Article:
Appelt, AL orcid.org/0000-0003-2792-9218, Andersen, RF, Lindebjerg, J et al. (1 more author) (2020) Prognostic Value of Serum NPY Hypermethylation in Neoadjuvant Chemoradiotherapy for Rectal Cancer: Secondary Analysis of a Randomized Trial. American Journal of Clinical Oncology, 43 (1). pp. 9-13. ISSN 0277-3732

https://doi.org/10.1097/COC.0000000000000609

Prognostic value of serum NPY hypermethylation in neoadjuvant chemoradiotherapy for rectal cancer: Secondary analysis of a randomised trial

Ane L Appelt, PhD[1,2], Rikke F Andersen, MSc[2], Jan Lindebjerg, MD[2], Anders Jakobsen, DMSc[2]

[1] Leeds Institute of Medical Research at St James's, University of Leeds and Leeds Cancer Centre, St James's University Hospital, Leeds, UK.

[2] Danish Colorectal Cancer Center South, Vejle Hospital, and Institute of Regional Health Research, University of Southern Denmark, Denmark.

The results of the current study have previously been presented as a proffered paper at the European Society for Radiotherapy & Oncology (ESTRO) annual conference in Barcelona, Spain, April 2018.

Corresponding author

Ane L Appelt

a.l.appelt@leeds.ac.uk, +44 0113 206 7685

Leeds Cancer Centre

St James's University Hospital, Bexley Wing, Beckett Street

Leeds, UK

Conflicts of Interest and Source of Funding

The authors report no conflicts of interest. Danish Colorectal Center South Is supported by the Region of Southern Denmark. Ane Appelt is supported by Yorkshire Cancer Research Academic Fellowship funding (grant L389AA). No funding bodies were involved in study design; in collection, analysis and interpretation of data; in writing of the study manuscript; or in the decision to submit the article for publication.

Abstract

Objectives: Long-term prevention of metastatic disease remains a challenge in locally advanced rectal cancer (LARC), and robust pre-treatment prognostic factors for metastatic progression are lacking. We hypothesised that circulating tumour specific DNA based on hypermethylation of the NPY gene (meth-ctDNA) could be a prognostic marker in the neoadjuvant setting; we examined this in a secondary, explorative analysis of a prospective trial.

Methods: Serum samples were prospectively collected in a phase III trial for LARC. Positivity for and fractional abundance of meth-ctDNA in baseline samples were estimated. Overall survival (OS) and rate of distant metastases were compared between meth-ctDNA positive and negative patients; other prognostic factors were controlled for in multivariate Cox regression. Importance of quantitative load was examined by considering the fractional abundance of meth-ctDNA relative to total circulating DNA.

Results: Baseline serum samples were available for 146 patients. Thirty patients had presence of meth-ctDNA, with no correlation with cT (p=0.8) or cN (p=0.6) stages. Median follow-up was 10.6 years for OS and 5.1 years for freedom from distant metastases. Patients with meth-ctDNA had significantly worse 5-year OS (47% vs 69%), even when controlling for other prognostic factors (HR=2.08, 95% CI 1.23-1.51). This appeared mainly driven by disparity in the rate of distant metastases (55% vs 72% at 5 years, p=0.01); HR=2.20 (1.19-4.07, p=0.01) in multivariate analysis. Increased quantitative load was highly significant for worse outcomes.

Conclusions: Meth-ctDNA could be a potential prognostic marker in the neoadjuvant setting and may, if validated, identify patients at increased risk of distant metastases.

Keywords

Rectal cancer; chemoradiotherapy; liquid biomarker; tumour specific DNA; hypermethylation

Introduction

Modern multimodality treatment of locally advanced rectal cancer results in excellent local control rates, with less than 10% of patients experiencing local disease recurrence with appropriate and selective use of neoadjuvant chemoradiotherapy (CRT) (1, 2). Long-term prevention of metastatic disease remains a challenge, however. The standard arm in the German CAO/ARO/AIO-04 study (3) had a cumulative incidence of distant metastasis of >25%, and despite some reduction with the addition of pre- and postoperative oxaliplatin, only a minority of patients benefitted. More recently, total neoadjuvant treatment (TNT) has gained popularity, although with limited prospective evidence (4-6). With the advent of neoadjuvant systemic treatment intensification, robust prognostic factors for metastatic progression are needed at the time of diagnosis to support treatment individualisation. The vast majority of the biomarker literature focuses on prediction of response to CRT at the time of surgery, though, leaving a clear clinical need for better baseline prognostic factors for long-term oncological outcomes (7).

