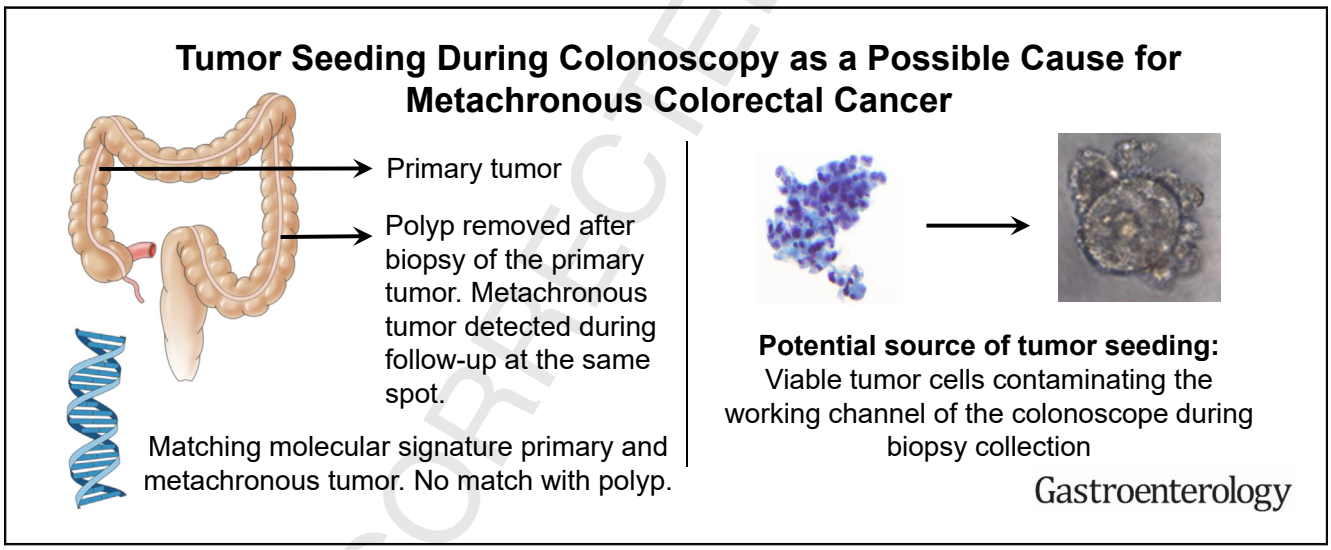
Tumor Seeding During Colonoscopy as a Possible Cause for Metachronous Colorectal Cancer

Yara Backes,[1] Tom C. J. Seerden,[2] Rosanne S. F. E. van Gestel,[1] Onno Kranenburg,[3] Inge Ubink,[3] Raymond M. Schiffelers,[4] Demian van Straten,[4] Malu S. van der Capellen,[1] Simone van de Weerd,[1] Wendy W. J. de Leng,[5] Peter D. Siersema,[1,6] G. J. A. Offerhaus,[5] Folkert H. Morsink,[5] W. Ramphal,[2] Jochiim Terhaar Sive Droste,[7] Anja U. G. van Lent,[8] Joost M. J. Geesing,[9] Frank P. Vleggaar,[1] Sjoerd G. Elias,[10] Miangela M. Lacle,[5] and Leon M. G. Moons[1]

[1]Department of Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Department of Gastroenterology and Hepatology, Amphia Hospital, Breda, The Netherlands; [3]Cancer Center, University Medical Center Utrecht, Utrecht, The Netherlands; [4]Laboratory of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands; [5]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands; [6]Department of Gastroenterology and Hepatology, Radboud University Medical Center, Nijmegen, The Netherlands; [7]Department of Gastroenterology and Hepatology, Jeroen Bosch Hospital, Den Bosch, The Netherlands; [8]Department of Gastroenterology and Hepatology, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands; [9]Department of Gastroenterology and Hepatology, Diakonessenhuis, Utrecht, The Netherlands; and [10]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, University Utrecht, Utrecht, The Netherlands



BACKGROUND AND AIMS: In patients who have undergone surgery for colorectal cancer (CRC), 3% have recurrence of (metachronous) CRC. We investigated whether tumor seeding during colonoscopy (iatrogenic implantation of tumor cells in damaged mucosa) increases risk for metachronous CRC. **METHODS:** In a proof of principle study, we collected data from the Dutch National Pathology Registry for patients with a diagnosis of CRC from 2013 through 2015, with a second diagnosis of CRC within 6 months to 3.5 years after surgery. We reviewed pathology reports to identify likely metachronous CRC (histologically proven adenocarcinoma located elsewhere in the colon or rectum from the surgical anastomosis). For 22 patients fulfilling the inclusion criteria, we ascribed the most likely etiology to tumor seeding when endoscopic manipulations, such as biopsies or polypectomy, occurred at the location where the metachronous tumor was subsequently detected, after endoscopic manipulation of the primary tumor. We collected clinical data from patients and compared molecular profiles of the primary and metachronous colorectal tumors using next-generation sequencing. We then examined the source of seeded tumor. We tested whether tumor cells stay behind in the working channel of the endoscope after biopsies of colorectal tumors, and whether these cells maintain viability in organoid cultures. **RESULTS:** In total, tumor seeding was suspected as the most likely etiology of metachronous CRC in 5 patients. Tumor tissues were available from 3 patients. An identical molecular signature was observed in the primary and metachronous colorectal tumors from all 3 patients. In 5 control cases with a different etiology of metachronous CRC, the molecular signature of the primary and metachronous tumor were completely different. Based on review of 2147 patient records, we estimated the risk of tumor seeding during

colonoscopy to be 0.3%–0.6%. We demonstrated that the working channel of the colonoscope becomes contaminated with viable tumor cells during biopsy collection. Subsequent instruments introduced through this working channel also became contaminated. These cells were shown to maintain their proliferative potential. **CONCLUSIONS:** In an analysis of primary and secondary tumors from patients with metachronous CRC, we found that primary tumor cells might be seeded in a new location after biopsy of the primary tumor. Although our study does not eliminate other possibilities of transmission, our findings and experiments support the hypothesis that tumor seeding can occur during colonoscopy via the working channel of the endoscope. The possibility of iatrogenic seeding seems low. However, our findings compel awareness on this potentially preventable cause of metachronous CRC.

Keywords: Colon Cancer; Recurrence; Metastasis; Complication.

After resection of colorectal cancer (CRC), patients undergo surveillance colonoscopy, as they are at risk for recurrent and metachronous CRC.[1] Patients operated on for CRC are at slightly increased risk for metachronous CRC, occurring in up to 3% of patients.[2] It is predominantly detected within the first 2 years after diagnosis of the primary CRC.[3] Different causes for metachronous CRC have been suggested, including incomplete polypectomies and missed or new lesions. However, the causative processes involved are not fully understood.[4]

Previous studies have shown that tumor cells exfoliate from CRC into the intestinal lumen, especially when the tumor is manipulated.[5,6] Yet, the risk of spontaneous tumor seeding, that is, implantation of tumor cells in damaged mucosa when polypectomy or biopsy is performed in the presence of CRC elsewhere in the colorectum, is estimated to be low.[7] However, the possibility of mechanical tumor seeding during colonoscopy, involving iatrogenic implantation of tumor cells in damaged mucosa, has never been explored.

In this proof-of-principle study, we investigated whether tumor cells stay behind in the working channel of the endoscope during biopsies from the tumor, and whether these cells could be implanted in the bowel wall when new instruments, such as injection needles, are introduced. In part I, we identified patients in whom the most likely etiology of metachronous CRC was mechanical tumor seeding. This was based on review of clinical data, and tested by comparing the molecular profile using next-generation sequencing (NGS). In part II, we examined whether the working channel of the endoscope may be the source of seeding.

