mutation markers, but could in the future be replaced or complemented by markers detecting CAEs. An important consideration in this respect is that the test should reliably detect very small quantities of marker analytes against large amounts of background DNA. Until recently, no reliable assay for the detection of SCNAs was available. According to the results presented in **chapter 3**, FAST-SeqS is able to detect copy number alterations in low purity samples. So, possibly, FAST-SeqS could also detect CAEs in stool samples. The analytical sensitivity however, will depend largely on the occurrence of identical LINE-1 sequences in bacterial DNA, the main constituent of stool DNA. LINE-1 sequences have been shown to be human specific[38] and our first explorations show poor alignment of the FAST-SeqS primers to the genomes of bacterial phyla most prevalent in human faeces.[39,40] Therefore, efforts investigating further CAE markers as basis for stool tests could be a meaningful next step.

As opposed to adenoma progression, the serrated pathway is not characterised by CIN, but by MSI. MSI might prove an appropriate progression marker, although it likely coincides with dysplasia in serrated polyps, from when it takes very little time to progress into cancer.[41] This indicates that the windows of opportunity to use genomic instability markers, *i.e.* CIN or MSI, are likely to differ for the traditional adenomas and serrated polyps, respectively. At the moment, research is conducted in which the whole exome, as well as the whole methylome of progressed and non-progressed serrated polyps is studied. This could help to identify appropriate progression biomarkers in serrated polyps.

The complexity of surveillance becomes clear when considering all the different aspects that need to be optimised and aligned. In this thesis, the focus has been on finding ways to reduce the colonoscopy burden of surveillance, by applying stool testing as diagnostic medium. In addition, I have sought to better understand which premalignant lesions should be the target for surveillance to prevent CRC, while avoiding overdiagnosis. Hopefully, the results of this thesis can in this way contribute to the development of new, more efficient surveillance strategies.

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“If I have seen a little further, it is because
I stood on the shoulders of others”

Isaac Newton

11.

Nederlandse samenvatting
List of publications
Curriculum vitae
Acknowledgements / Dankwoord

Nederlandse samenvatting

Darmkanker is een groot gezondheidsprobleem. In Nederland worden er ongeveer 14.000 nieuwe gevallen per jaar gediagnosticeerd en er sterven elk jaar meer dan 5.000 patiënten aan deze ziekte. Het is daarmee de op één na meest voorkomende vorm van kanker. De ziekte ontstaat uit voorloper afwijkingen, te weten adenomen en serrated poliepen, hierna samen poliepen genoemd, die naar schatting in 10 tot 15 jaar kunnen uitgroeien tot darmkanker. Dat gebeurt ongeveer in 1 op de 20 poliepen. Tijdens een inwendig onderzoek van de darm (coloscopie) kunnen deze poliepen opgespoord en verwijderd worden. Als darmkanker in een vroeg stadium gediagnosticeerd wordt, is de ziekte meestal nog zeer goed te genezen is en de belasting van de behandeling minder hoog.

In Nederland is in 2014 het bevolkingsonderzoek ingevoerd. Het doel van het programma is sterfte aan darmkanker te verminderen door zowel darmkanker in een vroeger stadium op te sporen, als darmkanker te voorkómen door het verwijderen van poliepen. In het bevolkingsonderzoek wordt gebruik gemaakt van de fecale immunochemische test (FIT), een test die bloed in de ontlasting detecteert. Als de test positief is, wordt de deelnemer een coloscopie aangeboden. Binnen het huidige bevolkingsonderzoek heeft ongeveer 5% van de deelnemers een positieve FIT uitslag. Tijdens de coloscopie wordt bij 7% van deze personen met een positieve FIT darmkanker gevonden en bij 39% gevorderde poliepen. Omdat deze personen ook na het verwijderen van die afwijkingen (samen *voortgeschreden neoplasie* genoemd) een verhoogd risico hebben op het krijgen van nieuwe poliepen, wordt hen geadviseerd zich te laten controleren en regelmatig een coloscopie te ondergaan. Het vervolgen van deze hoog-risico populatie wordt surveillance genoemd.

