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Quantification of Magnetic Nanoparticles in *ex vivo* Colorectal Lymph Nodes

Lennert Molenaar*, Melissa M. Horstman – van de Loosdrecht*, Lejla Alic*, Joop van Baarlen†, Jeroen J. H. J. Meijerink‡, Bennie ten Haken*, Ivo A. M. J. Broeders§,¶,** and Daan J. Lips‖

*Magnetic Detection & Imaging Group, Technical Medical Centre
University of Twente, Enschede, The Netherlands

†Department of Pathology, LabPON Hengelo, The Netherlands

‡Department of Operating Rooms and MITeC Technology Center
Radboud University Medical Center, Nijmegen, The Netherlands

§Department of Surgery, Meander Medical Center
Amersfoort, The Netherlands

¶Robotics and Mechatronics Group, University of Twente
Enschede, The Netherlands

‖Department of Surgery, Medisch Spectrum Twente
Enschede, The Netherlands
**IAMJ.Broeders@meandermc.nl

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En-bloc tumor resection is the standard treatment for locally advanced colorectal cancer (CRC). An extensive histopathological assessment is necessary to evaluate the metastatic spread and adjuvant therapy. Sentinel lymph node biopsy decreases the histopathological burden when only sentinel lymph nodes (SLNs) are examined. This study aims to evaluate the spread of a magnetic tracer throughout the lymphatic system after *ex vivo* injection in en-bloc resected specimens of patients with CRC. To achieve this, lymph nodes (LNs) were quantified using a new magnetic detection method. Fifteen patients with CRC diagnosed with clinically negative LNs were included in this study and received 2–4 *ex vivo* magnetic tracer injections (total volume of 2 mL). Magnetic sample series were acquired to create a look-up table for magnetic tracer quantification. In 80% of the patients, at least one magnetic LN was detected. A total of 33 LNs were marked as magnetic, containing an average of 8.1 μg iron. In 71% of the patients, metastases were found in nonmagnetic LNs. *Ex vivo* injection leads to sub-optimal tracer spread and therefore inaccurate diagnosis. This study presents a novel magnetic detection method to quantify magnetic tracer in lymph

**Corresponding author.

nodes. Detecting the SLNs in en-bloc resected specimens and involving only these LNs in histopathological investigation enable a decrease in healthcare costs or an increased diagnostic potential.

Keywords: Colorectal cancer; DiffMag; sentinel lymph node; superparamagnetic iron oxide nanoparticles; superparamagnetic quantifier.

1. Introduction

Worldwide incidence of colorectal cancer (CRC) in 2018 was 1.8 million, causing 861,000 associated deaths.[1] This number would have been larger if not for screening, where premalignant lesions are detected and treated, preventing CRC.[2] Standard care for patients with CRC is radical en-bloc tumor resection that includes regional lymph nodes (LNs).[3] Patients with confirmed metastatic LNs are consecutively treated with adjuvant radiotherapy. Although this method is effective, it involves substantial time, effort and finances required by histopathological examination of a large number of LNs.[4]

Sentinel lymph node biopsy (SLNB) limits the excision of LNs to solely sentinel lymph nodes (SLNs) using perioperative SLN mapping. SLNs directly drain from the tumor and have the highest probability of containing metastases.[5] Even with apparent advantages of perioperative SLN mapping, more evidence is necessary before the procedure could become standard practice. During the current routine en-bloc resection, a section of colon and mesocolon is removed including fatty tissue with (S)LNs.[6] *In vivo* tracer injection or *ex vivo* tracer injection can potentially facilitate the identification of SLNs. Although tracer spread of *in vivo* injections is expected to be superior, added patient burden is also anticipated. *In vivo* injections can be given prior to surgery with an extra colonoscopy or at the start of the surgery. For optimal tracer spread, it is necessary to apply at least 20 min for the tracer to spread, which extends the total surgery time. Therefore, investigating *ex vivo* injections as an alternative option could be beneficial, potentially enabling SLN identification without added patient burden and without extending surgery duration.