A major focus of clinical and translational cancer research in the last decade has been on the use of blood-based, tumour-specific markers, such as circulating tumour-specific DNA (ctDNA) for disease characterisation and prognostication (8, 9). Progress in non-metastatic rectal cancer has been slow, however, partly as identification of tumour-specific cell-free DNA in blood in non-metastatic rectal cancer has proven challenging. Cell-free DNA can be found in blood in healthy patients (10); thus even though absolute levels of cell-free DNA are usually elevated in cancer patients, this might not in itself indicate the presence of ctDNA. The current methods for detection of ctDNA generally rely on pre-identification of tumour-specific mutations in tissue, which may only be possible for a fraction of patients using traditional screening panels of known mutations (11, 12). Additionally, the majority of published studies have depended on surgical specimen tissue samples, making the methodology unsuitable for neoadjuvant treatment decisions (12).

Hypermethylation of the neuropeptide Y (NPY) gene has been proposed as a universal marker in colorectal cancer (14, 15), as methylation of the NPY gene is highly preferentially expressed in colorectal cancer compared to normal tissue (14). Analysis of hypermethylation of the NPY gene in circulating tumour specific DNA (meth-ctDNA) by droplet digital PCR may hence allow for

detection of ctDNA in non-metastatic rectal cancer patients (16). This has not previously been examined in detail in localised rectal cancer, however.

We hypothesised that meth-ctDNA could be a prognostic marker in the neoadjuvant setting and examined this in a secondary, explorative analysis of a prospective clinical trial.

Materials and methods

Patients took part in a phase III trial of radiotherapy dose escalation for locally advanced rectal cancer, for which main trial results have previously been reported (17, 18). In summary, patients with MRI-staged T3-4N0-2M0 rectal cancer and threatened circumferential resection margin in the lower two-thirds of the rectum were enrolled from 2005 to 2008 in two international centres (Denmark and Canada). They received 50.4Gy in 28 fractions with concomitant oral UFT and L-leucovorin, plus an additional 2x5Gy brachytherapy tumour boost in the experimental arm. Total mesorectal excision was performed eight weeks after the end of CRT, and adjuvant chemotherapy was delivered at the discretion of the treating physician and according to national guidelines. Patients were seen at surgical departments for on-protocol follow-up visits every 6 months for the first 3 years and once a year in the fourth and fifth years. Any further follow-up was at the discretion of the treating surgeon. All electronic patient records were reviewed at the time of final analysis of late trial outcomes (June 2013) to verify reported events and to identify disease relapse and death not otherwise reported. No differences between the trial arms were observed at the final analysis, for any of the late outcomes considered (18). For the current analysis, overall survival data were updated (Oct 2017) using the Danish Cause of Death Register. The trial protocol was approved by the research ethics committee for the Region of Southern Denmark (protocol ID VF20050006), the study was conducted in accordance with the Declaration of Helsinki, and all patients provided written informed consent for trial participation and blood sample collection for translational research.

Only patients in the Danish part of the trial were involved in the translational sub-study. Baseline blood samples were collected in the week prior to start of CRT. Serum was collected in 9 ml tubes, left to coagulate for at least 30 minutes and centrifuged at 2000g within 4 hours from sampling.