De huidige surveillance strategie voor patiënten met een verhoogd risico op darmkanker bestaat uit regelmatige coloscopieën. Echter zijn er een aantal problemen geassocieerd met de huidige strategie. Allereerst is er onduidelijkheid over de exacte meerwaarde van het surveillance programma. Aan de ene kant hebben meerdere epidemiologische studies laten zien dat patiënten die regelmatige coloscopie surveillance kregen een lager risico op darmkanker hadden dan de patiënten die geen surveillance ondergingen. Aan de andere kant stammen deze data uit de tijd vóór de invoering van bevolkingsonderzoeken. Of surveillance ook meerwaarde biedt naast een bestaand screeningsprogramma, is niet voldoende onderzocht. Ten tweede is de coloscopie een belasting voor de patiënt. Het is een invasieve procedure die in een klein gedeelte van de gevallen tot ernstige complicaties leidt. Bovendien ervaren patiënten de darmvoorbereiding vaak als onprettig en kan de procedure zelf oncomfortabel of pijnlijk zijn. Ten derde vormt het surveillance programma een belasting voor de samenleving. Door invoering van het landelijk bevolkingsonderzoek worden bij steeds meer personen poliepen gediagnostiseerd en verwijderd. Daarom komen ook steeds meer personen in surveillance programma's terecht en wordt een steeds groter gedeelte van de coloscopie capaciteit gebruikt voor deze surveillance coloscopieën. Dit terwijl bij slechts 10% van de surveillance coloscopieën voortgeschreden neoplasie gevonden wordt. Dat betekent dat de

beperkte coloscopie capaciteit niet maximaal effectief wordt ingezet en er veel onnodige kosten worden gemaakt. Tot slot worden met de coloscopie alle poliepen opgespoord en verwijderd. Gezien het feit dat naar schatting slechts 5% van de poliepen uit zou zijn gegroeid tot darmkanker, worden er veel poliepen onnodig behandeld.

Uit bovenstaande punten blijkt dat er ruimte is voor verbetering van de surveillance strategie. Het doel van dit proefschrift was inzichten te verschaffen in mogelijkheden tot verbetering van de surveillance strategie. Hiertoe heb ik enerzijds de moleculaire veranderingen bestudeerd die zich tijdens het uitgroeien van poliep tot kanker voordoen, en een techniek gevalideerd die deze veranderingen kan detecteren (**deel I**). Anderzijds heb ik verschillende surveillance strategieën in de klinische setting geëvalueerd, waarbij ik me in het bijzonder gericht heb op het toepassen van ontlastingstesten voor het selecteren van patiënten met voortgeschreden neoplasie voor behandeling met de coloscopie (**deel II**).

Deel I

In de review in **hoofdstuk 2** is uiteengezet hoe moleculaire kennis over de progressie van darmkanker heeft bijgedragen aan vroeg-detectie van dit type kanker. De ontdekking van biomarkers, alsmede de toenemende analytische sensitiviteit van laboratorium technieken, heeft de ontwikkeling van nieuwe diagnostische testen voor darmkanker in bloed en ontlasting gestimuleerd. Analoog aan deze benadering hebben inzichten in de onderliggende moleculaire veranderingen bij andere vormen van kanker, zoals slokdarmkanker en baarmoederhalskanker, ook geleid tot nieuwe strategieën voor vroeg detectie van deze vormen van kanker.
In **hoofdstuk 3** werd een nieuwe methode voor het detecteren van somatic copy number alterations (SCNAs) in het DNA gevalideerd. Deze methode genaamd FAST-SeqS maakt gebruik van één primer-paar om specifieke loci in het genoom te amplificeren. Analyse van de gegenereerde data vond plaats met de ontwikkelde software tool 'conliga'. Hiermee werden hoog-resolutie SCNA profielen verkregen. Ook bleek het mogelijk om SCNAs te detecteren in monsters met laag tumor percentage. Voordelen van FAST-SeqS ten opzichte van whole genome sequencing (WGS)-technieken zijn de versimpelde procedure ter voorbereiding van de DNA library en de lagere kosten. FAST-SeqS bleek een robuuste en simpele techniek, die geschikt is om toegepast te worden in de klinische praktijk, bijvoorbeeld in de screening op en surveillance van darmkanker.
In **hoofdstuk 4** hebben we het natuurlijke beloop van poliepen bestudeerd. Hiertoe hadden we beschikking over een cohort van 46 patiënten met in totaal 65 adenomen en serrated poliepen, die na detectie *in situ* waren gelaten en over een periode van ongeveer 3 jaar vervolgd waren. Aan het einde van deze periode werden alle poliepen endoscopisch verwijderd en deze weefselmonsters waren beschikbaar voor moleculaire analyse. We zagen dat de mate van poliep groei tijdens de follow-up periode gerelateerd was aan het optreden van specifieke

SCNAs (*cancer associated events* of CAEs) en het aantal mutaties in de poliep. Poliepen die in regressie waren gegaan vertoonden geen CAEs, maar wel enkele mutaties. De bevindingen van deze studie ondersteunen de functionele rol van deze moleculaire alteraties, die eerder vooral in cross-sectionele studies geïdentificeerd waren.