Methods

Part I: Identification of Potential Mechanical Tumor Seeding Cases

The Dutch National Pathology Registry was probed in October 2016. Patients with CRC diagnosed from 2013 to 2015 with a secondary CRC within 6 months to 3.5 years after surgery were identified. We reviewed the pathology reports to single out cases in which the secondary cancer concerned a metachronous CRC, defined as histologically proven adenocarcinoma located elsewhere in the colon or rectum than at the surgical anastomosis.[4] We collected pathology and endoscopy reports of these patients. Patients with a hereditary predisposition for CRC and patients with inflammatory bowel disease were excluded. For the 22 patients fulfilling the inclusion criteria, we ascribed the most likely etiology for metachronous CRC using criteria described previously (Supplementary Methods).[4] In particular, metachronous CRC was attributed to tumor seeding when endoscopic manipulation, such as biopsies or polypectomy at baseline endoscopy, occurred at the location where the metachronous tumor was subsequently detected, and this manipulation took place after endoscopic manipulation of the primary tumor. These cases were subjected to molecular fingerprinting as described below. Q8

Part I: Quantification of the Risk

In order to quantify the risk of mechanical tumor seeding (ie, what is the risk of tumor transmission when manipulation is performed after taking biopsies of the primary tumor?), we performed an additional search in 4 Dutch hospitals (3 nonacademic, 1 academic hospital). We identified all surgically treated patients diagnosed with CRC between 2013 and 2015 (n = 2147) (see Supplementary Methods). We reviewed the Q9 electronic patient reports and extracted the number of patients at risk for tumor seeding (denominator, ie, colonoscopy before surgery in which the primary tumor was biopsied or manipulated otherwise, and manipulation elsewhere in the colorectum subsequently in a segment that was not removed during colorectal surgery). We then extracted the number of patients in which tumor seeding had likely occurred (numerator). These cases were subjected to molecular fingerprinting as described described below. Q10

Part I: Next-Generation Sequencing on Suspected Tumor-Seeding Cases

From the suspected tumor-seeding cases, we collected formalin-fixed paraffin-embedded (FFPE) material from the primary tumor, the metachronous tumor, and from the polyp that was removed during baseline colonoscopy at the location where the metachronous tumor was subsequently detected. In addition, we collected FFPE material from 5 patients where the metachronous tumor was attributed to another cause as control group. We performed targeted NGS based on the Cancer Hotspot Panel v2+, as described previously, using the Ion Torrent PGM (see Supplementary Methods).[8,9] The incidence of co-occurrence of mutations was estimated using cBioportal for Cancer Genomics.[10]

Abbreviations used in this paper: **CRC, colorectal cancer; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing.**

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2019.07.062

Part II: Cytologic Assessment of Rinse Fluid Working Channel

Parallel to part I, we prospectively enrolled 26 patients with tumors located distal to the splenic flexure between June 2016 and September 2017 in 4 Dutch hospitals (University Medical Center Utrecht, Amphia Hospital, Diakonessenhuis, and Onze Lieve Vrouwe Gasthuis). After biopsies of the tumor were taken, the endoscope was withdrawn without flushing or suction. Any potential residual fluid/material on the exterior of the endoscope (including the tip) was removed with dry gauze. Ex vivo, we flushed the endoscope with 30 mL normal saline solution. The rinse fluid was mixed with 20 mL CytoLyt (Cytyc Corp, Marlborough, MA), centrifuged, and the sediment was used to make thin-layer preparations for cytologic examination. An expert gastrointestinal pathologist (MML) evaluated the cytology slides and scored the number of tumor cells as none (–), moderately present (+), or excessively present (++). In a subset of 7 cases, we repeated flushing of the working channel a second and third time with 30 mL saline solution and further processed and evaluated this in the same manner. In 3 cases, we introduced an injection needle through the working channel after tumor biopsies. We washed the tip of the needle in 20 mL CytoLyt, after which 10 mL saline was flushed through the needle.

Part II: Organoid Culture of Cells Collected in the Working Channel

From the cells contaminating the working channel, we attempted to grow an organoid, as described previously in 6 patients.[11] To assess whether the cultured organoid was derived from the tumor cells isolated from the working channel, we performed targeted NGS on the biopsies that were pulled through the channel. This was compared to the mutations found in the organoid with use of Sanger sequencing. Detailed information regarding organoid culture, targeted NGS, and Sanger sequencing can be found in the Supplementary Methods.

Results

Part I: Iatrogenic Tumor Cell Implantation as a Cause of Metachronous Colorectal Cancer

In the Dutch Pathology Registry, we identified 56 patients with a secondary CRC within 6 months to 3.5 years after surgery, among which 22 metachronous CRC and 34 anastomotic recurrences. Among the 22 patients with metachronous CRCs (50% male, median age 67 years), 3 were potentially caused by tumor seeding (Supplementary Figure 1). The clinical details regarding these 3 tumor-seeding cases and 5 control cases (ie, metachronous CRC attributed to missed lesions [n = 3] or to incomplete examinations [n = 2]) is presented in Table 1. From one suspected tumor-seeding case, no FFPE material was available, hampering molecular analysis. NGS on the primary tumor, metachronous tumor, and removed polyp of the other 2 suspected tumor-seeding cases showed an identical molecular fingerprint in the primary and metachronous tumor, whereas the removed polyp showed a different profile (Table 2). The incidence of co-occurrence of these specific molecular genetic variants is extremely rare (<1%). In the 5 control cases, the molecular fingerprint of the primary and metachronous tumor were, as hypothesized, completely different (Table 3).

Part I: Quantification of the Risk of Tumor Seeding

In order to quantify the risk, we reviewed the number of patients at risk for tumor seeding (denominator) and the number of patients in which tumor seeding had likely occurred (numerator) in 4 Dutch hospitals. A flowchart is presented in Figure 1. Among 2147 surgically treated CRC Q12 patients, 310 were at risk for mechanical tumor seeding. Among those 310 patients, 2 additional cases of tumor seeding were identified, among which FFPE material was available for 1 of them. The clinical characteristics of these cases are presented in Table 1. The case in which no material was available was labeled as possible tumor seeding or incomplete polypectomy. NGS on the primary tumor, metachronous tumor, and removed polyp of the other case showed an identical molecular fingerprint in the primary and metachronous tumor, whereas the removed polyp showed a different profile (Table 2). Based on these results, the risk of tumor seeding during colonoscopy when performing biopsy or polypectomy after taking a biopsy of a tumor was estimated to be 0.3%–0.6% (1 of 310 or 2 of 310, dependent on the case from which no material was available). When restricting the estimation to patients in whom follow-up colonoscopy was performed (71.3% of patients; 221 of 310), the risk was estimated to be 0.5%–0.9%.

Part II: Tumor Cells That Retain Their Proliferative Potential Were Identified in the Working Channel

To test our hypothesis that the working channel of the endoscope is the source of tumor seeding, we rinsed the working channel of the endoscope after taking biopsies from the tumor in 26 patients with CRC (69% male, median age 68 years). A median of 6 biopsies were taken (interquartile range, 6–10). After flushing, tumor cells were observed in the rinse fluid for 21 patients (81%; ++ in 12 cases; + in 9 cases) (Figure 2*A* and *B*). To test whether subsequently introduced instruments get contaminated, a needle was introduced through 3 contaminated working channels from 3 individual patients, after which tumor cells (+) were observed on the tip of the needle in all cases (Figure 2*C* and *D*). To test whether flushing decreased contamination, we repeated flushing of the working channel with 30 mL saline in the working channel of 7 patients (5 with ++, 1 with + and 1 with – atypical cells after the first flushing). Repeated flushing did only reduce the number of tumor cells in 1 patient (from ++ to +), whereas the number was unchanged in all others. A third flushing with 30 mL of saline also did not reduce the number of tumor cells (tested in 2 patients).