Samples were stored at -80°C until analysis. Serum was centrifuged 10 minutes at 10,000g prior to purification. DNA was purified from 2-4 ml serum on a QiaSymphony purification system (Qiagen, Hilden, Germany) using the Circulating nucleic acid kit. Fragments of CPP1 DNA were added prior to purification as exogenous control (17). DNA was eluted in 60 ul and water added to 400 ul. The amount and quality of DNA was determined by qPCR for B2M and CPP1 as previously described (19) except that 3 ul of template was analysed per well and a QuantStudio 12k Flex machine (Thermo Scientific, Waltham, MA, USA) was used. The remaining DNA was concentrated to 20 ul on Amicon Ultra-0.5 Centrifugal Filter Units (Millipore, Burlington, MA, USA). The entire eluate was bisulfite converted using EZ DNA Methylation lightning kit (Zymo research, Irvine, CA, USA) following the manufacturer's instructions and eluted in 15 ul. The following controls were included in the conversion protocol: water, genomic DNA from non-cancer controls and Universal methylated human standard (Zymo research, Irvine, CA, USA) as positive control. Samples and controls were analysed by droplet digital PCR together with an additional methylated, bisulfite converted control sample EpiTect control DNA, methylated (Qiagen, Hilden, Germany). Twelve ul of DNA from samples were mixed with primer/probe mix for NPY and Albumin (sequences from Garrigou et al 2016) and Supermix for probes (BioRad, Hercules, CA, USA) in a total volume of 48 ul. Two-point-five ul of control samples were used. Droplets were prepared on the Auto Droplet Generator (BioRad) in two wells with 20 ul per sample. PCR conditions were: 95°C/10 minutes followed by 40 cycles of 95°C/15 seconds, 56°C/1 minute and finally 98°C/10 minutes. Ramp rate was 1.5°C/second. Droplets were analysed in a QX100 Droplet reader (BioRad). Samples were considered positive for meth-ctDNA if >2 positive droplets/sample, and fractional abundance of meth-ctDNA was calculated as proportion of meth-ctDNA relative to total amount of circulating DNA. Serum analysis was performed blinded to patient outcomes.

The relationship between meth-ctDNA status and baseline disease staging (cT and cN stages) was examined using Fisher's exact test, as was the correlation between meth-ctDNA status and primary tumour regression grade (complete or major response, defined as TRG1 or TRG1-2) (20). For estimation of overall survival (OS), death from any cause was considered an event, and patients were censored if alive at the time of data update (Oct 2017). For estimation of freedom from distant metastasis, first incidence of distant metastasis was counted as an event, irrespective of any previous locoregional recurrence. Distant metastases were confirmed by either biopsy (preferred) or sequential imaging. Patients were censored at time of last clinical assessment prior

to final trial data analysis (June 2013). All times were calculated from date of trial enrolment. OS and freedom from distant metastases were compared between meth-ctDNA positive and negative patients using log-rank tests. Other baseline prognostic factors considered were clinical T and N stage as well as age for OS; multivariate Cox regression analysis was used to control for effect of these and treatment arm. The importance of quantitative load was examined by considering the fractional abundance of meth-ctDNA (i.e. the proportion of meth-ctDNA relative to total amount of circulating DNA) in multivariate analysis, controlling for the same factors as above. Based on this, five-year OS and freedom from distant metastases were estimated as a function of fractional abundance. The proportional hazards assumption was assessed by examination of Schoenfeld residuals, and potential non-linear dependence of continuous variables was examined by re-fitting models with restricted cubic splines. All analyses were conducted in R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria).

The REMARK (REporting recommendations for tumour MARKer prognostic studies) checklist was followed to ensure complete and transparent reporting (21).

Results

Baseline serum samples were available for 146 patients (out of 243 patients treated on trial); 24 patients were treated outside of Denmark, 71 patients had no baseline blood sample available (logistical reasons or samples used in previous research studies), and two patients had no follow-up data available (consent withdrawn). Table 1 summarises baseline characteristics for the cohort included in the current study as well as for all patients treated on trial. Median age was 64 years, 64% of patients were male, 81% of tumours were T3 (the remaining T4), and 88% of patients were N positive. Twenty-four patients in the current study cohort received adjuvant chemotherapy; corresponding to 22%, with 11 in standard arm, 14 in dose escalation arm, and 14 patients with no data available. No differences were observed between the current study and the full trial cohort.

Baseline blood sample collection was performed median 6 days (interquartile range, IQR, 6-7 days) before commencement of CRT. Thirty patients (20.5%) had detectable meth-ctDNA in baseline serum samples (i.e. samples were considered positive for meth-ctDNA), with median fractional

abundance in serum positive patients of 1.4‰ (IQR 0.6-3.7‰). We observed no correlation between meth-ctDNA and clinical T and N stages: Odds ratio (OR) for meth-ctDNA positivity for T4 vs T3 was 0.81 (95% confidence interval, CI, 0.22-2.49, p=0.8), and corresponding OR for N+ vs N0 was 0.63 (95% CI 0.19-2.48, p=0.6). Tumour regression grades in the pathology specimen at surgery were 22 TRG1, 18 TRG2, 82 TRG3, and 6 TRG4 (18 patients not assessed); with no correlation between meth-ctDNA status at baseline and complete (OR 0.64, 95% CI 0.11-2.49, p=0.76) or major (OR 1.13, 95% CI 0.40-3.54, p=1.0) tumour regression.