Deel II

Hoofdstuk 5 toonde een overzicht van het beschikbare bewijs voor de effectiviteit van de coloscopie als primaire screeningsmethode voor het reduceren van darmkanker incidentie en mortaliteit. Verschillende studies hebben laten zien dat het effect van coloscopie screening groter is voor distale dan voor proximale darmkanker. In de periode na coloscopie worden er vaker zogenaamde post-coloscopie darmkankers (PCCRCs) gevonden in het proximale colon dan in het distale colon. Een mogelijke verklaring kan zijn dat rechtszijdige tumoren een ongunstiger moleculair profiel hebben, maar ook potentieel vermijdbare factoren gerelateerd aan de kwaliteit van de uitgevoerde coloscopie spelen een rol. Surveillance programma's kunnen potentieel het beschermende effect van screening versterken. Benadrukt werd dat de verbeteringen in kwaliteit van de coloscopieën zoals deze in de afgelopen decennia heeft plaatsgevonden vragen om her-evaluatie van de huidige surveillance intervallen.
In **hoofdstuk 6** werd het studie protocol van een grote prospectieve, cross-sectionele studie uiteengezet. Deze studie wordt momenteel uitgevoerd in meerdere centra in het noordwesten en zuiden van Nederland. Het doel van deze MOCCAS (*Molecular stool testing for Colorectal CAncer Surveillance*) studie is om te evalueren of het aantal patiënten dat een surveillance coloscopie ondergaat, gereduceerd zou kunnen worden door gebruik te maken van ontlastingstesten. Hiertoe wordt de gevoeligheid van een moleculaire ontlastingstest (*multi-target stool DNA* test of mt-sDNA test) in het detecteren van voortgeschreden neoplasie vergeleken met die van FIT. Voor het afnemen van deze testen wordt aan patiënten gevraagd om voorafgaande aan hun routine surveillance coloscopie eenmalig ontlastingsmonsters te verzamelen.
In **hoofdstuk 7** werden de interim resultaten van de MOCCAS studie gepresenteerd. In de analyse werden 1.551 patiënten geïncludeerd. Uit de resultaten bleek dat de mt-sDNA test een hogere sensitiviteit had voor de detectie van voortgeschreden neoplasie dan FIT. Bij het verlagen van de cut-off om 50% test positiviteit te bereiken en dus de benodigde coloscopie-capaciteit te halveren, steeg de sensitiviteit van zowel de mt-sDNA test als de FIT. In dit scenario miste de mt-sDNA test 24% van de voortgeschreden neoplasieën, terwijl de FIT een significant hoger percentage van 40% miste. Hoewel een dergelijk hoog positiviteits-percentage in de screeningsetting niet acceptabel zou zijn, is dit in surveillance, waar coloscopie in 100% van de gevallen de huidige standaard is, mogelijk wel een optie. Modelleerstudies zijn nodig om de lange termijn effecten van dergelijke alternatieve surveillance strategieën in te schatten en de kosten en baten tegen elkaar af te wegen.

Alhoewel de FIT een lagere sensitiviteit had dan de mt-sDNA test, heeft deze test als voordelen dat het een stuk goedkoper, gebruiksvriendelijker en makkelijker te implementeren is, omdat slechts een klein monster van de ontlasting nodig is. In **hoofdstuk 8** hebben we daarom onderzocht of de prestatie van de FIT verbeterd kon worden door een aantal klinische en pathologische risicofactoren toe te voegen. Naast het verzamelen van ontlasting monsters, werd in de MOCCAS studie aan alle deelnemers gevraagd een vragenlijst met risicofactoren voor darmkanker in te vullen. Data uit de vragenlijsten werden voor deze analyse gecombineerd met de FIT resultaten en historische coloscopie bevindingen. Deze gegevens werden gebruikt om een model te herzien dat eerder in de screeningsetting ontwikkeld was om het risico op voortgeschreden neoplasie te voorspellen. De complete gegevens van 1.026 patiënten waren hiervoor beschikbaar. In het nieuwe, herziene model werden de variabelen FIT resultaat, leeftijd, calcium inname, rookgedrag, (tubulo)villeus adenoom of een grote poliep gevonden tijdens de vorige coloscopie opgenomen. Toepassing van het nieuwe model op de surveillance populatie zorgde voor een significante verbetering in de prestatie van de FIT. Bij een gelijk positiveits-percentage van 50% was de sensitiviteit van de FIT 68%, terwijl die van het model 75% was.