In a final step, we tested whether the tumor cells contaminating the working channel still have growth potential by culturing organoids from these cells in 5 patients. In 3 patients, no growth was seen, in 1 patient growth had

Table 1. Clinical Characteristics of the Suspected Tumor-Seeding Cases Identified in the Dutch Pathology Registry (Case Numbers 1, 2, and 3) and the Suspected Tumor Seeding Cases Identified in the 4 Dutch Hospitals (Case Numbers 4 and 5)

		Patient		Primary tumor		Removed polyp[a]					Metachronous tumor		
Variable	Case no.	Sex	Age, *y*	Location	TNM	Histology	R0/R1/Rx	Polyp size, *mm*	Morphology	Polypectomy equipment and technique	Location	TNM	Time interval, *mo*[b]
Suspected tumor-seeding cases identified in the Dutch Pathology Registry													
Tumor-seeding (cases)[c]	1	F	64	Distal rectum	pT3N0	TA, LGD	R0	13	Flat	Devices: snare, injection needle Technique: en-bloc	Sigmoid	pT3N1	8
	2	M	66	Ascending colon	pT3N2	TA, LGD	Rx	6	Sessile	Devices: snare. Technique: en-bloc	Transverse colon	pT3N0	15
Possible tumor seeding / incomplete polypectomy[d]	3	M	60	Sigmoid	pT3N2	TVA, LGD	Rx	5	NA	Devices: snare Technique: en-bloc	Rectum	ypT2N0	12
Suspected tumor-seeding cases identified in the 4 Dutch hospitals													
Tumor seeding (cases)[c]	4	F	48	Rectosigmoid	pT3N2	TVA, LGD	Rx	7	NA	Devices: snare, injection needle Technique: en-bloc	Rectum	pT2N0	16
Possible tumor seeding/incomplete polypectomy[d]	5	M	57	Sigmoid	ypT4N1	TA, LGD	Rx	5	Sessile	Devices: snare Technique: en-bloc	Rectum	NA	28

F, female; LGD, low-grade dysplasia; M, male; R0, resection margins negative for dysplasia; R1, resection margins positive for dysplasia; Rx, unable to determine resection margins; TA, tubulair adenoma; TNM, tumor/node/metastasis classification; TVA, tubulovillous adenoma.

[a]*Polyp* refers to the adenoma that was removed during baseline colonoscopy (ie, the colonoscopy in which the primary tumor was biopsied), which was located at the spot where the metachronous tumor was detected).

[b]Time interval between detection of the primary and metachronous tumor.

[c]Metachronous tumors were attributed to tumor seeding when endoscopic manipulation, such as biopsies or polypectomy at baseline endoscopy, occurred at the location where the metachronous tumor was subsequently detected, and this manipulation took place after endoscopic manipulation of the primary tumor.

[d]From 2 suspected tumor-seeding cases, no FFPE material was available, hampering molecular analysis. As molecular analysis differentiates between tumor seeding and incomplete polypectomy, this case was labeled as "possible tumor seeding/incomplete polypectomy."

FLA 5.6.0 DTD ■ YGAST62837_proof ■ 24 September 2019 ■ 4:23 pm ■ ce

Table 2. Targeted Next-Generation Sequencing on the 3 Suspected Tumor-Seeding Cases (Cases 1 and 2 Identified in the Dutch Pathology Registry, Case 4 Identified in the 4 Dutch Hospitals) Showed an Identical Molecular Fingerprint of the Primary and Metachronous Tumor, Whereas the Removed Adenomas Showed Different Molecular Fingerprints

Variable	Case no.	Specimen	Gene	Genomic variant	Protein variant
Suspected tumor-seeding cases identified in the Dutch Pathology Registry	1	Primary tumor	APC	c.3873_3877del	p.(Q1291Dfs*4)
			FBXW7	c.1513C>T	p.(R505C)
			KRAS	c.38G>A	p.(G13D)
			PIK3CA	c.1633G>A	p.(E545K)
			TP53	c.375 G>A	p.? Splice site mutation
		Metachronous tumor	APC	c.3873_3877del	p.(Q1291Dfs*4)
			FBXW7	c.1513C>T	p.(R505C)
			KRAS	c.38G>A	p.(G13D)
			PIK3CA	c.1633G>A	p.(E545K)
			TP53	c.375 G>A	p.? Splice site mutation
		Removed polyp	FBXW7	c.1513C>T	p.(R505C)
			KRAS	c.35G>T	p.(G12V)
			TP53	c.673-1G>A	p.? Splice site mutation
	2	Primary tumor	BRAF	c.1799T>A	p.(V600E)
			TP53	c.524G>A	p.(R175H
			PIK3CA	c.3140A>T	p.(H1047L)
			CTNNB1	c.94G>A	p.(D32N)
		Metachronous tumor	BRAF	c.1799T>A	p.(V600E)
			TP53	c.524G>A	p.(R175H)
			PIK3CA	c.3140A>T	p. (H1047L)
		Removed polyp	KDR	c.794C>T	p.(S265L)
			APC	c.4060_4064del	p.(S1355Rfs*18)
Suspected tumor-seeding case identified in the 4 Dutch hospitals	4	Primary tumor	ERB2	c.2119C>G	p.(P707A)
			TP53	c.159_166del	p.(W53*)
			APC	c.4132C>T	p.(Q1378*)
		Metachronous tumor	TP53	c.159_166del	p.(W53*)
			APC	c.4132C>T	p.(Q1378*)
		Removed polyp	APC	c.3921_3925del	p.(E1309Dfs*4)

NOTE. *Polyp* refers to the adenoma that was removed during baseline colonoscopy (ie, the colonoscopy in which the primary tumor was biopsied), which was located at the spot where the metachronous tumor was detected. Case numbers correspond to the case numbers in Table 1.

to be terminated due to fungal contamination. In the fifth patient, we succeeded in growing an organoid (Figure 3). To verify whether this organoid arose from the tumor cells, we performed NGS on the biopsy material from the corresponding patient (full molecular profile in Supplementary Table 1). The same mutations in the APC gene (c.4463delT, p.[L1488Yfs*19]) and TP53 (c.743G>A, p.[R248Q]) were detected in the biopsy and the organoid, which were not present in normal tissue of this patient (Supplementary Figure 2). The co-occurrence of these molecular genetic variants is extremely rare (<1%), and forms compelling evidence that the tumor cells contaminating the working channel formed the organoid and were cells from the tumor that was biopsied.

Discussion

The findings and experiments support proof-of-concept that mechanical tumor seeding during colonoscopy might occur. We identified patients in whom metachronous CRC was possibly caused by tumor seeding. In these patients, the molecular profile between the primary and metachronous tumor corresponded but did not match the molecular profile of the removed polyp, supporting the hypothesis of tumor seeding. We observed that the working channel of the colonoscope can get contaminated with tumor cells during biopsy collection, and that these cells are able to proliferate. It therefore might be that the pool of cancer cells within the working channel plays a role in tumor cell transmission. The possibility of iatrogenic seeding seems low (<1%), however, our findings compel awareness of this potentially preventable cause of metachronous CRC.

Importantly, the experiments conducted provide a proof-of-concept of mechanical tumor seeding, but do not eliminate other possibilities. An alternative explanation for our finding that the primary and metachronous tumor harbor the same molecular profile might be field cancerization. The evidence for this phenomenon in patients without inflammatory bowel disease is, however, very low.[12] One could also hypothesize that the common environmental and genetic background causes that 1 patient develops 2 molecular comparable tumors. However, this is not supported by previous studies that showed distinct molecular profiles in synchronous and metachronous tumors, nor is it supported by the distinct molecular profiles that we observed in our control group (Table 3).[13,14] Moreover, the experiments

Table 3. Targeted Next-Generation Sequencing on the Primary and Metachronous Tumor of the Control Cases (ie, Metachronous Colorectal Cancer Attributed to Missed Lesions [n = 3] or to Incomplete Examinations [n = 2]) Showed a Different Molecular Fingerprint of the Primary and Metachronous Tumor