Median follow-up for OS was 10.6 years (interquartile range, IQR, 9.2-11.5 years), with 75 events during follow-up. Median follow-up for freedom from distant metastases was 5.1 years (IQR 3.7-6.0 years), with 47 events during follow-up. Nine patients experienced locoregional recurrence during follow-up, of which three patients had locoregional recurrence as the first event. These three events were not included in any of the analyses for the current study. Patients with meth-ctDNA detectable in serum had significantly worse OS at 5 years (47% vs 69%, p=0.02), Figure 1a, and this difference appeared mainly driven by disparity in the rate of distant metastases (55% vs 72% at 5 years, p=0.01), Figure 1b. The prognostic importance of meth-ctDNA remained strong when correcting for other prognostic factors in multivariate analysis, with hazard ratio (HR) of 2.08 (95% CI 1.23-1.51, p=0.007) for OS and HR 2.20 (95% CI 1.19-4.07, p=0.01) for freedom from distant metastases. See Table 2 for full model fit.

Increased quantitative load was highly significant for worse outcomes; p<0.0001 and p=0.001 for OS and freedom from distant metastases, respectively. This held true in multivariate analyses, HR 1.24 (1.13-1.35, p<0.0001) per mille (‰) for OS and HR 1.17 (1.07 – 1.29, p=0.001) per mille for freedom from distant metastases; see Table 2. Figure 2 demonstrates the dependence of 5-year OS and freedom from distant metastases on quantitative meth-ctDNA load.

None of the multivariate models demonstrated deviation from proportional hazard, and none of the continuous variables (age and quantitative meth-ctDNA load) showed signs of non-linearity.

Discussion

Long-term prevention of metastatic disease remains a problem for locally advanced rectal cancer, and identification of patients who might benefit from systemic treatment intensification is challenging. The role of ctDNA in this setting is still unclear; this may be partly due to difficulties identifying ctDNA in blood when tumour-specific mutations are not readily identifiable in diagnostic tissue samples. We hypothesised that ctDNA might be generally detectable using hypermethylation of the NPY gene, and that presence of ctDNA in blood samples could be a prognostic marker. We tested this hypothesis in a cohort of patients with locally advanced rectal cancer, prospectively followed as part of a phase III trial. Our results demonstrated that meth-ctDNA may be detectable in baseline serum samples in 1 in 5 patients, and that presence of meth-ctNDA at baseline is a prognostic marker for outcome following chemoradiation and surgery. The correlation with survival outcomes appeared mainly driven by risk of metastatic disease; and it demonstrated a clear dose-response relationship, where higher quantitative load of meth-ctDNA was related to higher risk of death and distant disease progression.

This is, to the best of our knowledge, the first study to detect and assess the prognostic impact of pre-treatment meth-ctDNA in localised rectal cancer. The NPY gene encodes for a neuropeptide that is involved in a range of physiological processes, including vasoconstrictive effects in colon tissue. It is still unclear exactly how it might be involved in colorectal malignancy, although there is some indication that it could be related to invasive ability (22). It is evident, however, that methylation of the NPY gene is highly preferentially expressed in colorectal cancer compared to normal tissue (14, 23). This makes it a promising universal blood-based biomarker for colorectal cancer (14, 15, 24), avoiding many of the issues with alternative approaches to ctDNA detection, such as reliance on identification of patient-specific tumour mutations. A small number of studies of meth-ctDNA have previously focused on the metastatic setting (15, 25), but the importance for localised disease has not previously been studied in detail.