In **hoofdstuk 9** werd de patiëntenpopulatie die eerder een curatieve resectie voor darmkanker had ondergaan verder onder de loep genomen. De huidige richtlijn adviseert deze patiënten al één jaar na de chirurgische resectie een eerste surveillance coloscopie te ondergaan. Er is echter discussie of het interval van één jaar niet te behoudend is, onder andere omdat de kwaliteit van de coloscopieën in de afgelopen jaren sterk verbeterd is. In een retrospectieve studie met een cohort van 572 patiënten, vonden wij bij de één-jaars surveillance coloscopie bij 1.7% van de patiënten darmkanker. Het betrof hierbij lokale recidieven, maar ook metachrone kankers. Omdat de ogenschijnlijk metachrone carcinomen van een vergevorderd stadium waren, zouden het in werkelijkheid synchrone tumoren kunnen betreffen die tijdens de preoperatieve coloscopie gemist waren. De gerapporteerde hoge opbrengst rechtvaardigt de aanbeveling voor een surveillance interval van 1 jaar na chirurgische resectie van darmkanker.

Concluderend beoogt dit proefschrift bij te dragen aan het verbeteren van surveillance strategieën voor de vroeg detectie van darmkanker. Enerzijds heb ik geprobeerd inzichten te verschaffen in de moleculaire veranderingen die geassocieerd zijn met de progressie van darmkanker; anderzijds heb ik concrete alternatieve strategieën voor verschillende surveillance populaties in de dagelijkse praktijk bestudeerd. Met de verworven inzichten komen we hopelijk dichter bij de implementatie van nieuwe surveillance strategieën, die het aantal onnodige coloscopieën verminderen en de kosteneffectiviteit van surveillance verbeteren.

Curriculum Vitae

Meta van Lanschot was born on 22nd September 1988 in Boston, Massachusetts, USA. In 2006 she graduated *cum laude* from high school at the Gemeentelijk Gymnasium Hilversum. She went on to study Biomedical Sciences at the University of Edinburgh from 2006 to 2007 and started her studies in Medicine at the University of Groningen in 2007. She received her BSc degree in 2010, after which she became vice-president at the board of her student sorority Vindicat from 2010 to 2011. In 2011 she took up her MSc in Medicine, conducting junior residencies at the University Medical Center Groningen and Deventer Hospital. She performed her scientific internship in the laboratory of professor Rebecca Fitzgerald at the department of gastroenterology at the University of Cambridge in 2014. In 2015 she graduated from medical school and started a PhD research project under shared supervision of professor Gerrit Meijer at the department of pathology, Netherlands Cancer Institute (NKI) and professor Evelien Dekker at the department of gastroenterology, Amsterdam UMC, University of Amsterdam, with dr. Beatriz Carvalho as co-promotor (department of pathology, NKI). During her PhD she graduated *cum laude* from her MSc in Clinical Epidemiology at the University of Amsterdam. In August 2019 she will start working as strategist at Gupta Strategists.

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Dankwoord

Achter mij liggen een aantal zeer leerzame jaren als arts-onderzoeker, waarvan dit proefschrift de bekroning is. Dit werk was echter nooit tot stand gekomen zonder de inspanningen van heel veel mensen. Daarom wil ik op deze plek iedereen bedanken die in wat voor vorm dank ook heeft bijgedragen. Allereerst zijn dat de proefpersonen die hebben deelgenomen aan de verschillende onderzoeken die in dit proefschrift beschreven staan. Hoewel ik hen hier niet bij naam kan bedanken, zijn zij onmisbaar geweest.