Control no.	Specimen	Gene	Genomic variant	Protein variant	Conclusion
1	Primary tumor	APC	c.2626C>T	p.(R876*)	Incomplete examination
		NRAS	c.182A>G	p.(Q61R)	
	Metachronous tumor	KRAS	c.35G>A	p.(G12D)	
		PIK3CA	c.1633G>A	p.(E545K)	
2	Primary tumor	APC	c.3922A>T	p.(K1308*)	Incomplete examination
		PIK3CA	c.332_334del	p.(K111del)	
		TP53	c.1024C>T	p.(R342*)	
	Metachronous tumor	KRAS	c.35G>A	p.(G12D)	
		APC	c.4099C>T	p.(Q1367*)	
		TP53	c.637C>T	p.(R213*)	
3	Primary tumor	BRAF	c.1799T>A	p.(V600E)	Missed cancer
		ERBB4	c.898G>T	p.(D300Y)	
		MET	c.2888-1G>T	Unknown	
	Metachronous tumor	TP53	c.1146delA	p.(K382Nfs*40)	
		BRAF	c.1799T>A	p.(V600E)	
4	Primary tumor[a]	—	—	—	Missed cancer
	Metachronous tumor	ATM	c.3956A>G	p.(Y1319C)	
		PIK3CA	c.3140A>G	p.(H1047R)	
		PIK3CA	c.3062A>T	p.(Y1021F)	
5	Primary tumor	BRAF	c.1799T>A	p.(V600E)	
		CALR	c.1116_1118del	p.(E372del)	
		FBXW7	c.1393C>T	p.(R465C)	Missed cancer
		TP53	c.1146delA	p.(K382Nfs*40)	
	Metachronous tumor	APC	c.4285C>T	p.(Q1429*)	
		KRAS	c.35G>A	p.(G12D)	
		TP53	c.396G>C	p.(K132N)	

[a]No somatic mutations found

conducted do not exclude possibilities of tumor transmission other than working channel contamination, such as exfoliation of cancer cells passively being taken up into biopsied or polypectomy site tissue.[7] Studies on tumor regrowth based on exfoliated cells performed in rats showed that colon cancer cells have the ability to regrow on damaged mucosa in a minority of cases, whereas an intact mucosa was seen to be completely resistant.[15] This suggests that some extent of mucosal injury is necessary for impaction of cancer cells. From our study, it is impossible to discriminate whether it is merely the combination of mucosal injury due to mechanical manipulation and copresence of exfoliated cancer cells, or whether mechanical impaction by instruments is obligatory. The source of the exfoliated cells (ie, whether cancer cells derive from exfoliated cancer cells on the outside of the endoscope, from a pool of exfoliated cancer cells within the working channel, or from passing intraluminal exfoliated cancer cells) causing the regrowth is another point of uncertainty. Passive impaction of exfoliated cells has been observed after hemorrhoid band ligation below a tumor.[16] It remains challenging to provide compelling evidence for mechanical tumor seeding instead of passive impaction, as it is unethical to directly test whether injecting cancer cells from previous biopsies results in a higher risk of metachronous CRC in patients. In a recent CRC mouse model, intraluminal exposure of patient-derived human CRC organoids to an injured colon mucosa (dextran sodium sulfate–induced colitis) was shown to cause a time-dependent progression from a local adenocarcinoma to disseminated disease.[17] However, even animal experiments do not provide solid evidence, as it remains uncertain whether an immunocompetent human would respond the same way. Our study therefore does not provide compelling, but rather only circumstantial, evidence for the concept of mechanical tumor seeding.

Quantification of the risk of tumor seeding is challenging. We reviewed clinical data and performed molecular analysis in 4 Dutch hospitals, and calculated that the risk of tumor seeding was <1%. Although this risk might seem low, we believe the potential impact is high and awareness of this risk is therefore crucial. More importantly, it is likely preventable with minor alterations of the current procedures. Repeated flushing did not have a major impact on the number of cells contaminating the working channel. It might be that normal saline is unable to clean the clots of cells mixed with mucus sticking to the working channel. However, simple adaptations, such as changing the order of certain procedures (eg, submucosal tattooing before taking biopsies) and the placement of the tattoo at a safe distance from the tumor to avoid tumor penetration, might already prevent tumor seeding. It remains to be explored whether alternative strategies, for example, mechanical protection of the working channel or use of cytotoxic agents, could further minimize the risk of seeding when tumors are

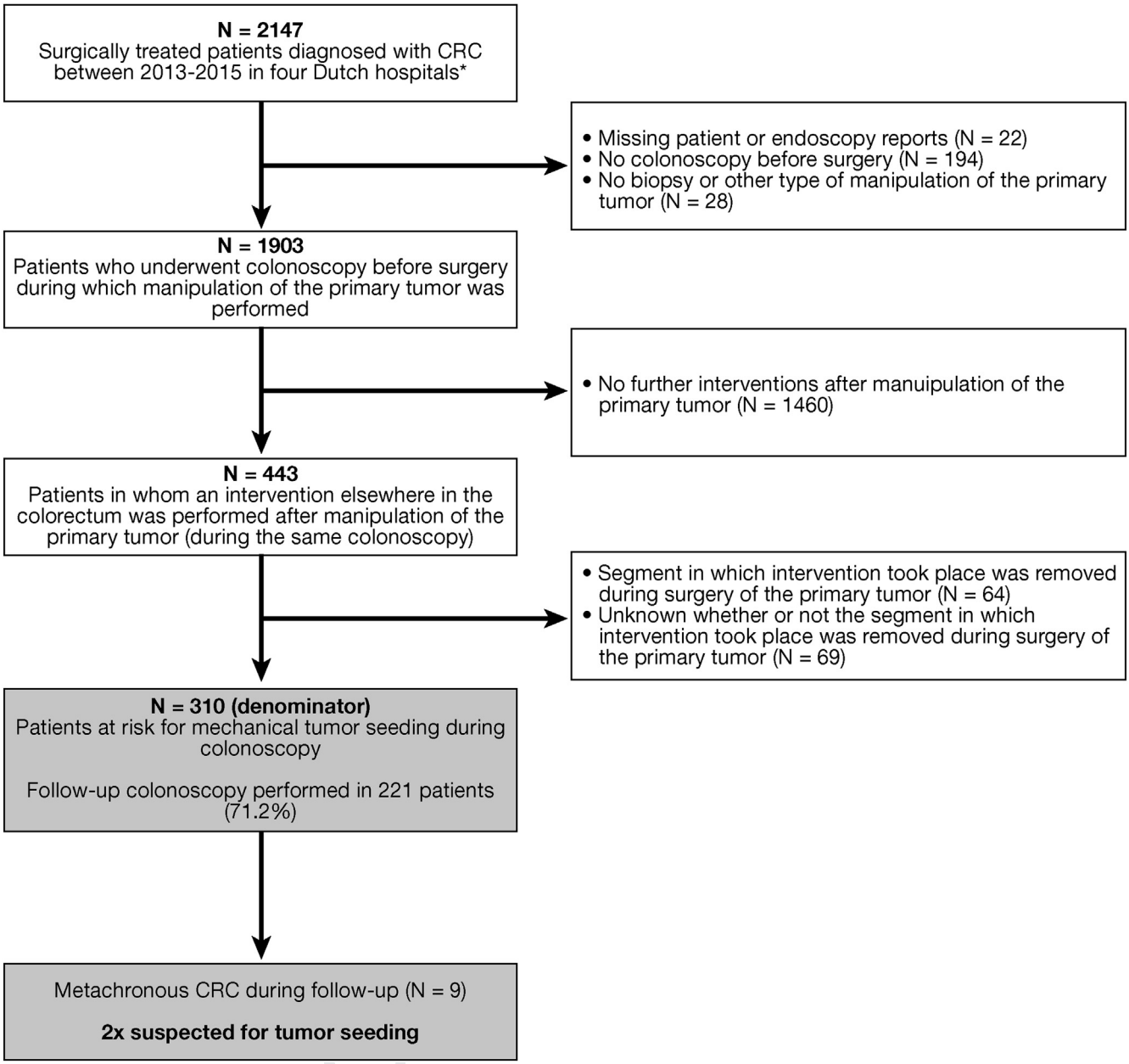


Figure 1. Quantification of the risk of mechanical tumor seeding during colonoscopy in 4 Dutch hospitals. The flowchart depicts the number of patients at risk for tumor seeding (denominator, n = 310) and the number of patients in which tumor seeding as cause of metachronous CRC was suspected.

manipulated during colonoscopy. With regard to cleaning techniques of the endoscope after colonoscopy, current guidelines recommend flushing of the endoscope channel with 70%–90% ethyl or isopropyl alcohol, followed by forced-air drying.[18] Although it could be that inadequate disinfection and sterilization might risk that atypical cells are not fully eliminated, it does not seem plausible that tumor cells are able to survive adequate cleansing methods with liquid chemical sterilants.

