On 5-fluorouracil therapy of colorectal cancer

Factors associated with prognosis and adverse reactions

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DOCTORAL THESIS

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INTRODUCTION

Colorectal cancer is the second most common malignant tumour in Western Europe and Northern America after breast cancer in women and lung cancer in men affecting 7% of the population and ranks as the second leading cause of cancer-related mortality.[1, 2] The annual incidence in Denmark is about 3300 cases that constitute 14% of all cancers.[3]

Colorectal tumours are staged according to the TNM-system into stage I (T1-2N0M0), II (A:T3N0M0, B:T4N0M0), III (A:T1,T2N1M0; B:T3,T4N1M0; C: anyTN2M0) and IV (anyTanyNM1). Other prognostic factors are histopathological type, degree of differentiation, perineural invasion, venous invasion, tumour budding and peritumoural inflammation. Also ileus and tumour perforation ahead of or during surgery are associated with poor prognosis.[IV, 4, 5]

In a Danish population study about half of the patients had distant metastases in the peritoneal cavity, liver or lung at the time of diagnosis or during follow-up.[6] Treatment with curative intent entails complete resection of the primary tumour and metastases. About 15% of patients having liver metastases are resectable at the time of diagnosis while another 10% may become resectable using e.g. chemotherapy or embolisation.[7]

Patients who are completely resected and at high risk of recurrence based on tumour stage and histopathologic characteristics (i.e. stage II T4, poor differentiation, perineural invasion, venous invasion, Ileus, perforation, and stage III and IV) are offered adjuvant chemotherapy.

Initially treatment with adjuvant 5-FU substantially reduced the recurrence rate and improved survival following complete resection of stage III colon cancer.[8] Addition of folinic acid and oxaliplatin has further reduced the 3-year recurrence rate and improved the survival.[9, 10] The benefit of chemotherapy in terms of improved recurrence free survival also applies to stage II colon cancer.[11]

Patients who cannot be completely resected are incurable and are offered palliative chemotherapy in order to control symptoms, maintain or improve quality of life and prolong symptom-free and overall survival.[12] 5-fluorouracil in combination with folinic acid and oxaliplatin is the mainstay of treatment in this setting.[13] Together with newer cytostatics such as irinotecan and incorporation of the novel targeted agents cetuximab and bevacizumab the chemotherapy has yielded substantial improvements in the management of metastatic colorectal cancer. In consequence, median overall survival from diagnosis may now approach 18-21 months.[14]

While the performance status and comorbidity of the patients is important to the feasibility of chemotherapy other factors relating to the tumour and the patient may be of significance to the efficacy and tolerability of the 5-FU treatment.

AIM

The aim of this thesis was to summarize the results of a series of investigations of tumour and patient characteristics and their association with the outcome and adverse reactions following 5-FU based chemotherapy of colorectal cancer. Three aspects of this topic were studied.

Enzymes related to pyrimidine homeostasis and 5-FU efficacy are significant to the tumor biology and sensitivity to chemotherapy.

Also enzymes involved in cancer cell invasion and host versus tumour immune response are of significance to cancer progression. Because the enzyme activities vary due to inherited traits or as a result of gene deregulation from microsatellite instability or chromosomal aberration these relationships were included in the studies.

Although the incidence of colorectal cancer increases with age the benefit and tolerance of such therapy in elderly ≥75 years is generally not well described. Therefore the outcome and toxicity experienced following adjuvant and palliative chemotherapy were investigated according to this age cut-off.

Special focus was directed to the adverse reaction of 5-FU on the heart in order to elucidate the pathophysiology, the long-term effect on cardiac function and the influence on the myocardial neuroendocrine axis by means of objective measures for ventricular hemodynamics and myocardial metabolism. In addition the significance of cardiovascular disease and renal impairment as potential risk factors for development of 5-FU cardiotoxicity have been investigated.

STUDY POPULATIONS AND METHODS

This thesis included the studies of three cohorts of patients who received chemotherapy for colorectal cancer at Department of Oncology, Rigshospitalet.

Cohort 1

The first group of retrospectively studied patients received first line palliative chemotherapy for inoperable colorectal cancer from January 2001 to September 2004. The therapy was intercurrently shifted from capecitabine 1250 mg × m^{-2} bid for two weeks every three weeks as monotherapy (n=178) to a combination with oxaliplatin 130 mg × m^{-2} on day one (XELOX) (n=82). Information on patient and tumour specific characteristics was obtained from surgical, pathological and oncological records. Data on survival were obtained from the national Central Registry on death recording. Blood count measurements, blood biochemistry, weight and toxicity evaluations were assessed at baseline and after each chemotherapy course. Toxicity evaluations were graded according to Common Toxicity Criteria (CTC) version 2.0. Disease status was assessed according to WHO response criteria at least every three treatment courses.

Primary objectives of the study were to compare capecitabine or XELOX treated patients using age cutoff 70 years, and capecitabine treated patients using age cutoff 75 years, with respect to progression free survival, overall survival times, response rates, biochemical function tests, performance status (Eastern Cooperative Oncology Group), weight change, tolerable dose and number of treatment courses received.

Cohort 2

The second group of retrospectively studied patients received adjuvant chemotherapy from February 1996 to December 2003 following complete resection of stage II (n=38), III (n=266) and IV (n=36) colorectal cancer. The chemotherapy was according to the Mayo Clinic regimen using 5-FU 425 mg × m^{-2} and leucovorin 20 mg × m^{-2} intravenous bolus on day 1-5 each 28 days for six treatment courses. Information on the chemotherapy, patient and tumour specific characteristics was obtained from surgical, pathological and oncological records. Data on recurrence and survival were obtained from surgical records and from the National Central Registry on hospital admissions and death recording.

The objectives of the study of patients with stage III colorectal cancer were to compare by age group with 75 year as cut-off the recurrence free survival rate and overall survival rate, toxicity levels, biochemical function tests, performance status and weight change, tolerable dose and number of chemotherapy courses received.

In addition various biochechemical analyses were performed on the primary tumours of stage II-IV colorectal cancer and associated with the recurrence free and overall survival of the patients. Hence the immunoreactivity of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase in the primary tumours of stage II and III disease was associated with outcome and induced toxicity following adjuvant chemotherapy.

The number of gene copies per nucleus of thymidylate synthase, thymidine phosphorylase and dihydrofolate reductase were assessed by fluorescence in situ hybridisation (FISH) using specific peptide nucleic acid probes in the primary tumour of stage II-IV disease and associated with the survival of patients.

Microsatellite instability in five reference loci and the immunoreactivity of four mismatch repair enzymes (hMSH2, hMSH6, hMLH1 and hPMS2) were assessed in primary tumours of stage II-IV disease and associated with outcome. The level of the collagenase Matrix Metalloproteinase 9 and its physiologic inhibitor Tissue Inhibitor of Metalloproteinases 1 were assessed by immunohistochemistry in cancer cells and supporting stroma cells of the primary tumours of stage II-IV disease and associated with outcome.

Cohort 3

The third group of prospectively studied patient (n=106) received adjuvant chemotherapy from August 2005 to September 2008. The chemotherapy was according to the FOLFOX-4 regimen consisting of oxaliplatin 85 mg × m^{-2} 2 hours infusion, folinic acid 400 mg × m^{-2} infusion and 5-FU 400 mg × m^{-2} bolus injection followed by 2400 mg × m^{-2} flat continuous infusion for 46 hours repeated every 2 weeks for intended 12 treatment courses. This study aimed to clarify the pathophysiology, risk factors and long-term effects of 5-FU influence on the heart. Serial measurements of systolic and diastolic features of the left ventricle by radionuclide ventriculography, plasma levels of brain natriuretic peptide (NT-proBNP) and lactic acid and ECG were sampled before chemotherapy, immediately after a treatment infusion and at follow-up two weeks after cessation of intended 12 treatment courses, and further evaluated by multivariate regression analysis including cardiovascular history and its risk factors. Furthermore the influence of 5-fluorouracil (5-FU) on the vascular endothelium was assessed using the levels of plasma von Willebrand factor (vWf), Urine Albumin to Creatinine Ratio (UACR), coagulation factor II+IIV+X and fibrin D-dimer and the association with 5-FU induced heart ischemia were evaluated.

The local research ethics committee approved these studies.

STATISTICAL METHODS

Relationships

Ratios were compared using Fisher's exact test or Chi-

square test. Non-parametric data were compared by Mann-Whitney U-test or Kruskal-Wallis test. Param-etric distributions were compared by Student's t-test or two-way analysis of variance (ANOVA) of repeated measures, based on sampling time (baseline, 5-FU treatment and follow-up) and groups (5-FU cardiotoxicity or not). A significant test for difference between repeated measures indicated a 5-FU treatment effect, whereas significant interaction between treatment and groups indicated different response to 5-FU treatment between groups.

Because the measuring range of vWf was truncated at >3 kU/l the repeated assessments were compared using non-parametric Friedmans analysis of variance. The ratios of individual values of vWf being inside versus outside the reference interval in patient subsets having cardiotoxicity or cardiovascular disease were compared using non-parametric statistics.

Kappa statistics was applied to assess correspondence between analyses for microsatellite instability and mismatch repair proteins.

Univariate survival analyses

Survival time was calculated from the time point of the complete resection of the tumour. Efficacy variables were relapse free survival (RFS), defined as time to relapse of primary disease or death or censoring, whichever occurred first, and death from any cause for overall survival (OS).

The distributions of RFS and OS were depicted as cumulated proportion survival using Kaplan-Meier methodology. Surviving patients without recurrence in the study period were censored at last follow up in the Central Registry. Univariate associations were tested using log-rank statistic and the actual size of difference as Hazard Ratio (HR) with 95% Confidence Interval.

Multivariate survival analyses

Cox multivariate proportional hazard linear regression was used to evaluate the association of clinical, pathological and biochemical characteristics to recurrence free survival and overall survival. All candidate prognostic variables were initially entered into the full model. Non-significant (P>0.1) variables were subsequently excluded backwards from the model (step-down variable selection). Graphical methods were used to ascertain underlying model assumptions as proportional hazards. The prognostic value of a given characteristic was quantified by the hazard ratio (HR) with 95% Confidence Interval. Significant variables were checked for interaction.