Gerrit, terwijl de verhuizing naar het AvL in volle gang was, startte ik bij jou als onderzoeker. Het was indrukwekkend om te zien hoe jij wetenschappelijke taken combineerde met klinisch werk en het leidinggeven aan de afdeling. Direct werd mij duidelijk dat ik veel kon leren van jouw visie op de pathologie en research. Jij hebt mij laten zien hoe je omvangrijke projecten kunt realiseren door duidelijke doelen en termijnen te stellen. Ook heb je mij geleerd structuur aan te brengen in mijn werk. Het duurde even voordat het kwartje viel, maar ik weet nu dat alle inclusiegrafieken, planningen en voortgangsrapportages die we tijdens onze promotiegesprekken hebben doorgenomen, mij de rest van mijn leven van pas gaan komen. Ik bewonder je vaardigheid om scherpe vragen te stellen, projecten op het juiste moment naar links of rechts bij te sturen en efficiënt tot de kern van een probleem te komen. Dank voor de interessante discussies, je scherpe correcties en de kans die je mij gegeven hebt om me ook op het gebied van de epidemiologie te ontwikkelen.

Evelien, mijn promotietraject begon ik in het AMC. Al tijdens mijn sollicitatie werd ik aangestoken door je enthousiasme en energie. Je liet me direct zelf aan de slag gaan met het opzetten van een grote studie en mij tijdens internationale congressen en bijeenkomsten proeven aan alle facetten van onderzoek. Ook jouw gevoel voor stijl kan ik erg waarderen. We hebben de afgelopen jaren veel complimenten uitgewisseld over rokjes, truitjes, oorbellen en kussentjes. En de promotiegesprekken zijn niet meer hetzelfde sinds ik maandelijks plaats neem op de met roze kussens bedekte bank! Het is opvallend hoe het je met al het komen en gaan van onderzoekers toch steeds weer lukt om zo'n hecht team te samen te stellen. Je zorgt er voor dat er bij jou in de groep altijd genoeg lol wordt beleefd, met als hoogtepunt het jaarlijkse kerstdiner. Tijdens mijn promotie was je er altijd om met me mee te denken. Dank voor al onze gesprekken, je kritische blik en de vrijheid die je me gegeven hebt.

Bea, zonder jou was dit proefschrift er niet geweest. Jij was voor mij een rots in de branding. Ik waardeer werkelijk alles aan jou. Je bescheidenheid, integriteit, intelligentie, optimisme en humor. Het is tekenend dat ik bij het schrijven van deze tekst twijfelde of ik dat nou in het Engels of Nederlands zou doen- we praatten altijd allebei de talen door elkaar. Tijdens de lunches in het AvL heb ik heel vaak dubbel gelegen om jouw verhalen. Je mediterrane temperament hebben mijn onderzoektijd opgefleurd en pit gegeven. Jij hebt mij geleerd

om complexe dingen simpel te maken en verbanden te leggen. Samen hebben we veel tijd onderweg doorgebracht; in de trein, het vliegtuig, op de fiets en, als het weer mee zat, op jouw boot. Dat waren altijd enorm gezellige momenten, waarop het voelde alsof ik met een vriendin op pad was. Ik kan je eigenlijk niet genoeg bedanken!

Anne-Marie, jij hebt mij intensief begeleid bij de OSCAR studie. Vanaf het allereerste moment ging onze samenwerking van een leien dakje. We hebben ons door heel wat patiëntendossiers, regelgeving, gastaanstellingen en artikelen heen geslagen, maar met een eindresultaat waar we trots op mogen zijn! Ik heb heel erg gewaardeerd dat je tussen je drukke klinische schema tijd voor onze telefoontjes vrij kon maken. Je was voorbereid, weloverwogen en samen kwamen we vaak weer tot nieuwe inzichten. Dank voor je oprechte interesse en de gelijkwaardige manier waarop je me altijd hebt benaderd.

Monique, ik ken weinig artsen of onderzoekers die zo trouw zijn aan zich zelf als jij. Dank je dat je er altijd voor me was en dat je met je adviezen onze onderzoeken naar een hoger klinisch en statistisch niveau hebt getild. Je bent voor mij een voorbeeld.

Dear members of the reading committee, thank you for your willingness to review this thesis and judge it on its content.

Professor Cees Lucas, dank voor uw hulp bij mijn master thesis en het vanaf de zijlijn meedenken met mijn promotietraject. Professor Rebecca Fitzgerald, you introduced me to research by taking me as a student in your lab. I couldn't have imagined a better place to first practice science. Seb, with your wit and creative mind, you were able to make complicated science accesible for an inexperienced student like me. Thank you for letting me make all those mistakes, while still trusting me with responsibilities.