Under comparable diagnostic accuracy, the identification of SLNs potentially reduces health costs since fewer LNs need to be histopathologically processed. Additional benefit involves a strategy for SLN ultrastaging, instead of single sectioning of all LNs.[7] This potentially increases the diagnostic value by an increased identification rate of metastases, micro metastases and isolated tumor cells. However, isolated tumor cells have limited predictive value, therefore having no influence on the adjuvant treatment. Considering micro metastases are associated with an increased recurrence rate and an inferior prognosis,[8] adjuvant treatment is advised. With this strategy, the total histopathological workload is expected to remain similar. It is unknown which strategy would be more beneficial and cost-effective; decreasing histopathological investigation by investigating fewer LNs or performing extensive histopathological investigation on only the SLNs. Regardless the strategy of preference, it is beneficial to identify SLNs for a more refined histopathological process.

A variety of SLNB tracers are currently clinically available: Radioactive, fluorescent and magnetic tracers.[9] Radioactive tracers have proven to be effective.[10] However, they suffer from serious drawbacks under strict regulations, a short decay and shelf life, varying availability per clinical site/country and a radiation dose, although minimal, for users and patients. Fluorescent tracers do not suffer from these drawbacks, but detection time is limited and they have a low penetration depth of 5–10 mm,[11] which is in many clinical cases insufficient. Magnetic tracers (also referred to as magnetic nanoparticles and, in this case, specifically superparamagnetic iron-oxide nanoparticles [SPIONs]) have been suggested to alleviate a variety of the issues constituted by radioactive or fluorescence tracers, such as being widely available, having a long shelf life, logistically easy to use and no half-life, can be detected with MRI and are radiation-free.[12] With a great potential, SPIONs need assessment of clinical relevance in terms of relation between the amount of injected tracer and amount of trapped tracer in the (S)LNs.[13,14]

The SuperParamagnetic Quantifier (SPaQ)[15] is used to accurately quantify the amount of magnetic tracer trapped inside LNs. A unique feature of the SPaQ is its relatively large sample holder for static samples (diameter 20 mm) compared to other magnetometers, such as vibrating sample magnetometry (VSM) and magnetic particle spectroscopy

(MPS).[16] This enables measurements on entire LNs. Due to the homogeneous magnetic field, precise orientation of the LN is irrelevant.

The aim of this study is the assessment of the clinical relevance for SPION-enhanced (S)LNs, which involves three research goals:

- Evaluation of tracer spread after an *ex vivo* peritumoral injection (with and without tissue massage).
- Quantification of the amount of tracer trapped inside the resected LNs.
- Evaluation of the performance of the SPaQ in a clinically relevant setting.

2. Methods

2.1. *The SuperParamagnetic quantifier*

The SPaQ is a relatively inexpensive in-house developed magnetometer with a sample holder placed in a homogeneous magnetic field (Fig. 1). The SPaQ uses an in-house developed control unit and laptop controlled acquisition software. The SPaQ is a mobile device that enables the use of the device in multiple clinical situations. In previous research, the SPaQ (in particle characterization mode) was used extensively for characterization of magnetic tracers.[15,17] Additionally, SPaQ functionality was extended to include a quantification mode, based on the patented differential magnetometry (DiffMag) detection principle.[18] This DiffMag principle (Fig. 2) is a nonlinear magnetic detection method specifically assessing magnetic tracer without influence of its surrounding. In case of a handheld device,[19] the height of the DiffMag signal reflects a combination of the amount of SPIONs and the distance between the detector and sample. Due to the homogeneous magnetic field in the SPaQ, the DiffMag signal (expressed in μV) is directly proportional to the amount of SPIONs, resulting in an accurate assessment uninfluenced by sample orientation. A DiffMag signal below three standard deviations of the average background noise, assessed prior to each acquisition, is labeled as noise. The following SPaQ settings are used for characterizing the applied magnetic field: AC amplitude of 0.83 mT, AC frequency of 2.5 kHz and DC field strength of 4.99 mT. The DiffMag signal is refreshed every 0.5 s.