We identified several case reports that support our findings. Tajika et al[19] described a case of tumor seeding from a rectosigmoid cancer at the polypectomy site of a synchronous rectal carcinoid and confirmed a clonal relationship between the primary and metachronous CRC. After endoscopy in the upper gastrointestinal tract, Asai et al[20] reported a case of implantation of esophageal squamous cell carcinoma into a gastric adenocarcinoma polypectomy scar. In addition, Kang et al[21] reported a case of tumor seeding through use of a contaminated submucosal injection needle during colonoscopy. Moreover, the phenomenon of tumor seeding is well recognized in other areas, for example, during percutaneous puncture of pancreatic and hepatocellular carcinoma.[22,23] In addition, one of the theories behind anastomotic recurrences after surgery is tumor seeding, supported by the finding that primary and anastomotic recurrent CRCs are often clonally related, and

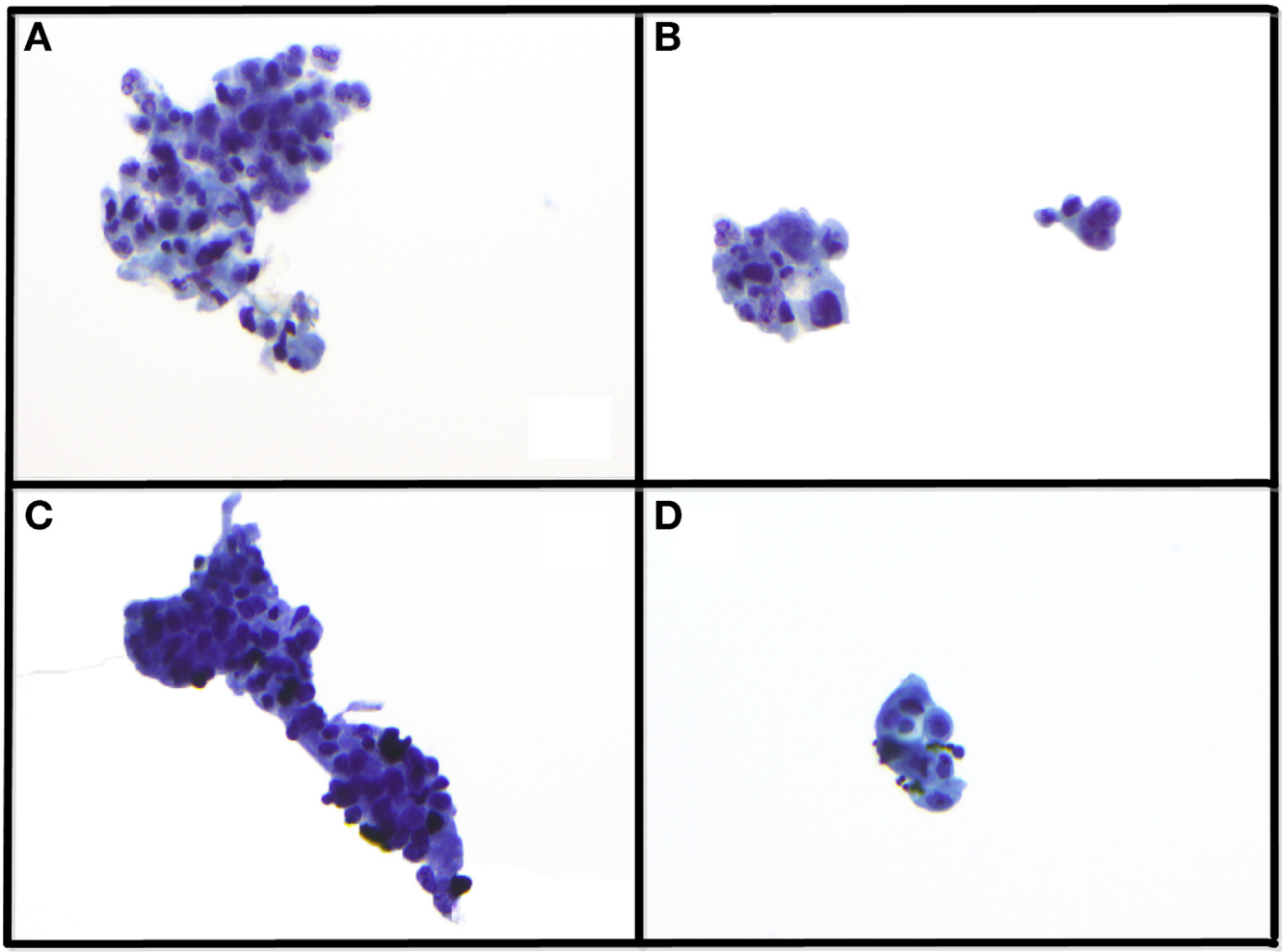


Figure 2. (*A, B*) Atypical cells in the rinse fluid of the working channel of the endoscope after biopsies taken from CRC were pulled through the channel (magnification: 40×). (*C, D*) Atypical cells on the tip of a needle after the needle had been pushed through the contaminated working channel (magnification: 40×).

that rectal washout reduces the risk for anastomotic recurrence.[24,25]

Tumor seeding as a cause of metachronous CRC sheds new light on acknowledged risk factors for metachronous CRC, such as proximal location of the CRC (eg, biopsies are taken first and synchronous polyps are removed during withdrawal risking tumor seeding), and synchronous polyps (eg, when manipulation of the bowel wall is performed after manipulation of CRC, especially when it concerns submucosal injection techniques, such as endoscopic mucosal resection).[26] Importantly, mechanical seeding of cells through contamination of the working channel might also affect other interventions during colonoscopy. For example, a recent review concluded that dye spillage into the intraperitoneal cavity occurs in about 2%–13% of patients when a resection site is tattooed during colonoscopy.[27] This means that dye was accidently injected transmural instead of submucosal. In addition, a case report described a tumor that was spotted with ink during colonoscopy, after which pigmented peritoneal cancer deposits were found at laparoscopy.[28] Tumor seeding might occur when fluid is mechanically injected through the bowel wall with a contaminated needle. This may occur when biopsies are taken before the needle is introduced, or when the needle penetrates through the tumor, and might explain why peritoneal metastasis occur in up to 2%–3% of patients without lymph node metastases even when sufficient (≥12) lymph nodes are retrieved.[29]

This study has limitations. Unfortunately, no FFPE material was available from 2 suspected tumor-seeding case. As molecular analysis differentiates between tumor seeding and incomplete polypectomy as the cause of metachronous CRC, these cases were labeled as "possible tumor seeding/incomplete polypectomy" (Table 1). Factors that might favor the likelihood of these polyps being incompletely resected (ie, size ≥10 mm and piecemeal resection) were absent.[30] However, submucosal injection before snare resection, which might favor tumor seeding, was also not performed. Therefore, we conclude that both incomplete polypectomy and tumor seeding could be the cause of metachronous CRC in these cases. A second limitation lies within the difficulty of estimating the risk of mechanical tumor seeding. Our estimate does not account for sources of variability, such as the type of hospital