Multivariate linear regression analyses

Multiple linear regression analysis was performed regressing NT-proBNP change from chemotherapy on clinicopathological parameters based on their association with cardiovascular disease. The dependent response variable was defined as ΔNT-proBNP (chemotherapy-baseline). The response variable was logarithmically transformed into approximate normal distribution to comply with statistical modelling assumptions. The contender predictor variables gender, age, cardiovascular disease, hypercholesterolemia, hypertension, diabetes, body mass index, smoking, and renal function were included forward stepwise in the model for evaluation, that each added predictor to the model the response variation thus explained was larger (P≤0.1 at entry) than the residual variation. As some patients received reduced 5-FU dose due to adverse events or reduced organ function the dose variable was kept in the model (forced entry) to adjust for this fact. Then only significant (P<0.05) predictors by backward stepwise regression were retained in the final model. The predictive capacity of the final model (goodnes-of-fit) is the proportion of the total variation that can be explained by the regression, which is expressed as the adjusted R^2.

Values of P<0.05 in two-sided tests were regarded significant.

Software

Statistics was performed with Statistica software release 6 (Statsoft Inc. Tulsa, OK, USA).

FACTORS ASSOCIATED WITH PROGNOSIS AND ADVERSE REACTIONS

5-FU is the cornerstone of adjuvant and palliative chemotherapy. The chemotherapy however is complicated by wide individual variability in antitumour efficacy and host toxicity. Determinants for some of this variation may be biochemical characteristics related to the tumours and clinical and genetic characteristics related to the patients.

Tumour biomarkers

Being a prodrug 5-FU requires activation by thymidine phosphorylase (TP). The major part of an administered dose of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) into 5,6-dihydrofluorouracil, before it can be converted to the active metabolite fluoro-2'-deoxyuridine-monophosphate that irreversibly inhibits thymidylate synthase. Dihydrofolate reductase (DHFR) provides reduced 5,10-methylenetetrahydrofolate for enhancing inhibition of Thymidylate Synthase (TS). Varying activity of TS,[IV, 4, 15, 16] DPD,[17] TP,[18-21] and DHFR[22] derives from inherited characteristics such as polymorph tandem repeat and single nucleotide polymorphism (SNP) in the promoter enhancer region of TS,[23] and individual differences in DPD[17] and TP[18] expression and short sequence deletions and SNP for DHFR[24].

In addition about 15-20% of colorectal cancers have

FIGURE 1

Recurrence free survival according to tumoural TS intensity (0-1 versus 2-3) in 303 patients after complete resection of colorectal cancer stage II-III and adjuvant chemotherapy. Censored data (+).[IV]

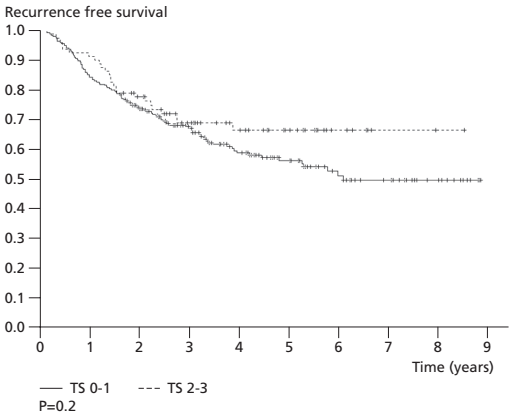
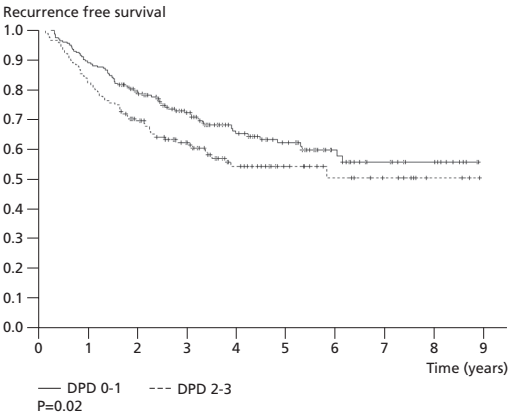


FIGURE 2

Recurrence free survival according to tumoural DPD intensity (0-1 versus 2-3) in 303 patients after complete resection of colorectal cancer stage II-III and adjuvant chemotherapy. Censored data (+).[IV]



microsatellite instability (MSI) leading to deregulation of the genes.[25-29] Colorectal cancers with MSI have characteristic tumour biology and 5-FU chemosensitivity.[25-29] To elucidate the molecular foundation for these characteristics the association of MSI with thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression in cancer cells was evaluated. Moreover deregulation of genes may also result from chromosomal anomalies (aneuploidy) that appear in the majority of colorectal cancers. Therefore the significance of somatic aberrations of the TS, TP and DHFR genes to the prognosis of colorectal cancer was studied as well.

Matrix metalloproteinase 9 (MMP-9) facilitates early steps in the metastatic cascade by degrading structural component of basement membranes,[30] while Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) is the main physiologic inhibitor of MMP-9.[31-36] Detailed studies of the underlying mechanisms of cancer invasion are complicated by the fact that additional functions are ascribed to MMP-9[37] and TIMP-1,[38] which may either enhance or impede cancer progression. Moreover the biological function of these molecules may differ whether they are expressed by cancer cells or by stromal cells.[39-45]

Age

Although the incidence of colorectal cancer increases with age several data suggest that treatment rates decline with age.[46-48] Patients at age 75 years and above are more likely to either receive lower dose or no chemotherapy.[49-53] This may relate to the fact that the benefit and tolerance of such therapy in elderly is generally is not well described as most clinical trials exclude these patients.[54, 55]

Cardiotoxicity of 5-Fluorouracil

5-FU is cardiotoxic leading to angina. Other symptoms of 5-FU cardiotoxicity such as hypotension and fainting indicate influence on the circulation and heart pump function.[II, 56] The pathophysiology, the long-term effect on cardiac function and on the neuroendocrine axis including proBNP secretion from cardiocytes[57, 58] is not clear. The significance of cardiovascular disease and renal impairment as risk factors for development of 5-FU associated cardiotoxicity remains to be clarified.[II, 56]

In the following separate accounts are made of the tumour related and the patient related factors being associated with the prognosis and the adverse reactions following 5-FU based chemotherapy.

FACTORS RELATED TO THE TUMOUR

Thymidylate Synthase and Dihydropyrimidine Dehydrogenase

Thymidylate Synthase (TS) and Dihydropyrimidine Dehydrogenase (DPD) synthesise and degrade thymidylate, respectively. The thymidylate homeostasis may have influence on tumour growth. Accordingly high TS[59-64] and low DPD expression[17, 65] have been associated with early disease recurrence and death in patients receiving surgery only, which suggests that TS and DPD expression can be considered prognostic for the outcome of colorectal cancer independently of chemotherapy.

The main mode of action of 5-FU is through irreversible blocking of TS, while the majority of an administered dose of 5-FU is catabolised by DPD. Therefore, individual expression of TS and DPD may be associated to varying toxicity and outcome of adjuvant 5-FU chemotherapy of colorectal cancer. To assess the predictive significance of TS and DPD expression to outcome pre-

supposes comparison of adjuvantly 5-FU treated patients with those not receiving chemotherapy.

TS and DPD may play key roles in the biology and 5-FU sensitivity of colorectal cancer. Previous studies evaluating prognostic and predictive significance of TS and DPD in completely resected colorectal cancer suggested that high TS [59-64] and low DPD [17, 65] expression may be associated to early spontaneous disease recurrence and death independent of chemotherapy, while also being related to improved outcome from adjuvant 5-FU chemotherapy.[59, 61-65] On the other hand low TS[59-64] and high DPD[17, 65] expression may be associated to low spontaneous recurrence rate and longer survival, while also indicating no improved outcome from adjuvant 5-FU chemotherapy.[59, 61-65]

Other studies compared outcome stratified by TS and DPD level among adjuvantly 5-FU treated patients, thus evaluating the integrated predictive and prognostic significance of these biomarkers. However, results have differed widely between studies as TS was reported to have no[15-17, 60-64, 66, 67] or direct correlation,[59, 68] and DPD to have no[67, 69] or inverse correlation[17, 65, 68, 70-73] to outcome. The differences between studies may relate to the various methods used to assess TS and DPD, and the criteria used to assign status.

The current study suggests that interaction between TS and DPD levels in completely resected and adjuvantly 5-FU treated colorectal cancer was associated with two-fold significant variation in risk of recurrence and death (**Figure 1** and **Figure 2**).[IV, 4] In keeping with previous studies[68] low DPD expression, confined to 59% of tumours, conferred low recurrence rate and longer overall survival. Further stratification identified a subset of 15% of patients also having high TS level, who had the significantly best outcome.[IV, 4] This subset corresponded to the patients who had improved outcome from adjuvant 5-FU in studies evaluating the predictive significance of TS and DPD.[59, 61-65]

Though differences in distribution of biomarkers according to age, tumour differention and ileus occurred (**Table 1**), confounding could not explain the associations of TS and DPD to the outcome, that were valid when adjusted for the multivariate context (**Figure 3**). Moreover, the associations were consistent in subgroup analysis of stage II and III, and colon and rectal cancer as well.

While in this study the marginally higher incidence of worse performance status in patients having high tumour DPD intensity was unexpected (**Table 2**), the lack of significant association of biomarkers in other respects to induced toxicity and chemotherapy makes this observation less valid. Also the TS levels in cells of normal mucosa, which appeared with varying pattern for 10% of cases, were without association to chemotherapy or adverse events. In contrast, significantly higher incidence of gastrointestinal toxicity has been demonstrated for patients with low TS expression in normal colonic mucosa, whereas other organ specific toxicity was not related to this.[74] Tumour heterogeneity and organ tissue specific distribution of TS[74] and DPD[75] may explain some of these inconsistencies.

A presumption critical to the inference of biomarkers at the site of sampling is that it reflects the situation in the entire tumour. However, TS level in the primary tumour may not exactly accord with that of matched samples taken from metastatic deposits.[60, 67, 76-82] It is likely that metastases represent subclones of cancer cells with altered genetics and growth features compared to the primary tumour.[60, 67, 76-80] Genetic instability also may account for the TS heterogeneity within primary tumours noticed in this study.[IV, 4] Tumour cells of micrometastatic deposits, being pathogenetic to recurrence after complete resection, may have elusive characteristics as well. The closer association to outcome suggests that biomarkers of invasive cells of the lateral part as opposed to the luminal part (data not shown) of primary tumours may better reflect characteristics and sensitivity to chemotherapy of micrometastasis.