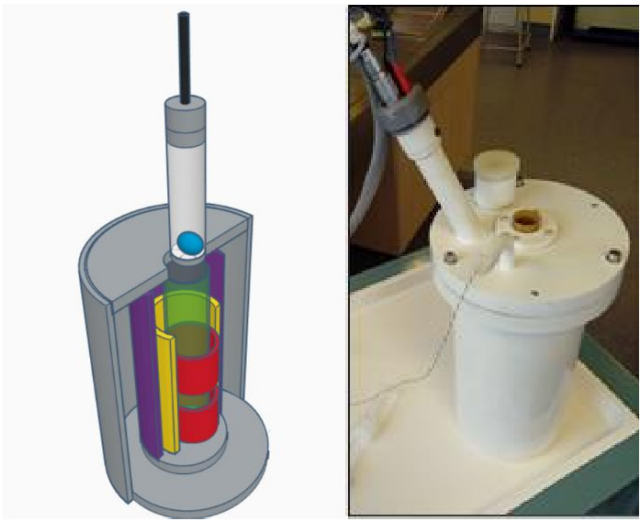

Fig. 1. (Color online) A simplified schematic cross-section and outer appearance of the SPaQ. The SPaQ has a height of 50 cm and width of 18 cm. The sample container has a diameter of 2 cm and a height of 2.5 cm. A vial with a LN (in blue) is lowered into the green shaft. An excitation coil (yellow) creates the homogenous magnetic field, and detection coils (red) measure the magnetic signal. The outer field coil (purple) serves as a shielding coil and improves the field homogeneity.

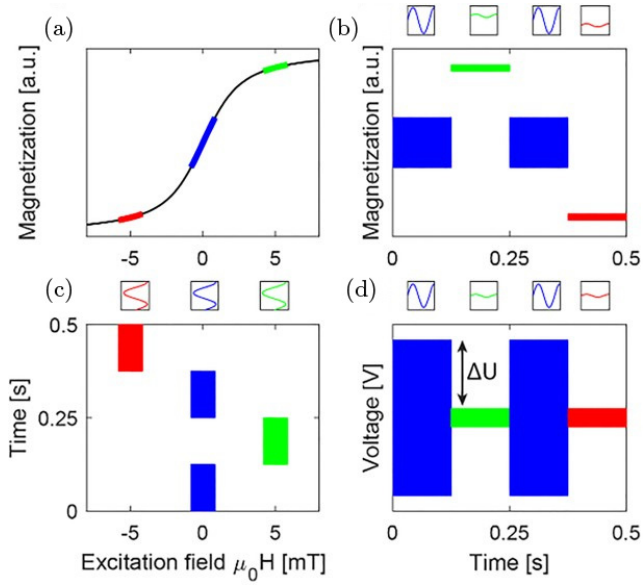

Fig. 2. (Color online) The DiffMag principle applied in the SPaQ. (a) Magnetization curve of magnetic particles. (b) AC field (blue) with DC offsets (red and green). (c) Magnetization of magnetic particles. (d) Measurement of nonlinear magnetic signal (ΔU).

2.2. *Clinical trial*

Fifteen adult patients diagnosed with primary CRC (cT1–4) and clinically negative LNs were included in this study if scheduled for a hemicolectomy (left/right), a transverse colon resection or a sigmoid resection. Written informed consent was obtained

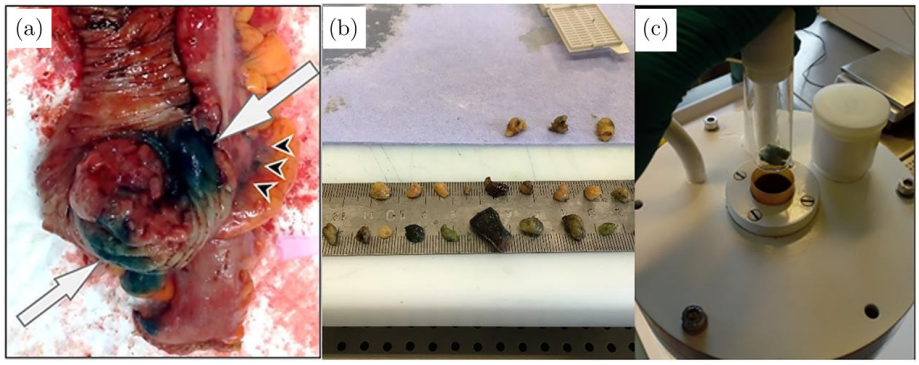

Fig. 3. (a) *Ex vivo* magnetic tracer injection in the primary tumor (white arrows) with visible tracer spread to lymph nodes (black arrows). (b) Resected lymph nodes during pathological research and (c) measurement of an individual lymph node.