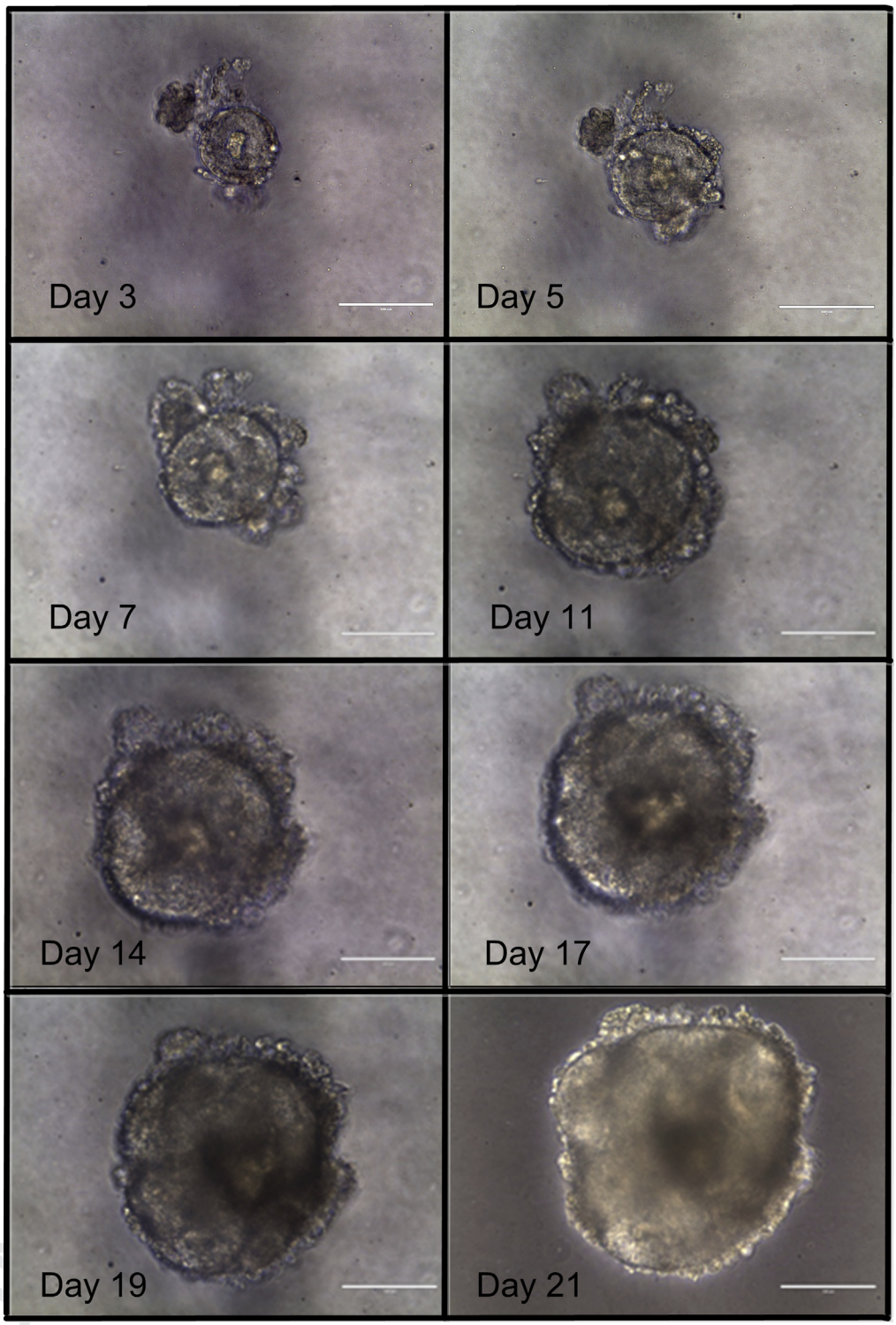


Figure 3. An organoid grew from tumor cells contaminating the working channel after biopsies had been taking from CRC indicating these cells are still viable with proliferative potential (magnification 40×; *white scale bar* = 100 μm).

examined, surgical expertise, and the instruments used. Previous studies examining the risk of seeding in hepatocellular carcinoma have shown that the estimated risk varies between studies, illustrating that estimating the exact risk remains a major challenge.[22]

In conclusion, the experiments conducted in this study provide a proof-of-concept of mechanical tumor seeding during colonoscopy. Although our study does not eliminate other possibilities of tumor transmission, our findings support the hypothesis that the working channel might play a role in tumor cell transmission. Based on review of clinical data, the possibility of iatrogenic seeding during colonoscopy seems low, however, our findings compel awareness on this potentially preventable cause of metachronous CRC.

Notably, our findings should not scare patients or clinicians away from the most important tool in CRC prevention, as colonoscopy indisputably remains the number one tool in early diagnosis and treatment of CRC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://dxdoi.org/10.1053/j.gastro.2019.07.062.

References

1. Kahi CJ, Boland CR, Dominitz JA, et al. Colonoscopy surveillance after colorectal cancer resection: recommendations of the US Multi-Society Task Force on Colorectal Cancer. Gastroenterology 2016;150:758–768 e11.
2. Hassan C, Wysocki PT, Fuccio L, et al. Endoscopic surveillance after surgical or endoscopic resection for colorectal cancer: European Society of Gastrointestinal Endoscopy (ESGE) and European Society of Digestive Oncology (ESDO) Guideline. Endoscopy 2019;51:C1.
3. Mulder SA, Kranse R, Damhuis RA, et al. The incidence and risk factors of metachronous colorectal cancer: an indication for follow-up. Dis Colon Rectum 2012;55:522–531.
4. le Clercq CM, Winkens B, Bakker CM, et al. Metachronous colorectal cancers result from missed lesions and non-compliance with surveillance. Gastrointest Endosc 2015;82:325–333 e2.
5. Inoue T, Fujii H, Koyama F, et al. Intraluminal lavage to remove exfoliated tumor cells after colorectal endoscopic submucosal dissection. Surg Endosc 2016;30:2773–2778.
6. Loktionov A. Cell exfoliation in the human colon: myth, reality and implications for colorectal cancer screening. Int J Cancer 2007;120:2281–2289.
7. Sheel AR, Artioukh DY. Endoscopic excision of synchronous large bowel polyps in the presence of colorectal carcinoma: is the fear of malignant cell implantation justified? A systematic review of the literature. Colorectal Dis 2015;17:559–565.
8. de Leng WW, Gadellaa-van Hooijdonk CG, Barendregt-Smouter FA, et al. Targeted next generation sequencing as a reliable diagnostic assay for the detection of somatic mutations in tumours using minimal DNA amounts from formalin fixed paraffin embedded material. PLoS One 2016;11:e0149405.
9. Hoogstraat M, Hinrichs JW, Besselink NJ, et al. Simultaneous detection of clinically relevant mutations and amplifications for routine cancer pathology. J Mol Diagn 2015;17:10–18.
10. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1.
11. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 2011;141:1762–1772.
12. Curtius K, Wright NA, Graham TA. An evolutionary perspective on field cancerization. Nat Rev Cancer 2018;18:19–32.
13. Zauber P, Huang J, Sabbath-Solitare M, et al. Similarities of molecular genetic changes in synchronous and metachronous colorectal cancers are limited and related to the cancers' proximities to each other. J Mol Diagn 2013;15:652–660.
14. Wang X, Fang H, Cheng Y, et al. The molecular landscape of synchronous colorectal cancer reveals genetic heterogeneity. Carcinogenesis 2018;39:708–718.
15. Hubens G, Lafullarde T, Van Marck E, et al. Implantation of colon cancer cells on intact and damaged colon mucosa and serosa: an experimental study in the rat. Acta Chir Belg 1994;94:258–262.
16. Abbasakoor F, Srivastava V, Swarnkar K, et al. Implantation anal metastases after out-patient treatment of haemorrhoids. Ann R Coll Surg Engl 2004;86:38–39.
17. O'Rourke KP, Loizou E, Livshits G, et al. Transplantation of engineered organoids enables rapid generation of metastatic mouse models of colorectal cancer. Nat Biotechnol 2017;35:577–582.
18. Kovaleva J. Endoscope drying and its pitfalls. J Hosp Infect 2017;97:319–328.
19. Tajika M, Niwa Y, Bhatia V, et al. A first report of tumor cell implantation after EMR in a patient with rectosigmoid cancer. Gastrointest Endosc 2012;75:1117–1118.
20. Asai S, Takeshita K, Kano Y, et al. Implantation of esophageal cancer onto post-dissection ulcer after gastric endoscopic submucosal dissection. World J Gastroenterol 2016;22:2855–2860.
21. Kang HJ, Lee BI, Kim BW, et al. Potential cancer cell inoculation of tattoo site through use of a contaminated needle. Gastrointest Endosc 2006;63:884–886.
22. Silva MA, Hegab B, Hyde C, et al. Needle track seeding following biopsy of liver lesions in the diagnosis of hepatocellular cancer: a systematic review and meta-analysis. Gut 2008;57:1592–1596.
23. Micames C, Jowell PS, White R, et al. Lower frequency of peritoneal carcinomatosis in patients with pancreatic cancer diagnosed by EUS-guided FNA vs. percutaneous FNA. Gastrointest Endosc 2003;58:690–695.
24. Matsuda A, Kishi T, Musso G, et al. The effect of intraoperative rectal washout on local recurrence after rectal cancer surgery: a meta-analysis. Ann Surg Oncol 2013;20:856–863.
25. Vakiani E, Shah RH, Berger MF, et al. Local recurrences at the anastomotic area are clonally related to the primary tumor in sporadic colorectal carcinoma. Oncotarget 2017;8:42487–42494.
26. Jayasekara H, Reece JC, Buchanan DD, et al. Risk factors for metachronous colorectal cancer or polyp: a systematic review and meta-analysis. J Gastroenterol Hepatol 2017;32:301–326.
27. Trakarnsanga A, Akaraviputh T. Endoscopic tattooing of colorectal lesions: is it a risk-free procedure? World J Gastrointest Endosc 2011;3:256–260.