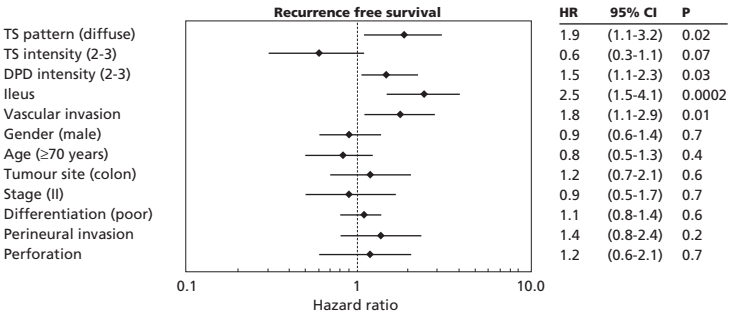
TABLE 1

Clinical, pathological and immunohistochemical characteristics in 303 consecutive patients with completely resected colorectal cancer receiving adjuvant chemotherapy.[IV]

		TS intensity		TS pattern		DPD intensity	
	No. (%)	(0-1;2-3) %	P	focal; diffuse %	P	(0-1;2-3) %	P
Proportions by score 0;1;2;3 %	–	56;20;15;9	–	71; 29	–	28;31;26;14	–
Gender							
Male	152 (50)	(78;22)	0.4	71; 29	0.8	(57;43)	0.4
Female	151 (50)	(74;26)		72; 28		(62;38)	
Age							
<70	239 (79)	(78;22)	0.1	72; 28	0.8	(63;37)	**0.04**
≥70	64 (21)	(67;33)		70; 30		(48;52)	
Tumour site							
Colon	250 (83)	(74;26)	0.2	72; 28	0.7	(60;40)	0.9
Rectum	53 (17)	(83;17)		70; 30		(58;42)	
Stage							
II	37 (12)	(73;27)	0.7	65; 35	0.4	(54;46	0.5
III	266 (88)	(76;24)		72; 28		(61;39)	
Differentiation							
Well	91 (30)	(85;15)	**0.0001**	77; 23	**0.0001**	(58;42)	0.5
Intermediate	129 (43)	(81;19)		81; 19		(64;36)	
Poor	83 (27)	(58;42)		51; 49		(55;45)	
Perineural invasion							
yes	56 (19)	(86;14)	0.1	77; 23	0.6	(55;45)	0.4
no	156 (51)	(75;25)		72; 28		(62;38)	
not assessed	91 (30)	–		–		–	
Vascular invasion							
V0	162 (54)	(76;24)	0.07	73; 27	0.5	(60;40)	1.0
V1	59 (19)	(88;12)		78; 22		(59;41)	
Vx	82 (27)	–		–		–	
Perforation							
yes	30 (10)	(90;10)	0.09	83; 17	0.2	(53;47)	0.6
no	273 (90)	(74;26)		70; 30		(60;40)	
Ileus							
yes	41 (14)	(85;15)	0.2	90; 10	**0.01**	(63;37)	0.6
no	262 (86)	(74;26)		68; 32		(59;41)	

FIGURE 3

Multivariate analysis of recurrence free survival according to TS and DPD expression, and clinicopathological characteristics of 303 consequtive patients completely resected for colorectal cancer and adjuvantly treated with chemotherapy.[IV]



Though the immunohistochemical assays are highly selective towards quantifying enzyme protein confined to tumour cells there are shortcomings. Considering the low prevalence of DPD deficiency in the general population,[83] the large proportion of tumours not staining for DPD may indicate low sensitivity for this assay. Moreover, based on comparison of matched samples taken before and after chemotherapy, enzyme activity adjust from transcriptional and translational regulation during chemotherapy,[81, 82] which may render the association of biomarkers with outcome obscure. An individually wide range of induction of gene expression following chemotherapy led to closer association of TS and DPD mRNA to outcome.[81, 82]

Assesment of tumour genotype may rather capture the range of adjustment of TS and DPD that occur during chemotherapy.[81, 82, 84]

TABLE 2

Percentages of patients according to number of chemotherapy courses received, tolerable dose in % of planned, induced toxicity, and worst performance status during chemotherapy, and immunohistochemical characteristics.[IV]

	TS intensity			TS pattern			DPD intensity		
	0-1	2-3	P	focal	diffuse	P	0-1	2-3	P
No. of chemotherapy courses									
1-2	10	19	0.1	9	18	0.06	10	14	0.8
3-4	4	12		6	8		9	4	
5-6	86	69		85	74		81	82	
Tolerable chemotherapy dose % of planned									
100	67	77	0.3	69	75	0.6	72	68	0.6
75	30	21		28	24		25	30	
50	3	2		3	1		3	2	
Worst CTC toxicity encountered									
0-2	65	70	0.5	68	63	0.3	69	63	0.2
3-4	35	30		32	37		31	37	
Worst performance status during treatment									
0-2	96	96	1.0	96	94	0.4	97	93	**0.02**
3-4	4	4		4	6		3	7	

CTC: Common Toxicity Criteria.

Conclusion

The results presented suggest that the immunohistochemical profiles of TS and DPD in the tumour together with clinical and pathological parameters may contribute to better prediction of outcome in patients with completely resected colorectal carcinoma receiving adjuvant 5-FU chemotherapy.

Mismatch Repair Deficiency and Microsatellite Instability

Deficient mismatch repair (MMR) promote malignant transformation as it allows accumulation of microsatellite instability (MSI), leading to inactivation of genes having key regulatory functions.[85-87] Colorectal cancer with MSI has characteristic biology and chemosensitivity, however the molecular basis remains unclarified.

In keeping with previous reports[25-27, 29] and a meta-analysis[88] we found favourable outcome associated with MSI as compared to MSS (stability) of completely resected colorectal carcinomas stage II-IV following adjuvant 5-FU treatment (**Figure 4**).[VI, 89] These findings were based on uniform criteria for categorization of microsatellite instability using the NCI recommended reference panel of five loci.[90] In addition, characterization of mismatch repair competency by assessing expression of four main mismatch repair proteins corroborated these results (**Figure 5**).[VI, 89] Others reported no prognostic significance of microsatellite instability in this setting, based on various criteria for microsatellite status.[28, 91-94]

Clinicopathological features of the MSI carcinomas may contribute to the better prognosis (**Table 3**). Hence the minor risk of bowel obstruction associated with right-sided tumours is an independent favourable prognostic variable. Moreover the generally lower staging at diagnosis is taken to indicate minor propensity of MSI tumours to metastasize.[88, 95-97] Possibly the decreasing frequency of MSI by stage was not as evident in this cohort because of selection. According to the current treatment algorithm most stage II cancers are not referred for adjuvant chemotherapy unless they have additional poor prognostic factors.

Initial investigations into the predictive role of microsatellite instability showed similar improvement in outcome from adjuvant 5-fluorouracil therapy irrespective of microsatellite status of the resected adenocarcinomas.[93, 98] Inadvertantly biased treatment groups for comparison may have accounted for this conclusion. In later reports improved outcome from adjuvant 5-fluorouracil related to patients with MSS tumours only,[25, 28, 94, 96, 99-101] whereas the subset having MSI cancers gained no similar beneficial effect from chemotherapy.[88] Hence the prevailing evidence suggests that 5-fluorouracil therapy should not be given to patients with MSI colorectal cancer.

FIGURE 4

Recurrence free survival by instable (MSI) and stable (MSS) microsatellites in tumors of colorectal cancer patients completely resected and adjuvantly treated with chemotherapy. Censored data (+).[VI]

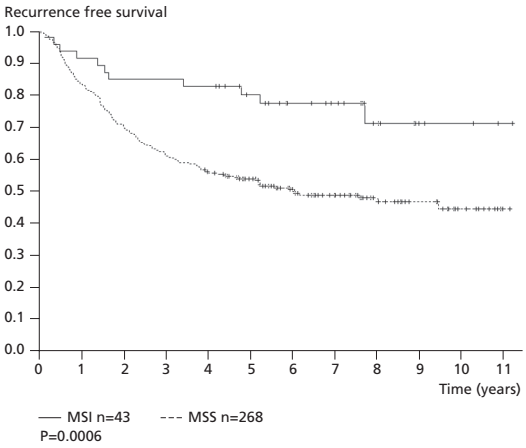


FIGURE 5

Recurrence free survival according to mismatch repair deficiency from loss either of hMSH2, hMSH6, or hMLH1 in colorectal cancers of patients completely resected and adjuvantly treated with 5-fluorouracil. Censored data (+).[VI]

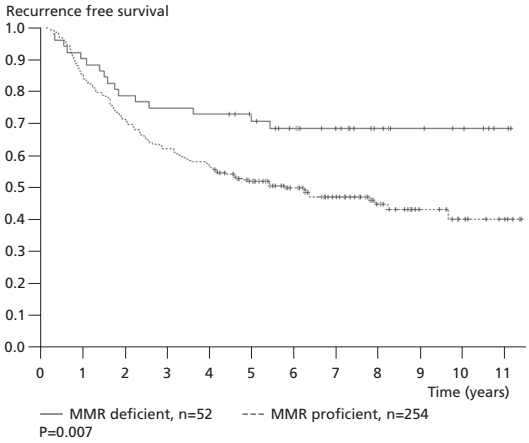


TABLE 3

Clinicopathological characteristics and tumor biomarker score according to microsatellite status.[VI]

	MSI* n=43		MSS* n=268		
	no.	(%)	no.	(%)	P
Gender					
Male	21	(49)	138	(51)	0.9
Female	22	(51)	130	(49)	
Age					
<70	28	(65)	217	(81)	**0.02**
≥70	15	(35)	51	(19)	
Tumor site					
Proximal colon	36	(84)	68	(25)	**0.001**
Distal colon	6	(14)	145	(54)	
Rectum	1	(2)	55	(21)	
Stage					
II	6	(14)	26	(10)	0.4
III	34	(79)	210	(78)	
IV	3	(7)	32	(12)	
Differentiation					
Well	5	(12)	88	(33)	**0.001**
Intermediate	7	(16)	121	(45)	
Poor	31	(72)	59	(22)	
Perineural tumor invasion					
yes	5	(12)	53	(20)	0.5
no	19	(44)	137	(51)	
not assessed	19	(44)	78	(29)	
Vascular tumor invasion					
V0	18	(42)	142	(53)	0.8
V1	8	(19)	58	(22)	
Vx	17	(39)	68	(25)	
Perforation					
yes	2	(5)	29	(11)	0.2
no	41	(95)	239	(89)	
Ileus					
yes	1	(2)	44	(16)	**0.01**
no	42	(98)	224	(84)	
Thymidylate synthase level					
0-1	12	(28)	218	(81)	**0.001**
2-3	30	(70)	47	(18)	
not assessed	1	(2)	3	(1)	
Dihydropyrimidine dehydrogenase level					
0-1	21	(49)	163	(61)	0.1
2-3	21	(49)	97	(36)	
Not assessed	1	(2)	8	(3)	

*) instable (MSI) or stable (MSS) microsatellites.

