

# Reduced folate and serum vitamin metabolites in patients with rectal carcinoma: an open-label feasibility study of pemetrexed with folic acid and vitamin B<sub>12</sub> supplementation

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The objectives of this single-center, open-label, phase II study were to evaluate (a) the feasibility and safety of neoadjuvant administration of pemetrexed with oral folic acid and vitamin B<sub>12</sub> (FA/B<sub>12</sub>) in newly diagnosed patients with resectable rectal cancer and (b) intracellular and systemic vitamin metabolism. Patients were treated with three cycles of pemetrexed (500 mg/m<sup>2</sup>, every 3 weeks) and FA/B<sub>12</sub> before surgery. The reduced folates tetrahydrofolate, 5-methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate were evaluated from biopsies in tumor tissue and in adjacent mucosa. Serum levels of homocysteine, cystathionine, and methylmalonic acid were also measured. All 37 patients received three cycles of pemetrexed; 89.2% completed their planned dosage within a 9-week feasibility time frame. Neither dose reductions nor study drug-related serious adverse events were reported. Reduced folate levels were significantly higher in tumor tissue compared with adjacent mucosa at baseline. After FA/B<sub>12</sub> administration, tissue levels of reduced folates increased significantly and remained high during treatment in both tumor and mucosa until surgery. Serum levels of cystathionine increased significantly compared with baseline after FA/B<sub>12</sub> administration, but then decreased, fluctuating cyclically during pemetrexed therapy.

## Introduction

Specific features of rectal cancer make curative treatment particularly challenging. Depending on the localization of the tumor and blood supply, its surgical removal can be difficult, and the metastatic pattern can differ from that of colon carcinoma. Clinically, local recurrence may have critical implications on a patient's continence function, pain, and quality of life [1–3].

The standard treatment for rectal cancer with a high risk for local recurrence (> stage II) consists of two elements: radiotherapy (RT) and chemotherapy (CT). The optimal combination is under discussion. Radiochemotherapy is the preferred option in many European countries, although the value of preoperative RT alone is currently

being debated [4]. The recommended dosing regimen for RT is 5 × 5 Gy [5]. Multimodality approaches of RT, CT, and surgery have led to enhanced sphincter preservation, tumor control, and reduction of treatment-related morbidity in patients with local rectal cancer [1, 6]. Although not specifically registered for rectal cancer, the fluoropyrimidines, capecitabine, or 5-fluorouracil are commonly used as chemotherapeutic drugs.

Homocysteine and methylmalonic acid levels decreased significantly after FA/B<sub>12</sub> administration, and remained below baseline levels during the study. These results indicate that administration of three neoadjuvant cycles of single-agent pemetrexed, every 3 weeks, with FA/B<sub>12</sub> in patients with resectable rectal cancer is feasible and tolerable. Tissue and serum vitamin metabolism results demonstrate the influence of pemetrexed and FA/B<sub>12</sub> on vitamin metabolism and warrant further study. *Anti-Cancer Drugs* 27:439–446 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.

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pemetrexed in colorectal cancer. A combination study conducted in Australia and Europe showed similar efficacy and safety of pemetrexed and irinotecan (ALIRI) compared with the conventional combination treatment of 5-fluorouracil and irinotecan in patients with metastatic or locally advanced colorectal cancer. However, progression-free survival was significantly shorter in the ALIRI group [9]. Recent pharmacogenomic analyses showed that polymorphisms correlated with the clinical outcome in both treatment groups [10].

Pemetrexed inhibits three folate-dependent enzymes: thymidylate synthase (TS), dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase. As a result, pemetrexed inhibits both pyrimidine and purine synthesis. The uptake of pemetrexed is driven by the reduced folate carrier (RFC) [11] and the proton-coupled folate transporter (PCFT) [12]. In addition, pemetrexed has a high binding affinity for folate receptor- $\alpha$  [13]. Once in the cell, it is rapidly converted to the active polyglutamate derivatives [14]. Polyglutamation prolongs the intracellular retention of pemetrexed and enhances its interaction with TS and other folate-dependent target enzymes [14–17]. These multiple mechanisms of action, along with the observed antitumor activity, suggest that pemetrexed may be efficacious in rectal cancer.