Ook alle auteurs die hebben meegewerkt aan de diverse onderzoeksprojecten wil ik graag bedanken. Met name de MOCCAS studie vormde een groot onderdeel van mijn promotietraject. Hoofdonderzoekers van de deelnemende centra, zonder u was het nooit gelukt om onlangs de 3.000^{e} MOCCAS patiënt te includeren. Uw bijdrage aan de uitvoering, als ook de inhoud van de MOCCAS studie is erg waardevol geweest. Hopelijk kunnen wij onze goede samenwerking tot het einde van de studie voortzetten.

Manon, hoewel de eerste MOCCAS patiënten geïncludeerd werden in de regio Amsterdam, volgde dankzij jouw inspanningen al snel uitbreiding naar de zuidelijke regio. De brede ervaring binnen jullie laboratorium met methylatie markers heeft mij in meerdere studies geholpen.

Wiesje, ook al zat je in Maastricht, van die afstand heb ik nooit iets gemerkt. Je bent ontzettend betrouwbare en gezellige partner om mee samen te werken. Het is aan jou te danken dat het inclusietempo in het zuiden steeds verder toenam. Ik hoop dat we elkaar nog vaker gaan zien als je straks in Amsterdam woont. Ook de andere leden van het team uit Maastricht – dank voor jullie werk!

Veerle, Patrick, Marjolein en Simone, na m'n studie epidemiologie beheerste ik de theorie, maar dankzij jullie heb ik die ook in de praktijk kunnen brengen. Ik had niet geweten waar ik had moeten beginnen met het maken van het risicopredictie model, als ik jullie heldere uitleg en R-kennis niet had gehad. Ik heb genoten van onze discussies en de analyses. Deze hebben zeker bijgedragen aan de beslissing om me straks in een andere richting te gaan ontwikkelen.

Pauline, je bent een belangrijke schakel geweest in de MOCCAS studie. Je behield het overzicht, liet ons over praktische punten nadenken en maakte van ons een hecht MOCCAS team. Van jouw gevoel voor taal heb ik veel geleerd. Ook was jij een spil in de planning van mijn promotie in het algemeen. Dank voor de tijd en energie die je in mij hebt gestoken.

Margriet, jouw tomeloze inzet voor de MOCCAS studie met het verwerken van alle pakketten en het registreren van de data in de kleurige Excel sheets zijn onevenaarbaar. Ik zal het missen om elke dinsdag tijdens de TGO meeting het pakket met de rode strik te zien, met daarbij jouw update over het aantal inclusies. Het is jammer dat we allebei weg zullen zijn als de koerier het laatste pakket komt brengen, maar laten we afspreken dat we dat moment alsnog samen gaan vieren met een riant stuk taart!

Anne, Pien en Marianne, er zijn ook heel wat MOCCAS samples door jullie handen gegaan. Jullie waren altijd bereid om pakketjes op te sporen en te helpen met het verwerken van de samples. Daarnaast hebben jullie mij geholpen om de analyses op de COCOS samples te doen. Het lab is met jullie en jullie muziekkeuze altijd een gezellige plek!

Aukje en Christine, het AMC en de Bergman Clinics waren de eerste centra waar patiënten zijn geïncludeerd voor de MOCCAS studie. Dat betekent dat jullie je al bijna vier jaar inzetten voor dit project. Ik heb veel van jullie geleerd over het opzetten en uitvoeren van klinische studies. En Aukje, we zijn een geoliede machine als het gaat om het samenstellen van site files!

Brenda, jij kwam pas in mijn laatste jaar in de TGO groep werken, maar in die tijd hebben we veel met elkaar samengewerkt. Zonder jou waren al die data nu niet zo netjes opgeslagen voor de eeuwigheid.

Ook alle studenten die week in, week uit geschikte patiënten hebben benaderd voor de MOCCAS, wil ik heel erg bedanken. Jullie daagden me uit met lastige vragen en ik was telkens

weer verbaasd hoe snel jullie behendig waren in alle ziekenhuissystemen. Anniek, Nienke, Sophie, Sally, Sharon, Wieteke, Annelies, Jessica, Bas, Malou en Ton, een *cheers* met de MOCCAS mok!