Tolerance of MMR deficient cancer cells to accumulate 5-fluorouracil adducts and conspicuous lymfocytic infiltration in tumours may account for the opposing trends of relative resistance of chemotherapeutic interventions, against the background of a favourable natural history.[102]

In multivariate analysis the outcome of MSI tumour patients was independent of the TS and DPD levels (**Figure 6**),[VI, 89] suggesting that differential expression of these enzymes could not account for the favourable natural history nor the resistance to chemotherapy.[25, 28, 94, 96, 99-101]

The correlation between high TS expression and microsatellite instability noticed in this (Table 3)[VI, 89] and other studies[103, 104] should be interpreted cautiously, as it may not explain prognostic and chemotherapeutic response characteristics of MSI and MMR deficient tumours. Accordingly high TS expression has generally been associated with early disease recurrence independently of chemotherapy,[59-64] while also being related to improved outcome from adjuvant 5-fluorouracil treatment.[59, 61-65] The low metastatic capacity[88, 95-97] and high apoptotic index[105] of MSI tumours may counterbalance metabolic features otherwise linked to poor prognosis.[59-64]

Earlier reports on the relationship between TS intensity and microsatellite instability have been conflicting[28, 103, 104, 106-111] arguing either for a direct correlation[103, 104] or no such association.[28, 106, 108, 109] The discrepancy may partly relate to lack of consistency in criteria defining microsatellite instability, as some studies were

FIGURE 6

Forest plots displaying multivariate Cox analysis of variables prognostic to recurrence free survival following complete resection of colorectal cancer and adjuvant chemotherapy. The prognostic variables included clinicopathological characteristics, tumor microsatellite status and expression of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).[VI]

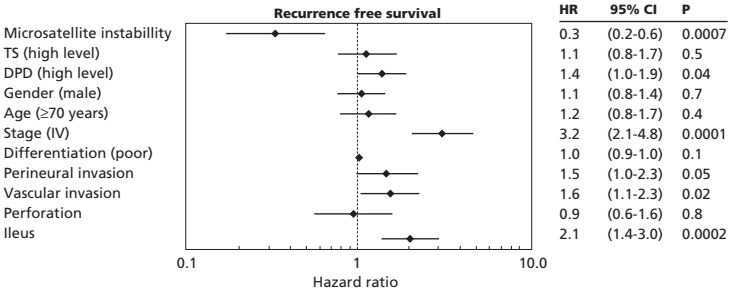


FIGURE 7

Thymidylate synthase expression score (0-3) assessed by immunohistochemistry according to number of TS alleles/nucleus above or below the median in colorectal cancer cells (n=302).[V]

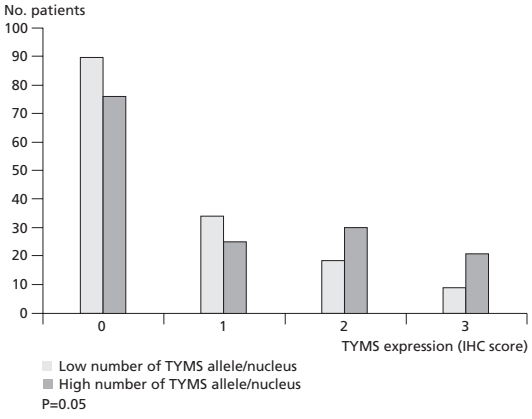


TABLE 4

Distribution of thymidylate synthase (TYMS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR) allele/nucleus below (low) or above (high) the median according to clinical and pathological characteristics in 314 colorectal cancer patients.[V]

	TYMS		TP		DHFR	
	low; high % n=303	P	(low; high) % n=300	P	(low; high) % n=216	P
Gender						
Male	(24; 27)	0.4	(25; 26)	1.0	(26; 25)	0.8
Female	(26; 23)		(25; 24)		(24; 25)	
Age						
<70	(42; 38)	0.09	(41; 39)	0.4	(39; 39)	0.9
≥70	(8; 12)		(9; 11)		(11; 11)	
Tumor site						
Colon	(38; 44)	**0.01**	(40; 42)	0.6	(40; 44)	0.2
Rectum	(12; 6)		(10; 8)		(10; 6)	
Stage						
II	(5; 6)	0.9	(5; 6)	0.1	(6; 6)	0.8
III	(41; 39)		(39; 40)		(40; 38)	
IV	(4; 5)		(6; 4)		(4; 6)	
Differentiation						
Well	(15; 17)	0.8	(15; 15)	0.9	(13; 17)	0.2
Intermediate	(21; 18)		(20; 21)		(24; 19)	
Poor	(14; 15)		(14; 15)		(13; 14)	
Perineural invasion						
yes	(9; 10)	0.8	(8; 9)	0.9	(8; 10)	0.7
no	(27; 26)		(26; 28)		(26; 25)	
not assessed	(14; 15)		(16; 13)		(16; 15)	
Vascular invasion						
V0	(27; 27)	0.6	(24; 29)	**0.04**	(26; 26)	0.8
V1	(12; 10)		(13; 8)		(10; 12)	
Vx	(11; 14)		(13; 13)		(14; 12)	
Perforation						
yes	(3; 5)	0.2	(3; 5)	0.3	(6; 4)	0.9
no	(47; 45)		(46; 45)		(44; 46)	
Ileus						
yes	(5; 8)	0.2	(6; 7)	0.8	(6; 8)	0.4
no	(45; 42)		(44; 43)		(44; 42)	

TABLE 5

Multivariate analysis of outcome according to allele/nucleus of TYMS, TP, and DHFR adjusted for the independent prognostic variables stage, vascular tumor invasion and bowel obstruction.[V]

	Recurrence free survival			Overall survival		
	hazard ratio	95% CI	P	hazard ratio	95% CI	P
TYMS*	1.6	1.1-2.2	**0.02**	1.6	1.1-2.3	**0.01**
TP*	1.0	0.7-1.5	0.9	1.1	0.8-1.6	0.6
DHFR*	0.7	0.4-1.1	0.08	0.7	0.7-1.1	0.1

*) higher relative to lower than median allele/nucleus.

based on single microsatellite marker [106, 107] whereas others [28, 103, 111] applied more restricted criteria.[90]

Also technical matters regarding immunohistochemical assessment of TS expression may have led to different results. While 24% of cases in this cohort had high TS score (2-3), the proportion in other studies has ranged between 19-77% of colorectal cancers in the adjuvant setting.[112]

As prospective trials usually exclude elderly patients,[28] also the age distributions of patient cohorts may have varied between studies. This raises the question whether high TS expression is confined to either inherited or sporadic MSI cancers that usually arise at an average age in the mid-forties or beyond the age of 70 years, respectively.[113] Difference in TS expression might be of significance to varying outcome of MSI tumours seen in the context of inherited repair deficiency.[101]

Significantly higher TS score in tumours deficient of hMLH1 as compared to those deficient of hMSH2 or hMSH6, actually did support the notion that TS expression may vary according to the etiology of mismatch repair deficiency.

Though the pattern of microsatellite instability and resultant influence on gene deregulation may depend on the mechanism of mismatch repair deficiency, no causal connection can be deduced from the high TS expression found in MSI tumours. The fact that microsatellite instability not being involved in recombinant events leading to TS gene variability [107, 110, 111] suggests that the phenomenon is rather of secondary character. Thus the somewhat paradoxical mucinous histology and

poor differentiation of MSI tumours [106, 108] (Table 3) have metabolic traits implying higher intensity and diffuse pattern of TS expression.[IV, VI, 4, 89]

Taken together there is no evidence to suggest direct influence of microsatellite instability on DPD or TS expression, nor that differential expression of these enzymes mediates the features of tumour biology or the 5-fluorouracil resistance of MSI carcinomas.

Conclusion

Microsatellite instability due to MMR deficiency is one of the main biomarkers in colorectal cancer as it not only indicates the pathogenesis but also provides information on prognosis and prediction of response to chemotherapy. Future investigations into the genes that are deregulated by microsatellite instability may clarify the molecular foundation for the distinct clinicopathological characteristics of MSI carcinomas.

ABERRANT GENES OF THYMIDYLATE SYNTHASE, THYMIDINE PHOSPHORYLASE AND DIHYDROFOLATE REDUCTASE

Most human cancer cells have structural aberrations of chromosomal regions leading to loss or gain of gene specific alleles. This study aimed to assess the range of gene copies per nucleus for a number of enzymes having significance to the efficacy of 5-FU therapy of colorectal cancer.[V, 114] Cytotoxicity of 5-FU is mediated through irreversible blocking of thymidylate synthase (TS). Being a prodrug 5-FU requires activation by thymidine phosphorylase (TP). Dihydrofolate reductase (DHFR) provides reduced 5,10-methylenetetrahydrofolate cosubstrate for enhanced TS inhibition.