A patient's folate and vitamin B<sub>12</sub> status will affect the toxicity of pemetrexed. Registration studies of pemetrexed in mesothelioma [18], and in second-line non-small-cell lung cancer [19], have demonstrated that supplementation with folic acid and vitamin B<sub>12</sub> (FA/B<sub>12</sub>) significantly reduces hematologic and nonhematologic toxicities, while maintaining efficacy. These results indicate that it is critical to include FA/B<sub>12</sub> with pemetrexed therapy [20,21].

The reduced folates tetrahydrofolate (THF), 5-methyl-tetrahydrofolate (mTHF), and 5,10-methylenetetrahydrofolate (5,10-mTHF) were evaluated in both tumor and adjacent mucosa.

Serum levels of homocysteine, cystathionine, and methylmalonic acid were also evaluated. Homocysteine is an amino acid that can either be remethylated to methionine or converted to cysteine in the trans-sulfuration pathway. The cobalamin-dependent remethylation process requires mTHF as the methyl donor and the enzyme methionine synthase. Elevated blood homocysteine levels can be the result of genetic defects, but they are most frequently due to folic acid deficiency [20,22]. Cystathionine is generated from homocysteine and serine in the synthesis of cysteine, and it is elevated during vitamin B<sub>6</sub> deficiencies [23,24]. Methylmalonic acid is a dicarboxylic acid, and increased levels may indicate a vitamin B<sub>12</sub> deficiency [23].

At the time our study was initiated in 2006, the activity of pemetrexed in rectal cancer had not been extensively

evaluated. For this reason, the primary objective of the study was to evaluate the feasibility of three cycles of pemetrexed in patients with resectable rectal cancer, before surgery. In addition, translational analyses were designed to provide an insight into the influence of pemetrexed therapy and concomitant FA/B<sub>12</sub> supplementation on folate and vitamin metabolism in tumor and adjacent mucosal tissue. This manuscript focuses on the translational research results.

## Methods

### Study design

This was a phase II, single-center, open-label feasibility study of single-agent pemetrexed with FA/B<sub>12</sub> supplementation as neoadjuvant treatment in chemo-naïve patients with newly diagnosed, resectable, nonmetastatic rectal cancer performed from 2006 to 2008. The primary objective was to evaluate three neoadjuvant cycles of pemetrexed before surgery with or without RT in patients with resectable rectal cancer. Feasibility was defined as the ability to receive the total planned dose of pemetrexed with FA/B<sub>12</sub> supplementation, administered for no longer than 9 weeks. This manuscript focuses on the translational secondary objectives to evaluate reduced folate levels in tumor tissue and adjacent mucosa, as well as serum vitamin levels.

### Patients

To be eligible, patients had to be at least 18 years of age and have a pathological or cytological diagnosis of adenocarcinoma of the rectum that was operable and amenable to curative surgery. No prior therapy for rectal cancer was allowed, and patients had to have an Eastern Co-operative Oncology Group performance status of 0 or 1, and an estimated life expectancy of at least 12 weeks. Patients with a second primary malignancy, an uncontrollable, clinically relevant third-space fluid collection, or those unable to interrupt the use of aspirin or other NSAIDs, were excluded from the study. All patients signed informed consent documents, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The study was approved by an institutional ethics review board (<http://www.clinicaltrials.gov> identifier: NCT00330915).

### Treatment plan

Patients were treated with three cycles of pemetrexed 500 mg/m<sup>2</sup> given on day 1, every 3 weeks, with FA/B<sub>12</sub> supplementation. Patients began vitamin supplementation with B<sub>12</sub> injections (1000  $\mu$ g) starting ~1–2 weeks before the first pemetrexed dose, and patients received daily oral folic acid (800  $\mu$ g) starting at least 5 days before the first pemetrexed dose. FA/B<sub>12</sub> supplementation continued for 3 weeks after the last pemetrexed dose. Preoperative or postoperative RT was optional.