prior to inclusion. Ethics committee approval was given by the Medical Ethical Committee Twente (Enschede, the Netherlands), under study number K19-13. The first 10 patients received two to four *ex vivo* peritumoral injections with a total of 2 mL Sienna+® (28 mg (Fe)/mL, Endomag, UK, Ltd.) diluted with Na Cl to a total volume of 4 mL and no tissue massage after injection (group 1). The next five patients received 3–4 *ex vivo* peritumoral injections with a total of 2 mL Magtrace® (28 mg (Fe)/mL, Endomag, UK, Ltd.) diluted with NaCl to a total volume of 4 mL and 1–2 min of tissue massage after injection (group 2). Figure 3(a) shows the resection specimen with the tracer injected after resection. For both groups, after a waiting period of at least 20 min (to promote tracer distribution), the specimen was fixated in formalin and sent to the pathology department.

All LNs were harvested from the resection specimen (Fig. 3(b)) 24–72 h after the *ex vivo* tracer injection. Subsequently, the spread of magnetic tracer was assessed for all LNs by using the SPaQ (Fig. 3(c)). The total acquisition time for one LN (putting a LN in the sample holder, SPaQ measurement and removing the sample) was under 30 s. After the SPaQ measurements, all nodes were processed in accordance with the following histopathology protocol: For every found LN, two adjacent central slices were sectioned and stained with H&E for a general overview and with Prussian blue to identify SPIONs (Figs. 4(a) and 4(b)). This provided data for each LN whether it contains metastatic cells and/or magnetic tracer.

2.3. *Look-up table acquisition*

Assessing the amount of iron trapped inside the resected LNs is facilitated by a predefined SPaQ look - up table (LUT) for both Sienna+® and Magtrace®.

A relation between amount of iron and corresponding DiffMag signal (μV) was experimentally established and modeled as a first order polynomial by in-house developed software (Matlab environment, R2020a, Mathworks, Inc., Natick, MA). The DiffMag signal corresponding to eight samples for both tracers were acquired 10 times using the SPaQ. The created LUT is available through a 4 TU repository for both Sienna+® and Magtrace®.[20]

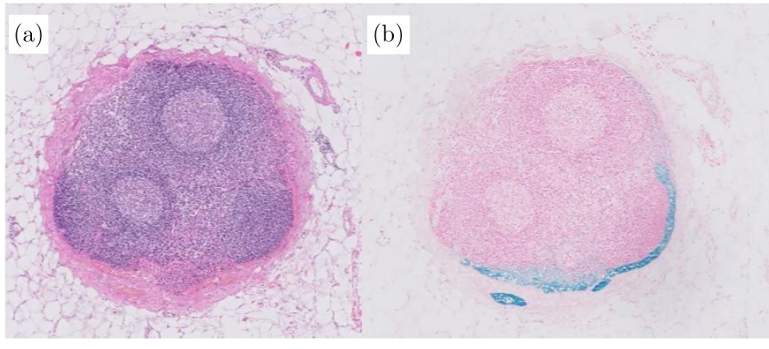

Fig. 4. (Color online) (a) Lymph node coupe with H&E staining and (b) Lymph node coupe with Fe staining (in blue the magnetic tracer).

Table 1. An overview of the obtained clinical results based on the SPaQ measurements.

	Group 1	Group 2
Patients (N)	10	5
Tracer type	Sienna+®	Magtrace®
Average number of LNs (range)	21.3 (5–36)	27.6 (10–44)
Average number of magnetic LNs (range)	1.9 (0–4)	2.4 (0–4)
SPION detection rate	8/10 (80%)	4/5 (80%)
Number of patients with metastases in LNs	3	4
Number of patients with metastases in SLNs	1	1

2.4. *Correction factor*

It is necessary to apply a correction to the μg Fe found, since the LUT is created with measurements on liquid samples. Riahi *et al.* previously investigated the effect of environmental factors on the differential magnetic susceptibility.[17] Increasing the viscosity or completely blocking the rotation of magnetic nanoparticles by freeze-drying reduced the magnetic signal from 7% to 74%. In the colorectal LN measurements, the tracer is trapped in the LNs. Therefore, the estimated amount of tracer is increased with the correction factor.