Using a panel of new probes that spanned the entire promoter/enhancer and encoding regions, target genes were quantified by fluorescence in situ hybridisation (FISH) that combines the advantage of providing an account of gene specific copy numbers in morphologically identified tumour cell nuclei. The tumoural gene dosage of TS, TP and DHFR basically covered a biological continuum with at most 4-fold range of numerical variation. Correlation of FISH signals for target genes and the corresponding centromeres indicated that little (13-32%) of this variability could be explained by the association with number of centromere per nucleus. Which suggests that deletion or independent multiplication was the main underlying pathofysiologic mechanism, whereas aneusomy and co-segregation with entire gene bearing chromosomes to a lesser extent explained the variation of gene copy numbers.[V, 114]

The cut off points based on the medians of gene copies in tumours (**Table 4**) should be considered arbitrary, as there is no uniform threshold for defining copy loss or amplification using the FISH technology. The indistinct criteria for categorizing the magnitude of gene specific copy number largely derive from the nuclear truncation that occurs during the sectioning process. Moreover, variable hybridization efficiency, interpretation of signal overlaps and nuclear borders, and interference from specimen autofluorescence, background probe staining, and necrotic tumour tissue are factors that might interfere with the specificity of the technique.

Within the range of TS gene aberration established, an association with varying protein expression (**Figure 7**) and outcome (**Table 5**, **Figure 8**a) was suggested.[V, 114] TS may have both prognostic and predictive significance in colorectal cancer with implications for the tumour biology and sensitivity to 5-FU as previously pointed out.[IV, VI 4, 89] There may be more explanations why this was not in accordance with the relationship between the number of TS genes per cell and the outcome (Table 5) (Figure 8a). While low gene copy number may restrict protein expression, wide ranges for correlation of these parameters appeared, stressing the role of posttranscriptional regulation of TS expression. In addition there are shortcomings to the FISH technology. Though it provides a quantitative estimate of gene specific copy number changes it cannot account for the allelic imbalance of polymorph alleles characteristic of this gene.

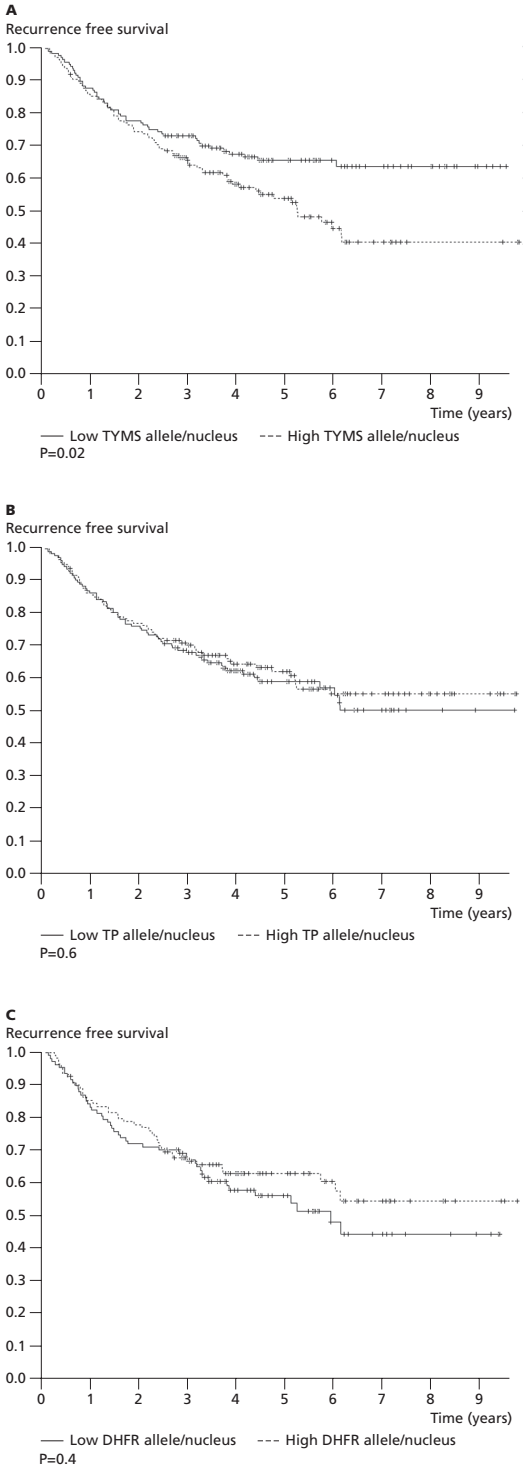
Due to a 28-basepair repeat polymorphism in the promoter region, individuals heterozygous for TS can acquire the loss or amplification from either the dominant overriding triple repeats or the recessive double repeat allele in their tumours.[115] Arguing that the composite genotype, covering the profile of alleles quantitatively and qualitatively, needs to be considered for prognostic purposes.

TP has previously been evaluated as a prognostic marker in colorectal [18, 20, 116] and rectal [84, 117] cancer. In the adjuvant,[116] neoadjuvant [84, 117] and palliative [18, 20] setting of 5-FU based chemotherapy, either inverse [18, 20, 84, no [116] or direct [117] association of tumour expression of TP mRNA [18, 20, 84] or protein [116, 117] with outcome have been found.

While increased TP activity is anticipated to lead to stronger exposure to activated 5-FU, these conflicting reports should be considered in the context that dual functions have been ascribed to this protein. Besides being a key enzyme in the activation of 5-FU and capecitabine, TP is identical to the angiogenic factor platelet-derived endothelial cell growth factor.[118] Although no association of TP gene copies and pathological parameters appeared in this study (Table 4), occurrence of lymphatic and venous invasion, and depth of tumour invasion have previously been related to high TP expression.[118] As a result, the therapeutic benefit due to greater sensitivity to chemotherapy conferred by high TP catalytic activation of 5-FU may to some extent have

FIGURE 8

Recurrence free survival following adjuvant chemotherapy of colorectal cancer stage II-IV by number of alleles/nucleus for thymidylate synthase (A), thymidine phosphorylase (B) and dihydrofolate reductase (C) in tumor cells. Censored data are indicated (+).[V]



been outweighed by a more malignant phenotype associated with enhanced tumour angiogenesis.

Although amplified gene copy and expression of DHFR is speculated to improve the effect of 5-FU, as increased availability of reduced folates synergistically produce tight binding of the active 5-FU metabolite FdUMP to TS [22] no such association was found (Table 5, Figure 8c). However, co-administration of folinic acid together with 5-FU, leading to equal availability of reduced folate, likely made this study inconclusive regarding the prognostic significance of the numerical variation of DHFR genes.

Gene aberration may also be a pathophysiological mechanism by which cancer cells acquire resistance to chemotherapy. Thus progression of colorectal cancer during 5-FU based chemotherapy was associated with increased number of DHFR gene copies.[119] Also induced TP gene expression [120] and TS copy number [119, 121] have been associated with reduced sensitivity to 5-FU under experimental conditions.

Conclusion

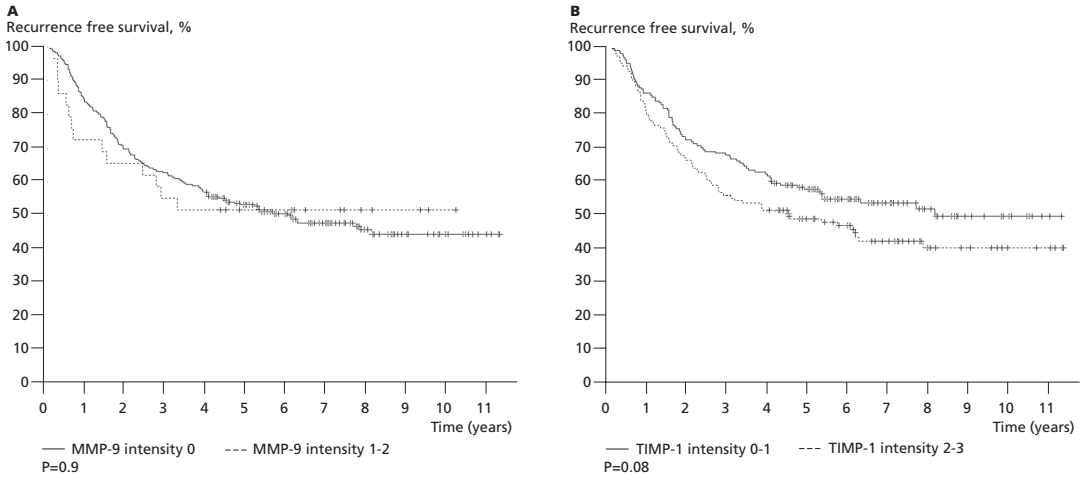
FISH analysis may provide novel aspects in the understanding of gene deregulation of colorectal cancer and its influence on the tumour biology and the sensitivity to 5-FU treatment.

MATRIX METALLOPROTEINASE-9 AND TISSUE INHIBITOR OF METALLOPROTEINASES-1

The matrix metalloproteinases are a group of proteolytic enzymes that collectively mediates degradation of fibrillar components of the extracellular matrix during maintenance and remodelling of tissue in physiological and pathological conditions such as growth, involution, inflammation, wound healing and cancer progression. [30-33, 35, 36, 39, 44, 122-129] Matrix metalloproteinase 9 (MMP-9), also termed gelatinase B or the 92-kDa type IV collagenase, is an extracellular protease that specifically degrades collagen type IV, which is the major structural component of basement membranes.[30] MMP-9 is particularly important in the context of cancer, as it allows cancer cells to infiltrate the adjacent stromal compartment, thereby facilitating a critical early step in the metastatic cascade. [31, 33, 35, 122, 123, 125-131] Moreover MMP-9 activates various growth factors and angiogenesis inhibitors like angiostatin with significance to tumour progression indicating a role as a general molecular switch in the microenvironment. [37]

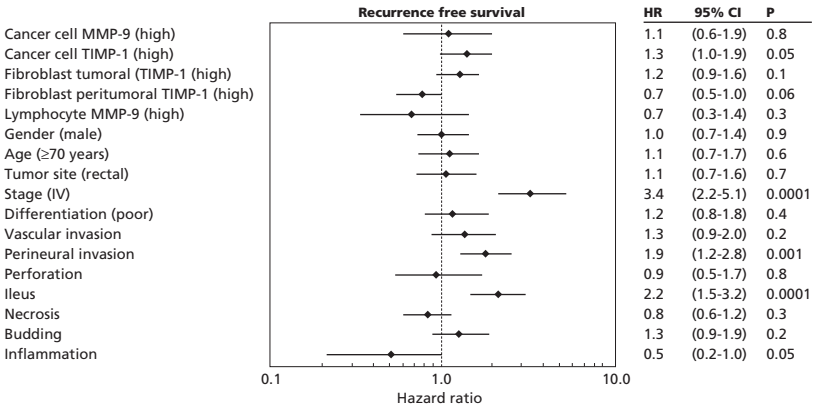
MMP-9 activity is tightly regulated at various levels including being secreted as an inactive zymogen that requires removal of an amino-terminal domain in order to acquire extracellular enzyme activity. [132] The secreted soluble glycoprotein Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) is the main physiologic inhibitor of MMP-9, as it forms high-affinity, noncovalent, but essentially irreversible 1:1 complexes with the active proteinase. [31-36]