### Baseline, efficacy, and safety assessments

Within 4 weeks of enrollment, tumors were measured using a radiologic method of measurement at the discretion of the investigator. Tumor assessments could be repeated before surgery using the same method as at baseline.

In addition to feasibility, efficacy assessments included pathological complete response (defined as the absence of any tumor cells), sphincter-saving surgery, and complete tumor resection rate (defined as radical tumor excision by total or partial resection of the mesorectum, including safety margins, preserving the anal sphincter system). Toxicities were assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE, version 3) [25], during the study and the 30-day post-therapy follow-up.

### Assessments of reduced folates and serum vitamin metabolites

Biopsies of tumor tissue and adjacent normal mucosa (~200 mg each) were obtained from each patient at three different time points: at baseline (before the patient started FA/B<sub>12</sub> supplementation), before the first pemetrexed administration (after the patient started FA/B<sub>12</sub> supplementation), and at surgery. All biopsies were obtained from an easily accessible site. The biopsies were snap-frozen in liquid nitrogen and stored at -70°C until analyzed. Surgical and pathological samples were examined for levels of the reduced folates THF, mTHF, and 5,10-mTHF. Blood samples for the analysis of vitamin metabolites were defibrinated, centrifuged at 1500g for 15 min, and the serum samples were stored at -70°C until analyzed. Serum levels of homocysteine, cystathionine, and methylmalonic acid were measured at baseline, before the first pemetrexed administration on days 8 and 15 of cycle 1, before pemetrexed administration on day 1 of cycles 2 and 3, and before surgery. The postbaseline biopsies and serum samples obtained before the first pemetrexed administration were taken at least 5 days after the start of FA/B<sub>12</sub> supplementation.

### Detection of reduced folates in tissue

A liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) method was used to evaluate levels of the reduced folates THF, mTHF, and 5,10-mTHF in tumor tissue and adjacent mucosa separately [26]. The extraction method involved homogenization, heat treatment, and folate conjugate treatment to hydrolyze polyglutamyl folates to monoglutamyl folates.

### Statistical analyses

The sample size calculation assumed that at least 85% of patients would receive the total planned dose, and the feasibility rate would be above 60%. To provide at least 91% power and a 95% confidence interval (CI) of

70.5–95.3%, it was determined that 36 eligible patients were needed. In addition, if a dropout rate of 10% occurred, 40 patients should be enrolled.

Feasibility and safety were evaluated for all patients who received at least one dose of study drug. The primary endpoint was the feasibility rate defined as the number of patients who received the planned total dose divided by the number of patients qualified for feasibility analysis. A two-sided exact binomial test was used to evaluate rates of feasibility and pathologic complete response, and the two-sided 95% CIs were calculated. At each time point, relative differences from baseline (before FA/B<sub>12</sub> supplementation) in reduced folate levels were described for tumor tissue and paired normal mucosal tissue. Baseline versus postbaseline means were compared using the Wilcoxon signed-rank test for pairwise comparisons. Statistical values were considered significant at *P* value less than 0.05.

## Results

### Baseline characteristics

Thirty-seven patients with a median age of 60 years entered the study and qualified for efficacy and safety analyses. Patients were predominantly male (70%) and White (97%), and all had an Eastern Co-operative