Alle leden van de TGO groep, we passen nauwelijks met elkaar aan een tafel in de kantine, laat staan in een lange lijst in dit dankwoord. Met mijn klinische achtergrond heb ik de afgelopen jaren ontzettend veel van jullie geleerd- op het gebied van bioinformatica en FAIR data, maar ook over het bakken van eigen brood, capoeira en sushi. Linda, dank voor je betrokkenheid en bevlogenheid. Meike, dank je dat je mij thuis hebt weten te maken in de wonderenwereld van de eiwitten. Christian, thank you for so patiently explaining me all about sequencing. Gosia, with you I could always have a laugh. Thank you for your stories and for showing me around in New York city. Iris en Karlijn, jullie positieve energie heeft me steeds erg opgevrolijkt.

Artsen van CRC groep, met jullie viel er veel te beleven. De bakjes pleur op dinsdag na de bespreking waren vaste prik en een goed moment om bij te praten. Dankzij jullie ben ik heel wat memorabele skireizen, WEO meetings, stepritten, 'meiden'-avonden (met daartussen altijd één man) en grapjes rijker. Frank en Jasper, met jullie kon ik gezellig kletsen, in het ziekenhuis of in de Jordaan. Arne, jij was mijn kamergenoot, moleculaire maat en sparringpartner. Jouw creatieve geest als het gaat om onderzoek of nouja, alles eigenlijk, is uitzonderlijk. Vic, ik zal die gezellige avonden op onze kamer in Wenen niet snel vergeten. De overgave waarmee je te werk gaat, waardeer ik enorm. Barbara, Manon, Joep, Maxime, Michael, Clasine, Joëlle, Britt, Lucinda, Liselotte, Willemijn en Karlijn, zonder jullie had ik nooit geweten dat er poliepen in zoveel kleuren en maten bestonden. Dank voor de avonturen!

Helmy en Suzie, naast Christine en Aukje verpleegkundigen van de CRC groep, het was altijd een feest om bij jullie op de kamer langs komen. Met jullie verstand van patiënten, kliniek en onderzoek, zijn jullie voor de CRC groep onmisbaar.

Karina, ondanks dat je in mijn geval te maken had met meerdere drukke agenda's, is het je gelukt gaten te vinden voor mijn promotie(team)-overleggen. Dank voor je inspanningen!

Dan mijn kamergenoten op B1. Het was een sip gezicht toen ik laatst mijn eigen naamplaatje nog als enige naast de deur zag hangen. Blijkbaar heb ik het op onze kamer lang volgehouden, ook al zijn alle uren die ik op B1 doorbracht voorbij gevlogen. Kam, Es, met jullie deelde ik niet alleen de kamer maar ook de collegezaal. De pit-burgers kwamen aan het eind onze neus uit, maar dankzij onze lange woensdagavonden hebben we het epidemiologie diploma wel op zak. Jeroen, je bent een echte Topper in de dubbele zin van het woord. Ik verwacht zo nu en dan nog een leuk YouTube filmpje te ontvangen in mijn inbox. Annefré, Sanne, Floris, Vincent en Kiki ook van jullie gezelschap heb ik genoten!

Er is ook een heel stel vrienden waar ik de afgelopen jaren veel aan heb gehad. Jullie hebben de verhalen over emmers met poep goed verdragen. Dank dat jullie er altijd voor me waren, mijn klaagzangen hebben aangehoord, maar vooral ook met mij de hoogtepunten hebben gevierd.

Lieve Soof, wat een geluk om jou als vriendin te hebben! Het is ongelofelijk hoe veerkrachtig, trouw en lief jij bent. Jouw psychologische inzichten zijn mij meer dan eens goed van pas gekomen. Hopelijk gaan wij de Domburg-traditie van onze moeders in de toekomst voortzetten!

Lieve Gees, jij bent altijd een luisterend oor en maakt dingen nooit moeilijker dan dat ze zijn. Ook de ellelange voicemails die je regelmatig achterlaat kan ik erg waarderen. Er gaan vast en zeker nog veel telefoontjes en natuurlijk bezoekjes aan Rotterdam volgen.

Lieve Mads, ik ben blij dat jij mij bij gaat staan als paranimf. Vanaf toen we heel klein waren werden wij al aangezien voor tweelingzusjes en we proberen dat zo min mogelijk te ontkennen. Inderdaad zitten wij altijd op één lijn. Onze wandelingen door het Vondelpark waren gezellige en bijzondere momenten, die mij vaak op nieuwe gedachten hebben gebracht. Voor mij ben jij het zusje dat ik nooit heb gehad.