FIGURE 9



MMP-9 (A) and TIMP-1 (B) immunoquantitation in carcinoma cells associated with recurrence free survival after complete resection of colorectal cancer stage II-IV and adjuvant chemotherapy. Censored data (+).[VII]

FIGURE 10



Forest plots displaying multivariate Cox analysis of recurrence free survival associated with clinicopathological characteristics and with MMP-9 and TIMP-1 immunoreactivity in carcinoma and stromal cells.[VII]

The previous observations that high levels of TIMP-1 in the primary tumour[31-36, 123, 125, 133] and in plasma of colorectal cancer patients[134-136] are correlated with poor outcome[31-35, 134, 135] was unexpected considering the well-established role of TIMP-1 as inhibitor of MMP-9-mediated matrix degradation during tumour cell invasion. However, as opposed to the invasion controlling effect of TIMP-1 linked to MMP-inhibition, recent studies have demonstrated that TIMP-1 possess additional functions including enhancement of malignant transformation,[137] stimulation of cell growth,[38] and inhibition of apoptosis,[138] as well as promotion of migration, invasion, and angiogenesis[139] indicating a potential tumour-promoting role of TIMP-1 in the early stages of tumourigenesis.[138] These observations suggest that TIMP-1 actually plays dual roles in cancer progression.

Detailed studies on the underlying mechanisms of cancer invasion into the supporting connective tissue are complicated by the fact that supporting stromal cells (e.g. fibroblasts, macrophages, granulocytes and lymphocytes) alter their expression of matrix degrading enzymes in neoplastic conditions. In response to colorectal cancer cell invasion the stromal cells express matrix-degrading enzymes more frequently than the cancer cells themselves, suggesting that matrix proteinases derived from the peritumoural stroma may be significantly more involved in tumour invasion than previously recognized.[39-45] The net matrix degrading activity in a tumour depends on the ratio between MMPs and TIMPs,[31-33] and the biological function of these molecules may differ whether they are expressed by cancer cells or by stromal cells.[39-45] These reflexions argue for a comparative study of the significance of MMP-9 and TIMP-1 expression in relation to prognosis of colorectal cancer, considering the cellular site of expression of the mentioned molecules.

We found strong expression of TIMP-1 (**Figure 9**b) by carcinoma cells to be associated with poor outcome of CRC patients (**Figure 10**), independently of the expres-

TABLE 6

Matrix Metalloproteinase 9 (MMP-9) and Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) expression in colorectal carcinoma cells and peritumoural lymphocytes (MMP-9) and fibroblasts (TIMP-1) according to clinicopathological characteristics.[VII]

		Carcinoma				Peritumoural			
	n=325 %	MMP-9 (0;1-2) %	P	TIMP-1 (0-1;2-3) %	P	MMP-9 (0-1;2-3) %	P	TIMP-1 (0-1;2-3) %	P
Proportions by score									
0;1;2;3 %	–	–	–	37;26;26;11	–	–	–	–	–
Gender									
Male	50	(92;8)	0.6	(58;42)	0.6	(66;34)	0.2	(49;51)	0.9
Female	50	(90;10)		(56;44)		(64;36)		(49;51)	
Age									
<70	79	(90;10)	0.1	(58;42)	0.4	(65;35)	0.3	(50;50)	0.2
≥70	21	(96;4)		(52;48)		(63;37)		(46;54)	
Tumour site									
Colon	81	(91;9)	0.9	(54;46)	**0.04**	(63;37)	0.2	(50;50)	0.9
Rectum	19	(90;10)		(69;31)		(72;28)		(46;54)	
Disease stage									
II	11	(91;9)	0.9	(49;51)	0.3	(70;30)	0.5	(53;47)	0.9
III	78	(91;9)		(57;43)		(64;36)		(49;51)	
IV	11	(92;8)		(66;34)		(69;31)		(43;57)	
Tumour stage									
T2	4	(77;23)	0.2	(62;38)	0.1	(62;38)	0.9	(33;67)	0.3
T3	59	(92;8)		(62;38)		(64;36)		(46;54)	
T4	37	(91;9)		(49;51)		(65;35)		(55;45)	
Differentiation (WHO)									
Well	31	(95;5)	0.2	(58;42)	0.8	(63;37)	0.4	(52;48)	0.5
Intermediate	42	(90;10)		(58;42)		(66;34)		(43;57)	
Poor	27	(87;13)		(53;47)		(66;34)		(54;46)	
Perineural invasion									
yes	19	(93;7)	0.7	(64;36)	0.3	(77;23)	**0.01**	(64;36)	**0.04**
no	51	(92;8)		(57;43)		(61;39)		(47;53)	
not assessed	30	–		–		–		–	
Vascular invasion									
V0	52	(93;7)	0.5	(58;42)	0.8	(65;35)	0.9	(50;50)	0.9
V1	22	(90;10)		(59;41)		(63;37)		(51;49)	
Vx	26	–		–		–		–	
Intestinal perforation									
yes	9	(90;10)	0.9	(55;45)	0.9	(76;24)	0.3	(52;48)	0.6
no	91	(91;9)		(57;43)		(64;36)		(49;51)	
Bowel obstruction									
yes	15	(91;9)	0.9	(53;47)	0.6	(72;28)	0.08	(52;48)	0.2
no	85	(91;9)		(57;43)		(64;36)		(48;52)	
Necrosis									
yes	37	(90;10)	0.3	(57;43)	0.9	(62;38)	0.2	(44;56)	0.06
no	63	(93;7)		(57;43)		(68;32)		(52;48)	
Budding									
yes	25	(93;7)	0.8	(53;47)	0.3	(71;29)	0.7	(43;57)	**0.01**
no	75	(92;8)		(59;41)		(65;35)		(51;49)	
Inflammation									
strong	37	(91;9)	0.9	(52;48)	0.1	(61;39)	**0.02**	(50;50)	0.3
weak	63	(92;8)		(60;40)		(69;31)		(49;51)	

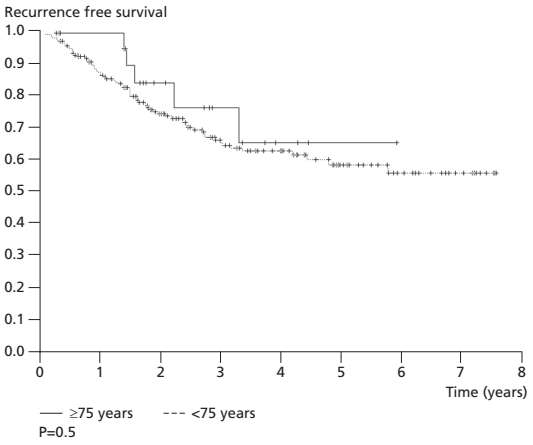
sion of MMP-9 (Figure 9a).[VII, 140] This finding is consistent with previous reporting that the levels of TIMP-1 mRNA,[35, 36] zymogen[31-34] and activity[123] in cancer cells or TIMP-1 protein plasma level[134, 135] are significantly related to the tumour stage at diagnosis[36, 123, 133-135, 141] and to the patient prognosis.[31-35, 134, 135]

The infrequent and low expression of MMP-9 by cancer cells (**Table 6**) suggests that this proteinase is of minor significance to tumour progression in later stages (II-IV) of colorectal cancer.[VII, 140] Besides MMP-2 having similar specific activity towards degradation of the basal membrane may substitute for MMP-9 regarding this function.[33] Conversely, in recents reports considerable higher rates in the range of 44-70% of tumour specimens had cancer cells expressing MMP-9, the level of which was inversely related to disease free survival.[126, 127, 142, 143] While the employment of different antibodies and technical procedures potentially may explain for such discrepancies between studies the fact that most specimens in the current study had MMP-9 immunostaining in stromal cells (Table 6) contradicted low sensitivity of the immunoreactivity in general.[VII, 140]

Varying distribution of clinicopathological characteristic between study cohorts may offer some explanation to the different profiles of MMP-9 expression being reported[142]. Hence the biomarker levels were significantly related to the localisation of the tumour as more intense MMP-9 expression was reported in lesions of the right colon as compared to left colon and rectum,[142] whereas the expression of TIMP-1 in the current

FIGURE 11

Recurrence free survival for patients of age <75 and ≥75 years after complete resection of colon cancer stage III and adjuvant 5-fluorouracil.[III]



study[VII, 140] was significantly stronger in colon as compared to rectum. Such distinct patterns of expression associated with the tumour site might reflect some basic differences in the tumour biology between the proximal and distal CRCs.[VI 34, 89]

In previous studies a direct relationship between the levels of MMP-9 mRNA,[35, 125, 128] protein,[123] enzyme activity[129, 131] or plasma level,[130] and advanced disease stage at diagnosis[123, 126, 130] and poor prognosis[35, 122, 125, 127, 128, 131] suggested a central role of MMP-9 for progression of colorectal cancer. However as the significance of MMP-9 to tumour progression may differ according to the site of expression, such data must be interpreted cautiously as they made no account of from which cell types the MMP-9 expression was derived.

The association between histopathological features and outcome of CRC and the adjacent stromal cells' expression of MMP-9 and TIMP-1 (Table 6) indicated their potential roles as important mediators of a host versus tumour reaction. Hence, the correlation between lymphocytic MMP-9 expression and the degree of inflammation suggests that matrix degrading proteinases may facilitate transmigration of lymphocytes, neutrophils and macrophages through blood vessels and extracellular matrix[144] to initiate a peritumoural host immune reaction leading to a better outcome.[VI, 39-42, 44, 89] Also the pronounced TIMP-1 expression by fibroblasts located at the border of the invading carcinoma nests and its association with a more favorable clinical outcome suggests a host response aiming at inhibiting tumour invasion. The founding mechanisms, however, remain unclarified considering the diverse functions of TIMP-1[38, 137-139] and the ambiguous relationship with growth characteristics such as perineural invasion and tumour budding (Table 6).