**Table 1** Baseline characteristics and surgery summary

Parameters	<i>n</i> (%)
Baseline characteristics	
Age [median (range)] (years)	61.0 (34.0–82.0)
Sex	
Male	26 (70.3)
Female	11 (29.7)
Race	
White	36 (97.3)
East Asian	1 (2.7)
BMI [mean (SD)] (kg/m <sup>2</sup> )	80.4 (15.2)
ECOG performance status 0	37 (100.0)
Histopathological subtype adenocarcinoma	37 (100.0)
Histopathological diagnosis grade	
Well differentiated	3 (8.1)
Moderately differentiated	25 (67.6)
Poorly differentiated	3 (8.1)
Not applicable	4 (10.8)
Unspecified	2 (5.4)
Time since initial pathological diagnosis [mean (SD)] (weeks)	3.2 (1.7)
Surgery summary	
Location of primary tumor	
Lower rectum	15 (40.5)
Middle rectum	16 (43.2)
Upper rectum	6 (16.2)
Total mesorectal excision	37 (100.0)
TNM staging at surgery: primary tumor	
T1	3 (8.1)
T2	9 (24.3)
T3	22 (59.5)
T4	3 (8.1)
TNM staging at surgery: regional lymph nodes tumor	
N0	22 (59.5)
N1	10 (27.0)
N2	5 (13.5)

BMI, body mass index; ECOG, Eastern Co-operative Oncology Group; *n*, number of patients; TNM, tumor, node, metastases.

Oncology Group performance status of 0 (Table 1). Most of the patients (68%) had a histopathological diagnosis grade of G2 rectal carcinoma (moderately differentiated), whereas 8% of patients had G1 (well differentiated) and 8% had G3 (poorly differentiated) rectal carcinoma.

### Tumor assessment, radiotherapy, and surgery summary

Tumors were measured with MRI in 36 patients and with computed tomography (including spiral computed tomography) in one patient. Metastatic disease could be excluded in all 37 patients. Twenty-eight (75.6%) patients received RT after CT. All 37 patients underwent surgery. The majority of the tumors were located in the lower (40.5%) or in the middle (43.2%) rectum (Table 1).

### Feasibility and other efficacy results

All patients were able to receive three cycles of CT; 33 patients completed their planned dosage within the 9 weeks, resulting in a feasibility rate of 89.2% (95% CI: 74.6–97.0). Thus, the primary objective was met.

All patients had complete tumor resection (R0). One pathologically proven complete response was reported. Eleven out of the 13 planned patients had abdominoperineal resection. All others [ $n=25$  (67.6%)], with the exception of one patient, had sphincter-saving surgery.

### Safety results

Neither dose reductions nor study drug-related serious adverse events were reported. Seven patients (18.9%) experienced grade 3/4 neutropenia. No patient deaths were reported during this study.

### Reduced folate levels in tumor tissue and adjacent normal mucosa

At baseline (before FA/B<sub>12</sub> supplementation), THF, mTHF, and 5,10-mTHF were significantly higher in tumor tissue compared with adjacent mucosa (Table 2).

After initiation of FA/B<sub>12</sub> supplementation (before the first dose of pemetrexed), levels of all three reduced folates significantly increased from baseline in both tumor tissue and adjacent mucosa (Table 3). In addition, levels of all three reduced folates were significantly higher in tumor tissue compared with adjacent mucosa during the entire treatment period (Table 2).

At the time of surgery, despite three cycles of pemetrexed therapy, the quantitative levels of all metabolites were numerically similar to the values after supplementation but before the first dose of pemetrexed. At the time of surgery, levels of all three reduced folates remained significantly higher in tumor tissue compared with adjacent mucosa (Table 2), and all levels remained significantly higher than baseline. For two patients, not all markers were measured because of insufficient tissue samples.

### Serum levels of homocysteine

The mean homocysteine level at baseline (before FA/B<sub>12</sub> supplementation) was 10.77  $\mu\text{mol/l}$ . After FA/B<sub>12</sub> supplementation (before the first dose of pemetrexed), homocysteine levels decreased significantly from baseline (Table 4). Throughout the course of treatment, postbaseline homocysteine levels remained significantly lower than those at baseline; however, the decreases that occurred after pemetrexed therapy were slightly smaller than the decreases observed after FA/B<sub>12</sub> supplementation but before pemetrexed therapy (Table 4). The change from baseline for each of the time points is shown.