28. Tutticci N, Cameron D, Croese J, et al. Peritoneal deposits with carbon pigmentation associated with endoscopic submucosal tattooing of a rectal cancer. Endoscopy 2010;42(suppl 2):E136.
29. Segelman J, Granath F, Holm T, et al. Incidence, prevalence and risk factors for peritoneal carcinomatosis from colorectal cancer. Br J Surg 2012;99: 699–705.
30. Hassan C, Quintero E, Dumonceau JM, et al. Post-polypectomy colonoscopy surveillance: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. Endoscopy 2013;45:842–851.

Received January 30, 2019. Accepted July 31, 2019.

Reprint requests
Address requests for reprints to: Leon M. G. Moons, MD, PhD, Department of Gastroenterology and Hepatology, University Medical Center Utrecht, Heidelberglaan 100, 3508 GA Utrecht, The Netherlands. e-mail: l.m.g.moons@umcutrecht.nl.

Acknowledgments
This study was approved by the Medical Ethics Review Committee of the University Medical Center Utrecht (approval for cytological examination rinse fluid working channel, reference number: 16-292; approval for growing organoids, reference number 17-168; approval for a search in the Dutch Pathology Registry and molecular analysis on FFPE-material of selected patients, reference number: 16-557). The study was performed in accordance with the Helsinki Declaration.

Author contributions: Study design: Yara Backes, Tom C. J. Seerden, Onno Kranenburg, Raymond M. Schiffelers, G. J. A. Offerhaus, Sjoerd G. Elias, Miangela M. Lacle, Leon M. G. Moons. Data collection: Yara Backes, Rosanne S. F. E. van Gestel, Inge Ubink, Demian van Straten, Malu S. van der Capellen, Simone van de Weerd, Folkert H. Morsink, W. Ramphal, Jochiim Terhaar Sive Droste, Anja U. G. van Lent, Joost M. J. Geesing, Miangela M. Lacle, Leon M. G. Moons. Data analysis: Yara Backes, Rosanne S. F. E. van Gestel, Malu S. van der Capellen, Wendy W. J. de Leng, Sjoerd G. Elias, Leon M. G. Moons. Data interpretation: all authors. Writing: Yara Backes, Leon M. G. Moons.

Conflicts of interest
The authors declare no conflict of interest.

Funding
This investigator-initiated study was supported by a grant from the Sacha Swarttouw-Hijmans Foundation. This funding source had no role in the design of this study, or in its execution, analyses, interpretation of the data, or decision to submit results.

Supplementary Methods

Criteria Used to Ascribe the Etiology of Metachronous Colorectal Cancer Cases

Among patients identified in the Dutch Pathology Registry with a second CRC within 6 months to 3.5 years after surgical resection of the primary CRC, it was considered a metachronous CRC when histologically proven adenocarcinoma was detected elsewhere in the colon or rectum than on the surgical anastomosis. It was considered a local recurrence if histologically proven adenocarcinoma was detected on the surgical anastomosis of the resection of the primary tumor. Among patients with metachronous CRC, the most likely etiology was ascribed using the following criteria:

- Tumor seeding was considered the most likely cause in case endoscopic manipulation (eg, biopsies or polypectomy) at baseline endoscopy occurred at the location the metachronous tumor was detected, and this manipulation took place after endoscopic manipulation of the primary tumor.
- Missed cancer was considered the most likely cause in case the metachronous tumor was detected in a segment previously (6–36 months) screened during colonoscopy.
- Incomplete resection was considered the most likely cause in case the metachronous tumor was detected at the location where a previously detected polyp had been biopsied or had been removed without previous manipulation of the primary tumor.
- Incomplete examination was considered the most likely cause in case the metachronous tumor was detected in a segment beyond the known furthest extent of endoscope insertion (eg, no cecal intubation due to stenotic primary tumor, with metachronous tumor detected in the segment proximal to the stenosis).
- Unclear: Insufficient information available to ascribe an etiology.

Quantification of the Risk of Tumor Seeding

We used the Dutch Surgical Colorectal Audit to identify all surgery-treated CRC patients diagnosed between 2013 and 2015 in 4 Dutch hospitals (3 non-academic and 1 academic). Patients with missing endoscopy or patient reports were excluded. Moreover, patients in whom no colonoscopy was performed before surgery or no manipulation (eg, biopsy, polypectomy) of the primary tumor was performed were excluded. We reviewed whether or not subsequent manipulation had been performed during the same colonoscopy. If manipulation had been performed, we checked whether or not the site of manipulation had been removed during subsequent surgery, as removal of this site would prevent potentially seeded tumor cells from growing out into metachronous CRC. This resulted in a cohort of patients at risk for mechanical tumor seeding during colonoscopy. Follow-up data were collected. FFPE material was collected from patients that developed metachronous CRC at the site of manipulation. Targeted NGS was performed on the primary tumor, metachronous tumor, and removed polyp, as described in the following section. Based on the number of patients at risk for tumor seeding (denominator) and the number of patients in which tumor seeding was confirmed (numerator), the risk of tumor seeding was quantified.

Next-Generation Sequencing

From all FFPE samples, 4-μm-thick H&E sections were prepared. Tumor percentage was determined by a pathologist and the most tumor-rich area was encircled for macrodissection. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. DNA concentration was determined using the Qubit Fluormeter (ThermoFisher Scientific, Waltham, MA). In order to reveal specific mutations, targeted NGS was performed using the Ion Torrent PGM platform (ThermoFisher Scientific), as described previously. NGS was based on the Cancer Hotspot Panel (consisting of 50 genes and supplemented with another 5 cancer-related genes; ThermoFisher Scientific).[1,2]

Organoid Culture

The working channel of the endoscope was flushed with 30 mL phosphate-based saline, which was collected in a 50-mL tube containing 20 mL warm (37°C) basal medium 2− (BM2−).[3] Organoids were cultured as described previously.[3] In short, the suspension was centrifuged and washed with phosphate-based saline multiple times. The pellet was filtered through a 40-μm filter to remove debris (eg, mucus and fecal content). The flow-through was resuspended in BM supplemented with Noggin, A83-01 and SB202190 (BM2+) and basement membrane extract in a 1:3 ratio. The mixture was plated in small drops in a 6-well plate with gridlines and placed upside down for 30 minutes in an incubator at 37°C to solidify. After this, 1 mL BM2+ was added per well. The medium was refreshed once per week and organoids were passaged if necessary, as described previously.[11] Organoids were photographed (Evos XL microscope; ThermoFisher Scientific) and size was measured using ImageJ (version 1.47; National Institutes of Health, Bethesda, MD).