If patients with locally advanced rectal cancer at high risk of metastatic progression are to be selected for upfront treatment intensification – such as induction, consolidation or intensified neoadjuvant chemotherapy (4-6, 26) – prognostic markers must be identifiable at treatment baseline. Previous work on ctDNA has primarily examined changes in ctDNA levels during and following treatment. In the largest and most involved study to date in this setting, Tie et al investigated sequential plasma samples from 159 patients undergoing chemoradiotherapy and

surgery, focusing on post-operative ctDNA for risk stratification (27). They were partly dependent on surgical tumour specimens for identification of somatic mutations for ctDNA detection. Others have focused on non-specific cell free DNA (cfDNA) and change in levels after neoadjuvant treatment (28–31). In a recent publication, Schou et al showed correlation between total cfDNA levels prior to treatment and long term disease outcome in 123 patients (31). Total cfDNA is disease non-specific, though, and may be elevated in patients with non-malignant disease (10). Based on the Schou et al study, we conducted a subsequent analysis of our own data, focusing on total cfDNA, and found no association with survival outcomes (data not shown).

The current study is, as far as we are aware, the largest publication so far to examining baseline ctDNA in localised rectal cancer. A previous study of tumour-specific mutations in cfDNA did not manage to demonstrate a relationship between baseline detection of ctDNA and prognosis in 97 locally advanced rectal cancer patients (8), possibly as too many patients were wild type for known KRAS and BRAF mutations.

The current study utilized a ctDNA detection methodology valid for all rectal cancer patients, irrespective of identification of patient-specific tumour mutations. Other strengths are systematic collection of blood samples and prospective follow-up of patients as part of a phase III trial. Conversely, it must be strongly emphasised that the analysis itself was retrospective and unplanned, thus purely hypothesis generating. Due to the timeframe of the original study, a number of factors known today to (potentially) impact patient prognosis were unavailable in our dataset, such as extra-mural venous invasion (EMVI) on diagnostic MRI and carcinoembryonic antigen (CEA) levels in serum. Note, however, that pathological factors were deliberately not included in the multivariate analysis, as these factors are generally not available at diagnosis, where upfront systemic treatment intensification may be of interest. All analyses were conducted on serum samples, where plasma today would be the preferred option, to avoid unspecific cfDNA from leucocytes (32). Finally, there are clearly additional questions to be answered on the dynamics of meth-ctDNA load during treatment, post-surgery, and in follow-up, but this was not the focus on the current study.

In summary, we have demonstrated that meth-ctDNA can be detected in pre-treatment blood samples in patients with locally advanced rectal cancer, and that this could potentially be a

prognostic marker for distant disease progression. Our findings will need validation in independent datasets, and any predictive value will have to be elucidated before application for treatment intensification selection can be considered.

Conclusion

Hypermethylation of circulating tumour specific DNA could be a potential prognostic marker in the neoadjuvant setting and may, if validated, identify patients at increased risk of distant metastases.

References

1. Engelen SME, Maas M, Lahaye MJ, *et al.* Modern multidisciplinary treatment of rectal cancer based on staging with magnetic resonance imaging leads to excellent local control, but distant control remains a challenge. *Eur. J. Cancer*. 2013;49:2311–20.
2. Glynne-Jones R, Wyrwicz L, Tiret E, *et al.* Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* 2017;28:iv22-iv40.
3. Rödel C, Graeven U, Fietkau R, *et al.* Oxaliplatin added to fluorouracil-based preoperative chemoradiotherapy and postoperative chemotherapy of locally advanced rectal cancer (the German CAO/ARO/AIO-04 study): final results of the multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol.* 2015;16:979–989.
4. Ludmir EB, Palta M, Willett CG, et al. Total neoadjuvant therapy for rectal cancer: An emerging option. Cancer 2017;123(9):1497-1506.
5. Cercek A, Roxburgh CSD, Strombom P, *et al.* Adoption of Total Neoadjuvant Therapy for Locally Advanced Rectal Cancer. *JAMA Oncol.* 2018;4:e180071.
6. Zaborowski A, Stakelum A, Winter DC. Systematic review of outcomes after total neoadjuvant therapy for locally advanced rectal cancer. Br J Surg 2019;106:979-987
7. Lim SH, Chua W, Henderson C, *et al*. Predictive and prognostic biomarkers for neoadjuvant chemoradiotherapy in locally advanced rectal cancer. Crit Rev Oncol Hematol 2015 Oct;96:67-80
8. Crowley E, Di Nicolantonio F, Loupakis F, *et al.* Liquid biopsy: monitoring cancer-genetics in the blood. *Nat. Rev. Clin. Oncol.* 2013;10:472–84.