### Serum levels of cystathionine

The mean cystathionine level at baseline (before FA/B<sub>12</sub> supplementation) was 231.29  $\mu\text{mol/l}$ . After FA/B<sub>12</sub> supplementation (before the first dose of pemetrexed), cystathionine levels increased significantly from baseline

**Table 2** Tetrahydrofolate, methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate levels in tumor versus adjacent mucosa at baseline, after supplementation, and at surgery

Parameters	Baseline <sup>a</sup>	After supplementation	At surgery
<b>THF</b>			
Number of patients, T/M	T: 37/M: 36	T: 35/M: 35	T: 37/M: 37
Levels T/M [mean (SD)] (pmol/g) <sup>b</sup>	T: 584 (256)/M: 463 (256)	T: 901 (365)/M: 620 (211)	T: 906 (456)/M: 704 (292)
Difference T/M [mean (SD)]	123 (294)	292 (408)	202 (459)
P-value <sup>c</sup> T/M	0.006	$\leq 0.001$	0.012
<b>mTHF</b>			
Number of patients, T/M	T: 37/M: 37	T: 36/M: 36	T: 37/M: 37
T/M [mean (SD)] (pmol/g) <sup>b</sup>	T: 436 (316)/M: 352 (199)	T: 903 (425)/M: 640 (286)	T: 1065 (676)/M: 639 (367)
Difference T/M [mean (SD)]	84 (227)	263 (352)	426 (585)
P-value <sup>c</sup> T/M	0.051	$\leq 0.001$	$\leq 0.001$
<b>5,10-mTHF</b>			
Number of patients, T/M	T: 37/M: 37	T: 36/M: 36	T: 37/M: 37
Levels T/M [mean (SD)] (pmol/g) <sup>b</sup>	T: 1018 (506)/M: 830 (610)	T: 2067 (1514)/M: 1405 (802)	T: 1871 (1052)/M: 1302 (925)
Difference T/M [mean (SD)]	188 (718)	662 (1272)	568 (1352)
P-value <sup>c</sup> T/M	0.018	$\leq 0.001$	0.004

5,10-mTHF, 5,10-methylenetetrahydrofolate; mTHF, methyltetrahydrofolate; T/M, tumor/mucosa; THF, tetrahydrofolate.

<sup>a</sup>Baseline = before FA/B<sub>12</sub> supplementation.

<sup>b</sup>pmol/g wet tissue weight.

<sup>c</sup>Wilcoxon signed-rank test was used for all pairwise comparisons.

**Table 3** Changes from baseline<sup>a</sup> in tetrahydrofolate, 5-methyltetrahydrofolate, and 5,10-methylenetetrahydrofolate levels (pmol/g)<sup>b</sup> in tumor and adjacent mucosa after FA/B<sub>12</sub> supplementation and at surgery

Parameters	After FA/B <sub>12</sub> supplementation		At surgery	
	Mucosa vs. baseline mucosa	Tumor vs. baseline tumor	Mucosa vs. baseline mucosa	Tumor vs. baseline tumor
THF [mean (SD)]	168 (294)	308 (401)	247 (412)	322 (493)
<i>P</i> -value <sup>c</sup>	< 0.001	< 0.001	< 0.001	< 0.001
mTHF [mean (SD)]	285 (274)	458 (491)	287 (432)	629 (621)
<i>P</i> -value <sup>c</sup>	< 0.001	< 0.001	< 0.001	< 0.001
5,10-mTHF [mean (SD)]	567 (897)	1031 (1561)	472 (1264)	852 (1029)
<i>P</i> -value <sup>c</sup>	< 0.001	< 0.001	0.010	< 0.001

5,10-mTHF, 5,10-methylenetetrahydrofolate; FA/B<sub>12</sub>, folic acid and vitamin B<sub>12</sub>; mTHF, methyltetrahydrofolate; THF, tetrahydrofolate.

<sup>a</sup>Baseline = before FA/B<sub>12</sub> supplementation.

<sup>b</sup>pmol/g wet tissue weight.