Conclusion

MMP-9 and TIMP-1 have diverse roles in cancer progression depending on their site of expression in carcinoma cells and peritumoural stromal cells. Increased level of TIMP-1 in cancer cells is associated with poor prognosis independently of its function as inhibitor of MMP-9. This recognition is essential for their application as prognostic markers and for development of rational anti-cancer therapies based on specific targeting of the matrix proteinase system.

FACTORS RELATED TO THE PATIENT

Age

The incidence of colorectal cancer increases with age. More than 40% of new cases of colorectal cancer appear in patients over 75 years of age.[145, 146] However several data suggest that treatment rates decline with age.[46-48] Patients at age 75 years and above are more likely to either receive lower dose or no adjuvant or palliative chemotherapy.[49-53] The benefit and tolerance of such therapy in elderly is generally is not well described as most clinical trials exclude these patients.[54, 55] Trial participants may also differ from the general population by medical comorbidity, performance status and different treatment demands that makes it unclear whether trial results pertain to elderly patients.[55]

We found similar recurrence free survival (**Figure 11**) and overall survival by comparing outcome of patients aged 75 years and above with their younger counterparts following adjuvant chemotherapy of colon cancer, which did not appear to be biased by unbalanced distribution of patient characteristics and prognostic variables at baseline (**Table 7**).[III, 147] No significant differences in toxicity rates by age group were found (**Table 8**), whereas performance status worsened in elderly during chemotherapy (**Table 9**). It is likely that decreasing performance status led to more cautious chemotherapy dosing to elderly, as these patients more frequently had dose reduction or discontinuation of chemotherapy before completion of the scheduled 6 treatment courses (Table 9). Likewise for patients <70 and ≥70 years receiving palliative chemotherapy (**Table 10**) we found comparable toxicity (**Table 11** and **Table 12**), progression free survival times (**Figure 12**).[I, 148] However this apparent uniformity covered up substantial differences among the elderly ≥70 years. In the intermediary age group of 70-74 years higher alkaline phosphatase (Table 10) and slightly worse performance status (Table 11) at baseline suggested these patients suffered higher tumour burden. As the toxicity from chemotherapy was similar between age groups (Table 12) lower response rates may have led to worsening of performance status (Table 11) and shorter survival in that age group. On the contrary, in the subset ≥75 years the significantly higher

TABLE 7

Gender, tumour characteristics and prognostic factors by age of patients completely resected for stage III colon cancer.[III]

	Age < 75 years (n=203) no. (%)	Age ≥75 years (n=24) no. (%)	P
Gender			
Male	102 (50)	10 (42)	0.4
Female	101 (50)	14 (58)	
Tumor site			
Ascendens	54 (27)	12 (50)	**0.04**
Transversum	24 (12)	1 (4)	
Descendens	16 (8)	0 (0)	
Sigmoid	109 (53)	11 (46)	
Tumor stage			
Tx	3 (1)	0 (0)	0.9
T2	5 (2)	1 (4)	
T3	129 (64)	15 (63)	
T4	66 (33)	8 (33)	
Lymph node stage			
N1	132 (65)	18 (75)	0.3
N2	71 (35)	6 (25)	
Differentiation			
Well	54 (27)	13 (54)	**0.01**
Intermediate	93 (45)	6 (25)	
Poor	56 (28)	5 (21)	
Perineural invasion			
not assessed	65 (32)	8 (33)	0.9
no	106 (52)	12 (50)	
yes	32 (16)	4 (17)	
Vascular invasion			
V0	110 (54)	13 (54)	0.9
V1	32 (16)	4 (17)	
Ileus			
no	174 (84)	23 (96)	0.1
yes	33 (16)	1 (4)	

TABLE 8

Worst toxicity according to Common Toxicity Criteria by age in patients with stage III colon cancer during treatment with adjuvant 5-FU.[III]

	Age < 75 years (n=203) no. (%)	Age ≥ 75 years (n=24) no. (%)	P
Anemia			
Grade 1-2	53 (26)	8 (33)	0.4
Grade 3-4	0 (0)	0 (0)	
Thrombocytopenia			
Grade 1-2	15 (7)	2 (8)	0.9
Grade 3-4	1 (0)	0 (0)	
Leucopenia			
Grade 1-2	71 (34)	4 (17)	0.9
Grade 3-4	4 (2)	1 (4)	
Infection			
Grade 1-2	38 (18)	4 (17)	0.8
Grade 3-4	8 (4)	2 (8)	
Bleeding			
Grade 1-2	47 (23)	6 (25)	0.9
Grade 3-4	0 (0)	0 (0)	
Nausia and vomiting			
Grade 1-2	133 (66)	17 (17)	0.7
Grade 3-4	11 (5)	0 (0)	
Mucositis			
Grade 1-2	134 (64)	17 (71)	0.4
Grade 3-4	32 (15)	4 (17)	
Diarrhoea			
Grade 1-2	128 (63)	18 (75)	0.8
Grade 3-4	31 (15)	3 (13)	
Cutaneous PPE			
Grade 1-2	28 (14)	5 (21)	0.6
Grade 3-4	4 (2)	0 (0)	
Myocardial ischaemia			
Grade 1-2	6 (3)	1 (4)	0.9
Grade 3-4	2 (1)	0 (0)	
Fatigue			
Grade 1-2	147 (72)	21 (88)	0.2
Grade 3-4	3 (1)	0 (0)	

PPE: palmar plantar erythrodysaesthesia.

TABLE 9

Worst performance status, weight gain, dose reduction and number of courses of adjuvant 5-FU treatment by age of patients with stage III colon cancer.[III]

	Age <75 years (n=203) no. (%)	Age ≥75 years (n=24) no. (%)	P
Performance status			
0	111 (55)	6 (25)	**0.002**
1-2	83 (41)	16 (67)	
3-4	9 (4)	2 (8)	
Weight gain			
<5% increase	106 (52)	20 (83)	**0.002**
5-10% increase	66 (33)	4 (17)	
10-20% increase	28 (14)	0 (0)	
>20% increase	3 (1)	0 (0)	
Dose % of baseline			
no dose reduction	146 (72)	12 (49)	**0.02**
75%	21 (10)	3 (13)	
66%	32 (16)	9 (38)	
50%	4 (2)	0 (0)	
Treatment courses			
1	10 (5)	5 (21)	**0.05**
2	11 (6)	2 (8)	
3	5 (2)	1 (4)	
4	6 (3)	0 (0)	
5	5 (2)	1 (4)	
6	166 (82)	13 (54)	

response rates from capecitabine may have maintained better performance status and weight gain (Table 11) during longer progression free interval (**Figure 13**), which in turn translated into longer survival.[I, 148]

Randomised trials addressing efficacy and toxicity of adjuvant 5-FU chemotherapy among elderly colon cancer patients based on cut-off age of 60 years,[149-151] 65 years,[152] 68 years,[98] and 70 years[51, 54, 153-157] demonstrated a significant improvement in outcome irrespective of age, which was achieved without excess toxicity.[54] However, the study sample did not reflect the age distribution of stage III colon cancer patients in the general population as 506 (15%) were aged ≥70 and 23 (<1%) patients were aged ≥80 years.[158, 159]

In addition three studies on palliative chemotherapy with 5-FU or capecitabine to colorectal cancer patients using age cut-off 60[160-162] or 65 years[160, 163] found no significant difference in time to progression.

Following 5-FU treatment increased rates of stomatitis, diarrhoea, nausea, vomiting, and leucopenia in elderly patients have previously been observed in some studies,[51, 54, 154, 157] whereas others reported no excess toxicity.[54, 152, 153] Among patients treated in randomised trials age was significantly related to higher rates of severe leucopenia in patients treated with 5-FU.[54] The eldest group of patients age ≥75 years, however did not experience worse toxicity compared to the younger patients receiving similar chemotherapy.[51] Pooled toxicity data from two multicentre phase III studies showed that there was an increased incidence of grade 3 or 4 overall adverse events, diarrhoea, stomatitis and hand-

TABLE 10

Clinicopathological characteristics and previous therapy according to age of patients with advanced colorectal cancer treated with capecitabine or XELOX.[I]

	Capecitabine or XELOX					Capecitabine		
	< 70 years (n=203) no. (%)	≥ 70 years (n=57) no. (%)	P	70-74 years (n=37) no. (%)	P*	< 75 years (n=160) no. (%)	≥ 75 years (n=18) no. (%)	P
Chemotherapy								
Capecitabine	135 (67)	43 (75)	–	25 (68)	–	160	18	–
XELOX	68 (33)	14 (25)		12 (32)		– –	– –	
Age								
Median, range	61, 24-69	73, 70-82	–	71, 70-74	–	63, 24-74	75, 75-82	–
Gender								
Male	114 (56)	35 (61)	0.48	23 (62)	0.39	86 (54)	10 (56)	0.88
Female	89 (44)	22 (39)		14 (38)		74 (46)	8 (44)	
Primary tumour site								
Ascendens	38 (19)	16 (28)	0.41	11 (30)	0.79	35 (22)	5 (28)	0.99
Transversum	10 (5)	4 (7)		3 (8)		10 (6)	1 (6)	
Descendens	13 (6)	5 (9)		4 (11)		10 (6)	1 (6)	
Sigmoid	71 (35)	15 (26)		9 (24)		47 (29)	5 (28)	
Rectum	71 (35)	17 (30)		10 (27)		58 (36)	6 (33)	
Tumour stage								
Locally advanced	28 (14)	5 (9)	0.31	2 (5)	0.42	28 (17)	4 (22)	0.57
Metastasized	175 (86)	52 (91)		35 (95)		132 (83)	14 (78)	
Liver	138 (68)	43 (75)		28 (76)		84 (53)	10 (56)	
Lung	53 (26)	12 (21)		9 (24)		48 (30)	4 (22)	
Tumour sites								
1	75 (37)	22 (39)	0.69	11 (30)	0.62	63 (39)	9 (50)	0.34
2	90 (44)	26 (46)		18 (49)		76 (48)	8 (44)	
≥3	38 (19)	9 (15)		8 (21)		21 (13)	1 (6)	
Tumour differentiation								
Unknown	110 (54)	21 (37)	0.08	18 (49)	0.23	52 (33)	1 (5)	0.63
High grade	5 (3)	0 (0)		0 (0)		5 (3)	0 (0)	
Intermediate grade	76 (37)	28 (49)		14 (38)		88 (55)	14 (78)	
Low grade	12 (6)	8 (14)		5 (13)		15 (9)	3 (17)	
Alk.Phosph. baseline								
Median, range	279, 45-1889	335, 18-2560	0.06	412, 77-2560	0.06	289, 45-2560	266, 18-1761	0.47
LDH baseline								
Median, range	365, 45-7629	376, 131-3290	0.90	385, 131-3290	0.90	432, 45-7629	397, 178-2173	0.73
Previous colon cancer								
Synchronous	116 (57)	35 (61)	0.56	24 (65)	0.25	101 (63)	10 (56)	0.39
Metachronous	87 (43)	22 (39)		13 (35)		59 (37)	8 (44)	
Previous therapy								
Adjuv. chemotherapy	57 (28)	12 (21)	0.29	6 (16)	**0.04**	43 (27)	6 (33)	0.52
Radiotherapy	30 (15)	5 (9)	0.72	3 (8)	0.12	26 (19)	2 (11)	0.53

*) compared to <70 years.