9. Khakoo S, Georgiou A, Gerlinger M, *et al.* Circulating tumour DNA, a promising biomarker for the management of colorectal cancer. *Crit. Rev. Oncol. Hematol.* 2018;122:72–82.
10. Spindler K-LG, Appelt AL, Pallisgaard N, *et al.* Cell-free DNA in healthy individuals, noncancerous disease and strong prognostic value in colorectal cancer. *Int. J. Cancer*. 2014;135.
11. Gaedcke J, Grade M, Jung K, *et al.* KRAS and BRAF mutations in patients with rectal cancer treated with preoperative chemoradiotherapy. *Radiother. Oncol.* 2010;94:76–81.
12. Sclafani F, Chau I, Cunningham D, *et al.* KRAS and BRAF mutations in circulating tumour DNA from locally advanced rectal cancer. *Sci. Rep.* 2018;8:1–9.
13. Reinert T, Henriksen TV, Christensen E, etl al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. JAMA Oncol 2019 (in press)
14. Garrigou S, Perkins G, Garlan F, *et al.* A study of hypermethylated circulating tumor DNA as a universal colorectal cancer biomarker. *Clin. Chem.* 2016;62:1129–1139.
15. Garlan F, Laurent-Puig P, Sefrioui D, *et al.* Early evaluation of circulating tumor DNA as marker of therapeutic efficacy in metastatic colorectal cancer patients (PLACOL study). *Clin. Cancer Res.* 2017;23:5416–5425.
16. Andersen RF. Tumor-specific methylations in circulating cell-free DNA as clinically applicable markers with potential to substitute mutational analyses. Expert Rev Mol Diagn 2018;18:1011-1019
17. Jakobsen A, Ploen J, Vuong T, *et al.* Dose-effect relationship in chemoradiotherapy for locally advanced rectal cancer: A randomized trial comparing two radiation doses. *Int. J. Radiat. Oncol. Biol. Phys.* 2012;84.
18. Appelt AL, Vogelius IR, Pløen J, *et al.* Long-term results of a randomized trial in locally advanced rectal cancer: No benefit from adding a brachytherapy boost. *Int. J. Radiat. Oncol. Biol. Phys.* 2014;90:110–118.
19. Pallisgaard N, Spindler KLG, Andersen RF, Brandslund I, Jakobsen A. Controls to validate plasma samples for cell free DNA quantification. Clin Chim Acta 2015;446:141-6
20. Mandard AM, Dalibard F, Mandard JC, *et al.* Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer*. 1994;73:2680–6.
21. McShane LM, Altman DG, Sauerbrei W, *et al.* Reporting recommendations for tumor marker prognostic studies (REMARK). *J. Natl. Cancer Inst.* 2005;97:1180–4.
22. Ogasawara M, Murata J, Ayukawa K, *et al.* Differential effect of intestinal neuropeptides on

invasion and migration of colon carcinoma cells in vitro. *Cancer Lett.* 1997;119:125–130.
23. Mitchell SM, Ross JP, Drew HR, *et al.* A panel of genes methylated with high frequency in colorectal cancer. *BMC Cancer*. 2014;14:1–15.
24. Roperch JP, Incitti R, Forbin S, *et al.* Aberrant methylation of NPY, PENK, and WIF1 as a promising marker for blood-based diagnosis of colorectal cancer. *BMC Cancer*. 2013;13:1–10.
25. Boeckx N, Op de Beeck K, Beyens M, *et al.* Mutation and Methylation Analysis of Circulating Tumor DNA Can Be Used for Follow-up of Metastatic Colorectal Cancer Patients. *Clin. Colorectal Cancer*. 2018;17:e369–e379.
26. Nilsson PJ, van Etten B, Hospers GA, *et al.* Short-course radiotherapy followed by neo-adjuvant chemotherapy in locally advanced rectal cancer – the RAPIDO trial. *BMC Cancer*. 2013;13:279.
27. Tie J, Cohen JD, Wang Y, *et al.* Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. *Gut* 2018;68(4):663-671.
28. Zitt M, Müller HM, Rochel M, *et al.* Circulating cell-free DNA in plasma of locally advanced rectal cancer patients undergoing preoperative chemoradiation: a potential diagnostic tool for therapy monitoring. *Dis. Markers*. 2008;25:159–65.
29. Agostini M, Pucciarelli S, Enzo MV, *et al.* Circulating cell-free DNA: A promising marker of pathologic tumor response in rectal cancer patients receiving preoperative chemoradiotherapy. *Ann. Surg. Oncol.* 2011;18:2461–2468.
30. Carpinetti P, Donnard E, Bettoni F, *et al.* The use of personalized biomarkers and liquid biopsies to monitor treatment response and disease recurrence in locally advanced rectal cancer after neoadjuvant chemoradiation. *Oncotarget*. 2015;6.
31. Schou J V., Larsen FO, Sørensen BS, *et al.* Circulating cell-free DNA as predictor of treatment failure after neoadjuvant chemo-radiotherapy before surgery in patients with locally advanced rectal cancer. *Ann. Oncol.* 2018;29:610–615.
32. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: Preanalytical considerations. Clin Chim Acta 2013;424:222-30.