3. Results

3.1. *Clinical results*

The clinical results of colorectal LNs using the SPaQ are summarized in Table 1 and further detailed in the Appendix A (Tables A.1 & A.2). The average number of magnetic LNs per patient was 1.9 (0–4) for group 1 and 2.4 (0–4) for group 2. Both patient groups showed an 80% SPION detection rate (i.e., in 80% of the patients at least one LN containing SPIONs was detected). During histopathological examination, LN metastases were found in a total of seven patients. In two of those patients, metastases were found in the LNs containing magnetic tracer, which we defined as SLNs (29%).

3.2. *Magnetic look-up table*

Figure 5 shows the DiffMag signals for both tracers (Sienna+® and Magtrace®) with the first-order polynomial fit.

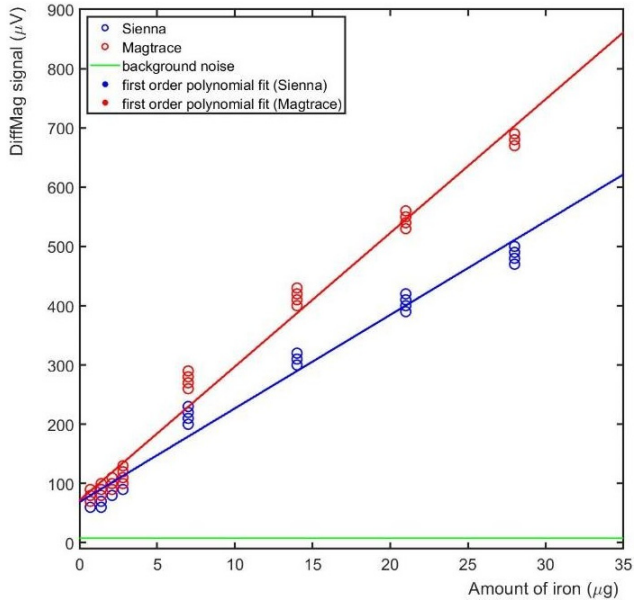

Fig. 5. (Color online) An overview of the two sample series with their corresponding first order polynomial line. The blue line represents the DiffMag signal for Sienna+® and the red line represents the DiffMag signal for Magtrace®. The green line represents the mean background noise.

3.3. *Amount of tracer trapped in LNs*

For the Sienna+® group, a total of 21 LNs were identified, with an average DiffMag signal of 249 μ V (range: 100–530) resulting in an average of 11.0 μg Fe (range: 2.0–29.2). For the Magtrace® group, a total of 12 LNs were identified, with an average DiffMag signal of 136 μV (range: 90–360) resulting in an average of 2.9 μg Fe (range: 0.8–12.8). After applying the correction factor, the average amount of Sienna+® was 11.8–19.1 μg Fe and 3.1–5.0 μg Fe for Magtrace®. When all 33 LNs were combined, the average μg Fe was 8.3 (8.9–14.4) μg Fe after correction. Further details can be seen in Table 2.

4. Discussion

The spread of magnetic tracer throughout the lymphatic system after *ex vivo* injection was seen in 80% of the patients, despite the absence of natural lymph flow. No difference was observed in the SPION detection rate between the groups with and without tissue massage. In 29% of the patients with LN metastases, the metastases were detected in SLNs (magnetic LNs). Consequently, histopathological investigation of only these SLNs would result in inadequate medical treatment for 71% of

Table 2. The clinically measured colorectal nodes with the corresponding calculated first polynomial based on the measured sample series.

Sienna+®			Magtrace®		
LN #	DiffMag signal (μV)	First polynomial (μg Fe)	LN #	DiffMag signal (μV)	First polynomial (μg Fe)
1	420	22.2	22	360	12.8
2	520	28.6	23	240	7.5
3	200	8.3	24	120	2.1
4	410	21.6	25	120	2.1
5	440	23.5	26	90	0.8
6	160	5.8	27	90	0.8
7	190	7.7	28	90	0.8
8	100	2.0	29	90	0.8
9	170	6.4	30	110	1.7
10	200	8.3	31	90	0.8
11	440	23.5	32	110	1.7
12	160	5.8	33	120	2.1
13	100	2.0			
14	130	3.9			
15	200	8.3			
16	160	5.8			
17	280	13.4			
18	120	3.2			
19	200	8.3			
20	530	29.2			
21	100	2.0			
Average	249	11.0		136	2.9
After correction		11.8–19.1			3.1–5.0

patients. To enable successful clinical implementation of *ex vivo* SLN dissection, improved tracer spread is necessary.