<sup>c</sup>Wilcoxon signed-rank test was used for testing changes from baseline.

**Table 4** Changes from baseline in serum levels of metabolites by time point

Time point	Baseline <sup>a</sup> value (μmol/l) (SD)	Change from baseline	<i>P</i> -value <sup>b</sup>
Homocysteine	10.77 (2.33)	Mean (SD) (μmol/l)	
After supplementation		-4.77 (1.96)	< 0.001
Cycle 1, day 8		-4.17 (1.81)	< 0.001
Cycle 1, day 15		-3.49 (1.75)	< 0.001
Cycle 2, day 1		-4.63 (2.07)	< 0.001
Cycle 3, day 1		-4.59 (2.28)	< 0.001
Cycle 3, day 15 (before surgery)		-3.86 (2.00)	< 0.001
Cystathionine	231.29 ± 104.51	Mean (SD) (nmol/l)	
After supplementation		206.24 (214.14)	< 0.001
Cycle 1, day 8		24.44 (86.37)	0.150
Cycle 1, day 15		12.31 (76.95)	0.289
Cycle 2, day 1		176.58 (182.65)	< 0.001
Cycle 3, day 1		154.79 (174.39)	< 0.001
Cycle 3, day 15 (before surgery)		-7.75 (112.19)	0.967
Methylmalonic acid	186.19 ± 53.32	Mean (SD) (nmol/l)	
After supplementation		-24.00 (44.11)	0.012
Cycle 1, day 8		-21.96 (51.84)	0.042
Cycle 1, day 15		-18.46 (53.60)	0.086
Cycle 2, day 1		-11.08 (54.18)	0.427
Cycle 3, day 1		-9.58 (43.46)	0.608
Cycle 3, day 15 (before surgery)		-20.71 (45.94)	0.035

<sup>a</sup>Baseline = before FA/B<sub>12</sub> supplementation.

<sup>b</sup>Wilcoxon signed-rank test was used for pairwise comparisons.

(Table 4). On days 8 and 15 of cycle 1, the cystathionine levels remained higher than baseline (before FA/B<sub>12</sub> supplementation); however, the increase was not significant (Table 4). After completion of cycles 1 and 2 of pemetrexed therapy, the increases in cystathionine were again significant (Table 4). On day 15 of cycle 3 (before surgery), cystathionine levels had decreased from baseline, but not to a significant degree (Table 4).

### Serum levels of methylmalonic acid

The mean methylmalonic acid level at baseline (before FA/B<sub>12</sub> supplementation) was 186.19 μmol/l. After FA/

B<sub>12</sub> supplementation (before the first dose of pemetrexed), methylmalonic acid levels decreased significantly from baseline (Table 4). After initiation of pemetrexed therapy, throughout the course of the study, and before surgery, methylmalonic acid levels remained lower than baseline; however, the differences were not significant except at cycle 1, day 8, and cycle 3, day 15 (Table 4).

### Discussion

In this study, single-agent pemetrexed with FA/B<sub>12</sub> supplementation was given as neoadjuvant CT in patients with resectable rectal cancer. RT was optional. All 37 patients were able to receive three cycles of CT; 89.2% completed their planned dosage within the defined 9-week feasibility time frame, and therefore the primary objective was met. All patients had complete tumor resection and 67.6% had sphincter-saving surgery. There were no dose reductions or study drug-related serious adverse events. There was only one kind of drug-related grade 3/4 toxicity (neutropenia) that was reported in seven patients (18.9%).

This is one of the first studies to use the LC-MS/MS method to evaluate intracellular folates in tumor and adjacent mucosa tissue of patients with rectal carcinoma undergoing neoadjuvant therapy. In addition, this study was the first to evaluate changes of intracellular reduced folates and systemic vitamin B<sub>12</sub> metabolites following FA/B<sub>12</sub> supplementation and pemetrexed therapy.