Figures

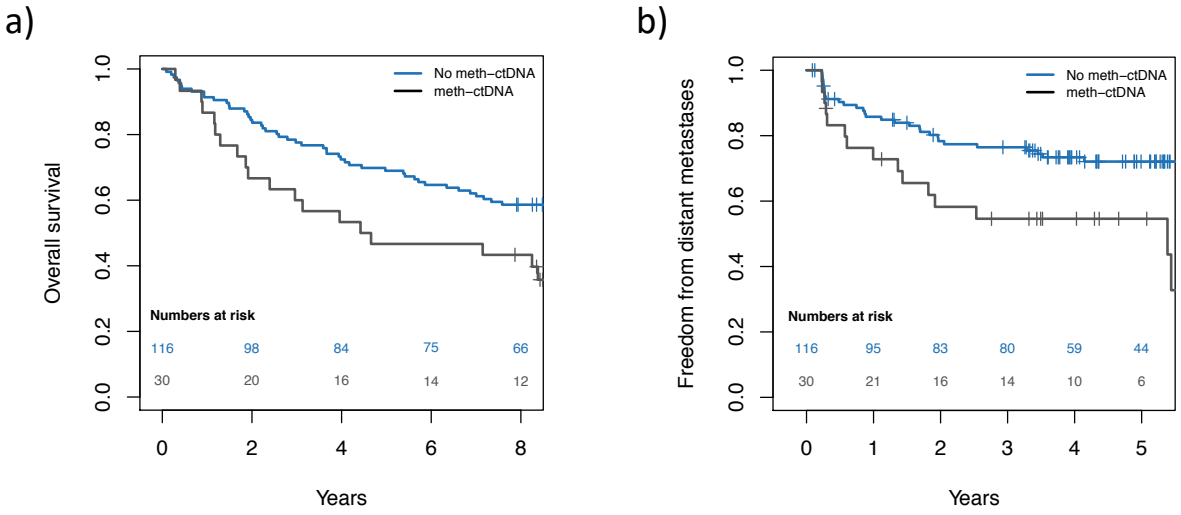

Figure 1: Long term outcomes with/without detectable meth-ctDNA

Overall survival (a) and freedom from distant metastases (b) for patients with locally advanced rectal cancer treated with preoperative chemoradiotherapy. Black curves indicate patients with hypermethylated circulating tumour specific DNA (meth-ctDNA) detected in baseline blood serum samples; blue curves indicate patients with no meth-ctDNA.