An average amount of 8.9–14.4 μg of tracer was found trapped in LNs, after correction. When analyzing the standard deviation of both sample series with the SPaQ, good repeatability is suggested. When converted to μg Fe with the presented LUT, we can conclude that measurement deviations with the SPaQ are practically negligible. To our knowledge, there is only one previous study (breast cancer) reporting the amount of magnetic tracer (Resovist) trapped in the dissected LNs as assessed by a handheld magnetometer.[21] This study reported a mean of 140 ± 40 μg Fe, which is significantly higher than that in our study (33 LNs with an average of 8.1 [8.7–14.1] μg Fe). Besides the differences (different tracer and cancer type), the main difference is the *ex vivo* injection, which presumably resulted in a much lower tracer spread throughout the tissue.

We established five unique aspects on valorization of the SPaQ as a clinical device. First, it is a relatively large sample holder, evaluating a complete LN instead of single plaques. Second, the SPaQ is relatively small and low in cost. Third, the device can easily be transported to a desired location, such as a pathology department. Fourth, the acquisition takes less than a second, making it possible to incorporate in current workflows without significant time investment. Last, LUT data were very concise, despite the high number of repeated acquisitions, proving the high reproducibility of the SPaQ.

To utilize the SPaQ to its full potential in a clinical setting, we envision an additional study using *in vivo* injections and a larger patient group. As illustrated earlier during SLN mapping by a fluorescent tracer,[22] changing the injection from *ex vivo* to *in vivo* is expected to lead to increased tracer spread. Until recently, *in vivo* tracer injections for patients with CRC were only considered feasible prior to the surgery during an additional colonoscopy or at the start of the surgery (extending surgery time). Currently, extended intended use allows the magnetic tracer to be injected up to 30 days prior to surgery. This leads to the opportunity to inject the tracer during the standard diagnostic colonoscopy. Comparing a time window of 30 days versus 12 h when using a radioactive tracer, the magnetic tracer provides great flexibility and has a major advantage regarding surgical planning.

5. Conclusion

This study presents a novel magnetic detection method to quantify magnetic tracer and assess magnetic tracer spread in colorectal LNs. With the expectation that *in vivo* tracer injection would greatly improve the tracer spread, the principle of *ex vivo* SLN dissection still has a great diagnostic potential. In regards to quantitative assessment of iron amount trapped in LNs, identification of the SLN(s) from a large number of resected LNs enables two options viable in histopathological investigation: Decrease of the ever increasing healthcare costs or increased diagnostic value.

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The authors declare no conflicts of interest related to this paper. Written informed consent was obtained prior to inclusion. Ethics committee approval was given by Medical Ethical Committee Twente (Enschede, the Netherlands), under study number K19-13.

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Appendix A

Table A.1. Sample series used to acquire the look-up table.

Sienna+®				
μg Fe	0.7	1.4	2.1	2.8
Volume (μL)	2.5	5	7.5	10
Concentration (μg/μL)	0.28	0.28	0.28	0.28
μg Fe	7	14	21	28
Volume (μL)	2.5	5	7.5	10
Concentration (μg/μL)	2.8	2.8	2.8	2.8
Magtrace®				
μg Fe	0.7	1.4	2.1	2.8
Volume (μL)	2.5	5	7.5	10
Concentration (μg/μL)	0.28	0.28	0.28	0.28
μg Fe	7	14	21	28
Volume (μL)	2.5	5	7.5	10
Concentration (μg/μL)	2.8	2.8	2.8	2.8

Table A.2. Overview of the demographic data of all included patients.

	Group 1	Group 2
Patients (N)	10	5
Number of males/females	7/3	3/2
Mean age	61.9	63
Mean BMI	27.6	25.1
Number of left/right hemicolectomies	1/6	0/2
Number of sigmoid resections	2	3
Number of transversum resections	1	0
Tumor stage (radiologists) cTx	4	2
Tumor stage (radiologists) cT1	1	0
Tumor stage (radiologists) cT1/2	1	0
Tumor stage (radiologists) cT2	1	1
Tumor stage (radiologists) cT2/3	2	1
Tumor stage (radiologists) cT3	1	1
Tumor location - coecum	4	2
Tumor location - colon ascendens	2	0
Tumor location - colon transversum	2	0
Tumor location - sigmoid	2	3

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