Before FA/B<sub>12</sub> supplementation, levels of THF, mTHF, and 5,10-mTHF were all significantly higher in the tumor tissue compared with adjacent mucosa. These findings may indicate a differential regulation of reduced folate levels in tumor tissue and the adjacent mucosa. This may be explained by a variety of mechanisms, including dysregulated folate transport receptors or carriers such as PCFT and RFC, differential functioning of export pump systems in tumor cells [27,28], and/or variable levels and activity of intracellular enzymes. In a

previous study, the folylpolyglutamate synthase:γ-glutamyl hydrolase ratio indicating the folate turnover rate was significantly lower in tumor tissue than in adjacent mucosa [26]. Inside the cells, folates are polyglutamated by folylpolyglutamate synthase. In contrast to folylmonoglutamates, polyglutamates are retained in the cells [29] and are considered to be better substrates for most cellular enzymes than monoglutamates [30]. Thus, different levels of these folate-associated enzymes in the tumor tissue affect the rates of polyglutamation and hydrolysis of polyglutamates, and, in turn, may indirectly affect DNA synthesis, DNA repair, and DNA methylation [26].

After initiation of FA/B<sub>12</sub> supplementation (before the first dose of pemetrexed), all three reduced folates (THF, mTHF, and 5,10-mTHF) increased significantly in both tumor tissue and adjacent mucosa, and all three reduced folates remained significantly higher in tumor tissue compared with adjacent mucosa. After pemetrexed therapy, the folate levels remained increased from baseline; the differences between adjacent mucosa and tumor tissue remained significantly different. These results are consistent with the expectation that folate supplementation would increase the levels of the three reduced folates in both the tumor tissue and adjacent mucosa [14, 31], and demonstrate that for both types of tissue the transcellular folate transport systems were not saturated. These results support previous findings that THF is the primary intracellular metabolite after folic acid supplementation (before reduced folates are built) and that conversion of folic acid to intracellular folates is a multi-step process that involves many enzymes and membrane transport processes [14,31].

The reduced folate results from this study are also consistent with what is known about the properties of pemetrexed. The uptake of pemetrexed is mainly driven by the RFC and PCFT and pemetrexed is then very rapidly converted to active polyglutamate derivatives that are retained for long intervals and reach high levels within the cell. Pemetrexed is a potent inhibitor of its major target enzyme TS, sensitive to folate levels within cells, and inhibits additional enzyme targets [14]. Pemetrexed suppresses THF-dependent reactions by directly blocking the THF cofactor-dependent enzymes, without an effect on the level of cellular THF pools [14]. As a result, there is no redistribution of folates within cells and the levels of folate substrates are not changed [14].

The elevation of serum levels of homocysteine, cystathionine, and methylmalonic acid reflect disturbances in folate metabolism and deficiencies in vitamins B<sub>6</sub> and B<sub>12</sub>. These three markers act as early and sensitive surrogate indicators for blood levels of the vitamins [20]. For the rectal cancer patients in our study, the median baseline values of all three metabolites fell within other

reference ranges published for general populations [23, 24,32]. After FA/B<sub>12</sub> supplementation, the reduction of homocysteine levels was comparable with other published data for patients who were only supplemented with FA/B<sub>12</sub> and were not treated with CT [33]. As such, the baseline levels of homocysteine that we observed were comparable to those reported for the general population [33], and the observed response to FA/B<sub>12</sub> supplementation by comparable reduction of homocysteine may suggest a universal systemic mechanism in humans as a response to FA/B<sub>12</sub> supplementation.