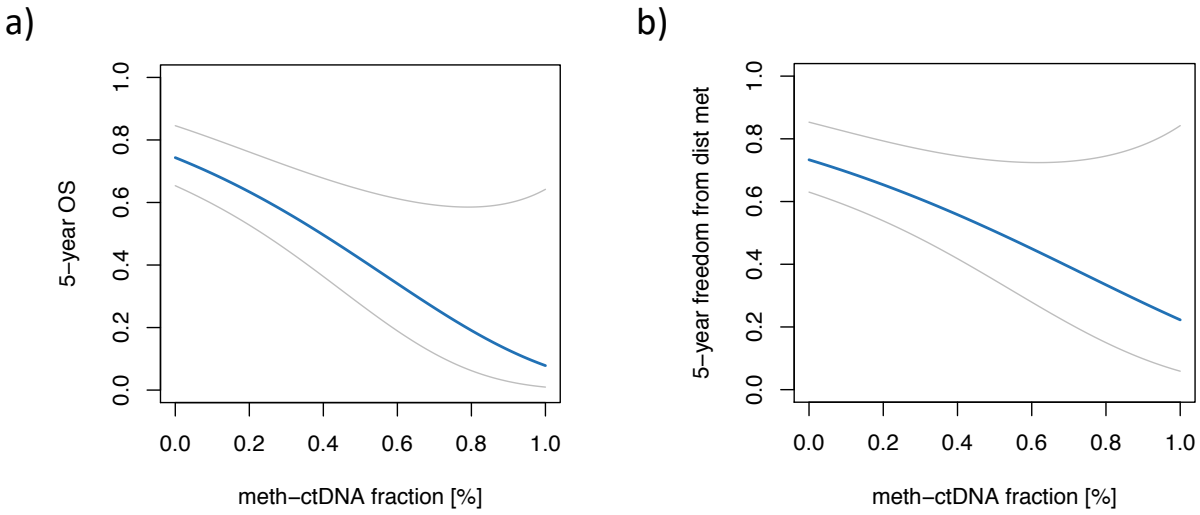

Figure 2: Five-year outcomes as function of fractional abundance of meth-ctNDA

Five-year overall survival (OS, a) and freedom from distant metastases (b) as function of fractional abundance of hypermethylated circulating tumour specific DNA (meth-ctDNA) for an average patient (cT3cN1, 64 years old), based on multivariate Cox model. Solid blue lines show model fit, while light grey lines represent 95% confidence intervals.

Tables

Table 1: Patient characteristics

	Meth ctDNA cohort (n=146)	**Full trial population (n=243)**
Age, years (median, IQR)	64 (57-69)	63 (56-69)
Gender		
- Women	53 (36%)	87 (36%)
- Men	93 (64%)	156 (64%)
cT		
- cT3	118 (81%)	204 (84%)
- cT4	28 (19%)	39 (16%)
cN		
- N0	18 (12%)	26 (11%)
- N1-2	128 (88%)	217 (89%)
Tumour diameter [cm] (median, IQR)	3.5 (3.1-4.1)	3.6 (3.0-4.2)
Tumour length [cm] (median, IQR)	4.8 (4.0-5.6)	4.7 (3.9-5.5)
Distance to mesorectal fascia [mm] (median, IQR)	0 (0-2)	1 (0-2)
Distance from anal verge [cm] (median, IQR)	2.2 (0.5-3.7)	2.4 (0.6-4.0)
Treatment arm		
- Standard	76 (52%)	123 (51%)
- Dose escalated	70 (48%)	120 (49%)

IQR: Interquartile range. Meth-ctDNA: Hypermethylated tumour specific circulating DNA.

Table 2: Multivariate Cox regression analyses

	OS		Distant metastases	
	HR	p value	HR	p value
Hypermethylation presence				
Meth-ctDNA present	2.08 (1.23 – 3.51)	0.007	2.20 (1.19 – 4.07)	0.01
cT4 (vs cT3)	1.50 (0.86 – 2.61)	0.15	1.15 (0.56 – 2.33)	0.71
cN+ (vs cN0)	1.01 (0.50 – 2.06)	0.97	1.26 (0.49 – 3.22)	0.63
Boost treatment arm	1.34 (0.85 – 2.13)	0.21	1.08 (0.61 – 1.91)	0.81
Age (continuous)[†]	1.04 (1.01 – 1.07)	0.02		
Hypermethylation quantitative load				
Meth-ctDNA [‰]*	1.24 (1.13 – 1.35)	<0.0001	1.17 (1.07 – 1.29)	0.001
cT4 (vs cT3)	1.58 (0.91 – 2.76)	0.11	1.22 (0.60 – 2.47)	0.59
cN+ (vs cN0)	0.89 (0.44 – 1.81)	0.76	1.13 (0.44 – 2.90)	0.80
Boost treatment arm	1.42 (0.89 – 2.26)	0.14	1.15 (0.64 – 2.06)	0.64
Age (continuous)[†]	1.04 (1.01 – 1.07)	0.02		

All values in brackets are 95% confidence intervals. Meth-ctDNA: hypermethylated circulating tumour specific DNA. NPY: neuropeptide Y. HR: Hazard ratio. OS: Overall survival. *The HR for quantitative NPY hypermethylation load represents the change in risk with an increase of one ‰ (0.1%) in the fractional abundance of meth-ctDNA in serum cell free DNA. [†]The HR for age represents the change in risk with an increase in age of one year.