Lieve Yvie, paranimf, als Hillie én MDL-collega kon jij op alle fronten met mij meeleven. We hebben de afgelopen jaren onze gezamenlijke passie ontdekt (poep) en van elkaar kunnen leren tijdens praatjes op congressen. Je bent een vriendin door dik en dun, met wie ik veel feest heb gevierd maar ook mijn twijfels heb kunnen delen. Ik bewonder je onverstoorbaarheid en de passie waarmee je het doktersvak uitoefent.

Lieve Hillies, Mighty Mike, de groene cobra en de Barney Army- ze staan nog vers in mijn geheugen. Met jullie kan ik heel hard lachen én verhit discussiëren. Die combi is een *one-hundred-and-eighty*!

Familie Koolen, Paula, Jan, Wouter, Eva, Sarah en Steve, en eigenlijk horen daar natuurlijk ook een beetje de Strijboschen bij, ik ben bij jullie ondergedompeld in een warm bad. Ik ben blij dat ik jullie dankzij Martijn in mijn leven heb gekregen. De komende jaren hoop ik nog heel veel gezelligheid met jullie te beleven. Maar met alle activiteiten die er bij jullie altijd georganiseerd worden, is dat gegarandeerd. Tijn en Lizzy, jullie zijn nu nog twee kleine frummels, maar ik kan niet wachten om jullie te zien opgroeien.

Lieve Willem, als oude broer heb jij mij vele fijne kneepjes bijgebracht. Letterlijk, in de pijnlijke zin, maar ook figuurlijk, als het aankomt op boeken, films of vakantiebestemmingen. Met welke vraag ik ook bij jou aankom, jij lijkt er altijd al een keer over te hebben nagedacht en kan mij daarom de juiste afwegingen laten maken. Jij en Rien zijn een onverwoestbaar duo. Ik

heb geluk dat jullie elkaar hebben gevonden, want nu kan ik bij één en hetzelfde adres ook nog terecht voor interior design en romcom advies. Rientje, je bent een echte bikkel en ik heb groot respect voor jou.

Lieve Luud, jij bent een *golden boy*, ondanks dat je je gouden tand al twee keer hebt verloren. Je doet alles op je eigen manier en dat lijkt je gemakkelijk af te gaan. Omdat je van alles en nog wat combineert, heb je het ook eigenlijk altijd druk. Toch weet je je in alle oprechtheid om mij te bekommeren. Als oudere zusje hoopte ik jou van adviezen te kunnen voorzien, maar de afgelopen tijd heb jij mij als doorgewinterde consultant juist veel geleerd. Sanneman, jij voelt je thuis in elk gezelschap. Met je spankelende persoonlijkheid, zorgzaamheid en niet te vergeten je kookkunsten, ben je niet alleen een aanwinst voor Ludo, maar voor ons allemaal!

Lieve Tino, je weet maar half hoeveel van dit boekwerk ik aan jou te danken heb. Jouw nuchterheid, grapjes en altijd vrolijke humeur hebben me er doorheen gesleept. Er zijn maar weinig mensen te vinden die zelfs schaterlachen in hun slaap. Als jij thuis komt, kom ik meteen tot rust. Je staat werkelijk open voor alles en iedereen en bekijkt alles van de positieve kant. Ik kan me geen groter geluk voorstellen dan jou en ben je dankbaar hoe onvoorwaardelijk jij er voor mij geweest bent. Er staan ons vast nog vele vakanties naar Albanië te wachten!

Lieve Pouge en Moeshki, wat jullie voor mij betekenen en aan mij hebben gegeven kan ik eigenlijk niet in een bedankje kwijt. Moesh, jij bent mijn morele (en stylistische) kompas en je voelt met jouw neusje altijd haarfijn aan hoe het met me gaat; daar kan geen dokter tegenop. Pouge, jij bent rotsvast, een raadgever op wie ik kan vertrouwen en voor mij een voorbeeld van integriteit. Jullie wijze woorden, en dan vooral 'doe het nu', heb ik tijdens mijn PhD vaak in mijn hoofd herhaald. Tijdens al het wikken en wegen en de dipjes hebben jullie mij gesteund en tegelijkertijd vrijgelaten om tot eigen keuzes te komen. Zonder jullie was dit boekje er nooit gekomen- daarom is dit werk voor jullie.