Vitamin B<sub>6</sub> acts as a cofactor for cystathionine-β-synthase that converts homocysteine to cystathionine [32]. This conversion results in a decrease of homocysteine and an increase of cystathionine. In our study, after FA/B<sub>12</sub> supplementation, there was a significant decrease in both homocysteine and methylmalonic acid levels accompanied by an increase of cystathionine levels, which is consistent with previously published results [32]. After three cycles of pemetrexed therapy before surgery, both homocysteine and methylmalonic acid levels predictably remained decreased. However, cystathionine levels also decreased to nearly baseline levels within the first 2 weeks of the first pemetrexed therapy cycle (cycle 1, day 15). After 3 weeks of pemetrexed therapy (before cycle 2), cystathionine levels again increased. After three full cycles of pemetrexed therapy (before surgery), cystathionine levels had decreased from baseline, but not significantly. This cyclical phenomenon has not been described previously. Most literature describes the increase in cystathionine after an increase of folate or protein uptake [24], but the decrease of cystathionine levels following pemetrexed therapy has not been described. The results observed here may reflect an intracellular effect of pemetrexed during CT in which a 'recovery' appears to occur after some time, as demonstrated by the increase of cystathionine before the next cycle.

This study has limitations, including the number of biopsies taken and the type of tumor studied. For ethical reasons, only three biopsies were taken from each patient. The first time point for tissue analysis (i.e. before FA/B<sub>12</sub> supplementation) was chosen because the influence of FA/B<sub>12</sub> supplementation was expected to be significant [22]. The evaluation of the influence of pemetrexed therapy on tumor tissue compared with adjacent mucosa was performed only at surgery. This was done to avoid complications due to biopsies during CT. Therefore, there are no data to describe changes in intracellular folate levels by pemetrexed therapy throughout the three cycles of CT. However, at the time of surgery, relative changes from baseline of mean values in mucosa or tumor tissue of THF, mTHF, and 5,10-mTHF remained significantly higher compared with baseline, supporting a continuous influence of folate supplementation in the mucosa, as well as in tumor

tissue. It is not known whether the quantitative changes are tumor specific for rectal carcinoma, or whether these results may be translated to other tumor types, in which folate levels and responses may be different. There are currently no data to enable comparison with the folate metabolism for other tumor types.

In conclusion, in this open-label, phase II, feasibility study of pemetrexed conducted in chemo-naïve patients with resectable rectal cancer, all 37 patients enrolled were able to receive three cycles of CT and 89.2% of patients received the treatment as planned within a defined 9-week feasibility time frame. Thus, the primary objective was met. All patients had complete tumor resection and more than 60% had sphincter-saving surgery. Neither dose reductions nor study drug-related serious adverse events occurred. These results indicate that three neoadjuvant cycles of single-agent pemetrexed at 500 mg/m<sup>2</sup> plus FA/B<sub>12</sub> supplementation every 3 weeks before surgery with or without subsequent RT were feasible and safe in patients with resectable rectal cancer.

In addition, serum and tissue markers of vitamin metabolism were evaluated throughout the study. Metabolites measured during this study were influenced by FA/B<sub>12</sub> supplementation and pemetrexed therapy. At all time points, analyzed levels of the reduced folates THF, mTHF, and 5,10-mTHF were higher in tumor than in mucosal tissue, which might be attributed to a dysregulation in folate metabolism, as suggested previously. Reduced folate levels increased after FA/B<sub>12</sub> supplementation and remained higher than baseline in tumor and adjacent mucosal tissue.

After FA/B<sub>12</sub> supplementation, serum homocysteine and methylmalonic acid levels decreased and remained lower than baseline throughout the study. Cystathionine levels fluctuated cyclically first increasing and then decreasing within the first 2 weeks of the first pemetrexed cycle and then increasing again, probably indicating a recovery effect following pemetrexed CT. Thus, pemetrexed might have had an influence on cystathionine levels as shown by the observed ‘recovery’ cycle. The results, we observed, provide valuable insights into the influence of pemetrexed and FA/B<sub>12</sub> supplementation on vitamin metabolism and prompt further study of these mechanisms.

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## Conflicts of interest

Dr Clemens Stoffregen is an employee of Eli Lilly and Company, and as such has stock ownership in said company; Dr Bengt Gustavsson received a research grant from Eli Lilly; Maria Tångefjord was an employee of Eli Lilly Sweden, Solna, Sweden. For the remaining authors there are no conflicts of interest.

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