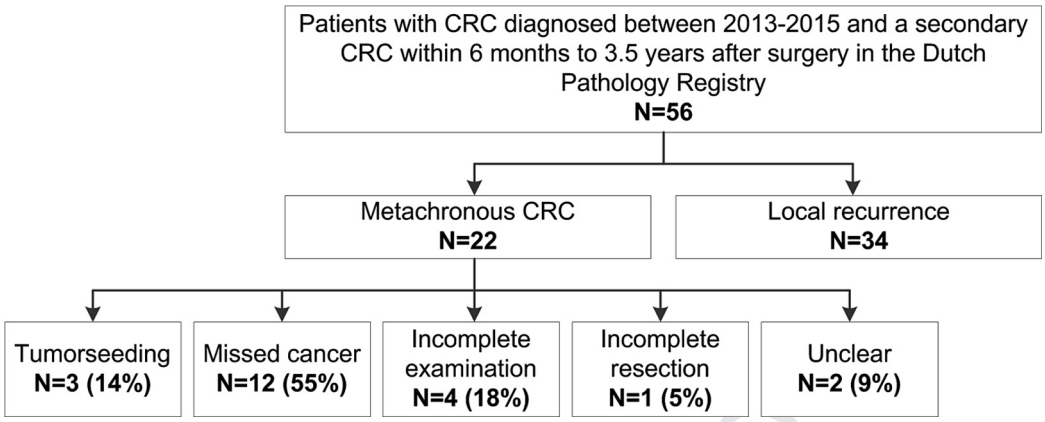
Sanger Sequencing

Sanger sequencing on the organoid was performed using polymerase chain reaction primers (APC forward primer: AGAGAGAGAGTGGACCTAAGC, APC reverse primer: GTATAAATGGCTCATCGAGGCT; TP53 forward primer: CCTGCTTGCCACAGGTCT, TP53 reverse primer: GTCAGCGGCAAGCAGAG). The correct product was

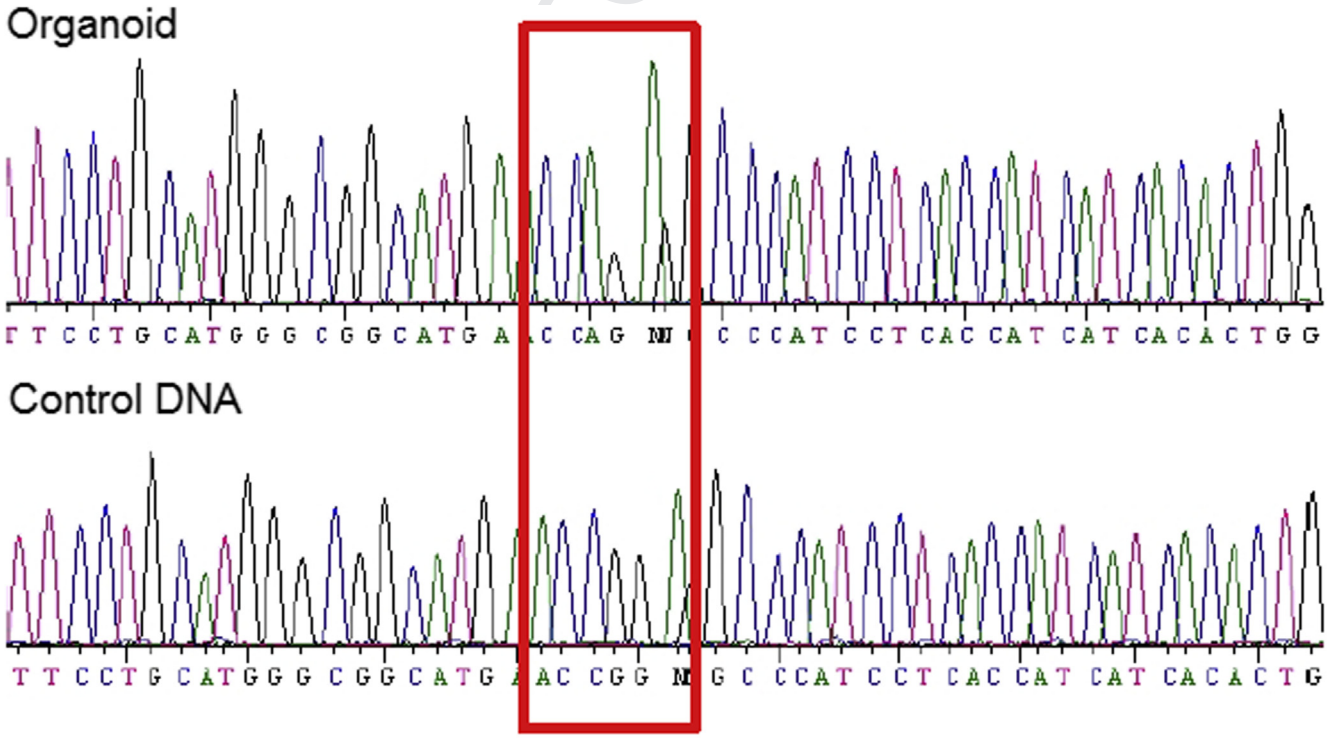
confirmed with sequencing of the polymerase chain reaction product of the biopsy. The sequence reaction was carried out using the Big Dye Terminator Cycle Sequencing Kit (Life Technologies, Grand Island, NY). Samples were run on a 3730XL DNA analyzer (ThermoFisher Scientific) and analyzed using DNASTAR's SeqMan pro software.

References

1. de Leng WW, Gadellaa-van Hooijdonk CG, Barendregt-Smouter FA, et al. Targeted next generation sequencing as a reliable diagnostic assay for the detection of somatic mutations in tumours using minimal DNA amounts from formalin fixed paraffin embedded material. PLoS One 2016; 11:e0149405.
2. Hoogstraat M, Hinrichs JW, Besselink NJ, et al. Simultaneous detection of clinically relevant mutations and amplifications for routine cancer pathology. J Mol Diagn 2015;17:10–18.
3. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 2011;141:1762–1772.



Supplementary Figure 1. Etiology of metachronous CRC based on information derived from endoscopy and pathology reports. The most likely etiology was ascribed using the following criteria: Tumor seeding was considered the most likely cause in case endoscopic manipulation (eg, biopsies or polypectomy) at baseline endoscopy occurred at the location the metachronous tumor was detected, and this manipulation took place after endoscopic manipulation of the primary tumor. Missed cancer was considered the most likely cause in case the metachronous tumor was detected in a segment previously (6–36 months) screened during colonoscopy. Incomplete resection was considered the most likely cause in case the metachronous tumor was detected at the location where a previously detected polyp had been biopsied, or had been removed without previous manipulation of the primary tumor, with metachronous tumor detected in the segment proximal to the stenosis).



Supplementary Figure 2. The TP53 (c.743G>A, p.(R248Q)) mutation present in the biopsy was also present in the organoid, confirming that the organoid grew out of tumor cells contaminating the working channel (CGG(Arg) → CAG (Gln)). The *top tracing* shows the DNA sequence from the organoid, the *bottom tracing* shows a normal DNA sequence from a control patient.

Supplementary Table 1. Targeted Next-Generation Sequencing on the Biopsy From Which an Organoid Was Cultured, in Order to Verify Whether This Organoid Grew Out of Tumor Cells

Gene	Genomic variant	Protein variant
APC	c.3920T>A	p.(I1307K)
APC	*c.4463delT*	*p.(L1488Yfs*19)*
FBXW7	c.1513C>T	p.(R505C)
NOTCH1	c.7369C>G	p.(L2457V)
TP53	*c.743G>A*	*p.(R248Q)*

NOTE. Mutations in italics were Sanger sequenced on the organoid to assess the clonal relationship between the biopsy and the organoid.