TABLE 11

Baseline and worst performance status and weight change during capecitabine based chemotherapy for advanced colorectal cancer according to age groups.[I]

	Capecitabine or XELOX					Capecitabine		
	<70 years (n=203) no. (%)	≥70 years (n=57) no. (%)	P	70-74 years (n=37) no. (%)	P*	<75 years (n=160) no. (%)	≥75 years (n=18) no. (%)	P
Performance status, baseline								
0	131 (65)	27 (47)	0.17	17 (46)	0.08	89 (56)	8 (44)	0.59
1	56 (27)	25 (44)		16 (43)		54 (34)	9 (50)	
2	16 (8)	5 (9)		4 (11)		17 (10)	1 (6)	
Performance status, worst								
0	68 (35)	12 (21)	0.68	7 (19)	**0.003**	48 (30)	4 (22)	0.38
1-2	102 (53)	35 (61)		20 (54)		89 (56)	14 (78)	
3-4	15 (8)	5 (9)		5 (14)		20 (12)	0 (0)	
5	7 (4)	5 (9)		5 (13)		3 (2)	0 (0)	
Weight change								
5% loss	19 (9)	6 (11)	0.94	6 (16)	0.60	21 (13)	0 (0)	**0.004**
–5-5%	94 (46)	28 (49)		17 (46)		95 (59)	9 (50)	
5-10% increase	29 (14)	12 (21)		7 (19)		22 (14)	5 (28)	
10-20% increase	11 (5)	5 (9)		3 (8)		8 (5)	2 (11)	
> 20% increase	2 (1)	1 (2)		0 (0)		2 (1)	1 (6)	

*) compared to <70 years.

foot syndrome from age 65 years on, that were particularly high in patients aged 80 years or older.[162] However, this was explained by age-related impairment of renal function, as multivariate analysis adjusting for creatinine clearance showed age per se not to have significant influence on the toxicity profile of capecitabine.[162]

TABLE 12

Worst CTC grade toxicity during capecitabine based chemotherapy of advanced colorectal cancer according to age of patients.[I]

	Capecitabine or XELOX					Capecitabine		
	< 70 years (n=203) no. (%)	≥70 years (n=57) no. (%)	P	70-74 years (n=37) no. (%)	P*	< 75 years (n=160) no. (%)	≥ 75 years (n=18) no. (%)	P
Anemia								
Grade 1-2	57 (28)	22 (39)	0.20	14 (38)	0.38	64 (40)	7 (39)	0.34
Grade 3-4	2 (1)	0 (0)		0 (0)		1 (1)	0 (0)	
Thrombocytopenia								
Grade 1-2	25 (12)	9 (16)	0.31	5 (14)	0.92	5 (3)	0 (0)	0.42
Grade 3-4	0 (0)	1 (2)		0 (0)		0 (0)	1 (6)	
Leucopenia								
Grade 1-2	14 (7)	4 (7)	0.95	2 (5)	0.85	15 (9)	2 (11)	0.39
Grade 3-4	1 (0)	0 (0)		0 (0)		1 (1)	0 (0)	
Infection								
Grade 1-2	16 (8)	9 (16)	**0.03**	5 (14)	0.15	19 (12)	4 (22)	0.83
Grade 3-4	9 (5)	5 (9)		5 (14)		8 (5)	0 (0)	
Nausia and vomiting								
Grade 1-2	94 (47)	28 (49)	0.48	18 (49)	0.72	2 (1)	2 (11)	0.50
Grade 3-4	6 (3)	0 (0)		0 (0)		0 (0)	0 (0)	
Mucositis								
Grade 1-2	66 (33)	23 (40)	0.37	14 (38)	0.65	66 (41)	9 (50)	0.88
Grade 3-4	1 (1)	0 (0)		0 (0)		3 (2)	0 (0)	
Diarrhoea								
Grade 1-2	92 (46)	23 (40)	0.30	12 (32)	0.24	56 (35)	9 (50)	0.43
Grade 3-4	11 (6)	10 (18)		10 (27)		1 (1)	0 (0)	
Cutaneous PPE								
Grade 1-2	99 (50)	30 (53)	0.74	15 (41)	0.08	73 (46)	10 (56)	0.80
Grade 3-4	29 (15)	6 (11)		3 (8)		9 (6)	0 (0)	
Myocardial ischaemia								
Grade 1-2	3 (2)	3 (5)	0.57	2 (5)	0.86	4 (3)	1 (6)	0.98
Grade 3-4	4 (2)	0 (0)		0 (0)		4 (3)	0 (0)	
Fatigue								
Grade 1-2	147 (74)	45 (79)	0.06	27 (73)	0.06	4 (3)	1 (6)	0.95
Grade 3-4	11 (6)	4 (7)		4 (11)		4 (3)	0 (0)	
Pain								
Grade 1-2	26 (13)	5 (9)	0.93	4 (11)	0.89	117 (73)	16 (89)	0.81
Grade 3-4	0 (0)	0 (0)		0 (0)		12 (8)	0 (0)	
Sensoric neuropathy								
Grade 1-2	54 (27)	7 (12)	**0.02**	7 (19)	0.10	64 (40)	7 (39)	0.34
Grade 3-4	2 (1)	0 (0)		0 (0)		1 (1)	0 (0)	

*) compared to <70 years.

Conclusion

The inference of our studies has limitations because retrospective data cannot fully control for the distribution of important covariates among treatment groups. The elderly patients included likely represent a somewhat selected group in good performance and with low comorbidity. Considering the age specific incidence of colorectal cancer a great number of elderly patients were never referred to the department of oncology for evaluation of the feasibility of chemotherapy. Moreover colorectal cancer may have different natural history depending on age, that comparison between age groups may be misleading. With these reservations the results of these studies do not suggest increasing age per se to be associated to lower efficacy nor to increased toxicity of chemotherapy.

5-Fluorouracil induced cardiotoxicity

Incidence

5-fluorouracil (5-FU) and its prodrug capecitabine are cardiotoxic[164-171] leading to chest pain with projection to the left arm or neck, arrhythmias, heart failure, myocardial infarction or cardiogenic shock.[164-171] An overall incidence of 1.2%-18% for 5-FU associated cardiotoxicity has previously been reported.[164-171]

The incidence of 5-FU induced cardiotoxicity may depend on dose, route of administration and schedule of chemotherapy. We reported an overall incidence of 4.3% among patients treated with various regimens which covered increasing incidences for capecitabine (XELOX), Mayo or de Gramont (FOLFOX-4) regimens.[II, 56] In a later study an incidence of 8.5% was found for patients treated with FOLFOX-4 only.[VIII, 172] Major clinical trials using the adjuvant FOLFOX protocol made no account of cardiac adverse reactions.[10]

Etiology

The pathogenesis of 5-FU induced cardiotoxicity may involve influence on the cardiac vasculature leading to reduced myocardial blood perfusion as suggested by the electrocardiographic changes being characteristic of ischemia.[II, VIII, 56, 172-178] Experimental histological studies

FIGURE 12

Progression free survival from start of palliative capecitabine or XELOX therapy for advanced colorectal cancer according to age <70 and ≥70 years.[I]

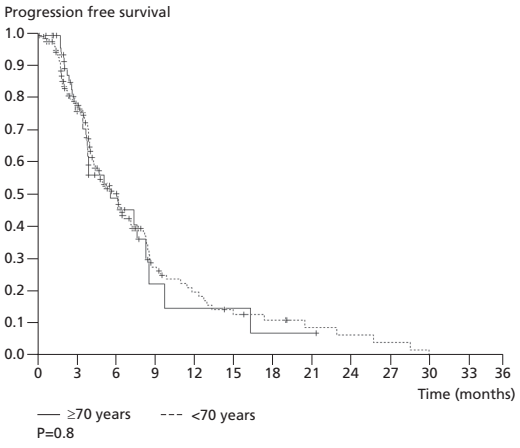


FIGURE 13

Progression free survival from start of palliative capecitabine therapy for advanced colorectal cancer according to age <75 and ≥75 years.[I]

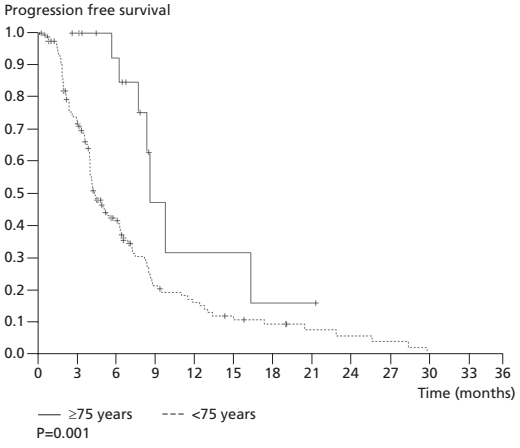
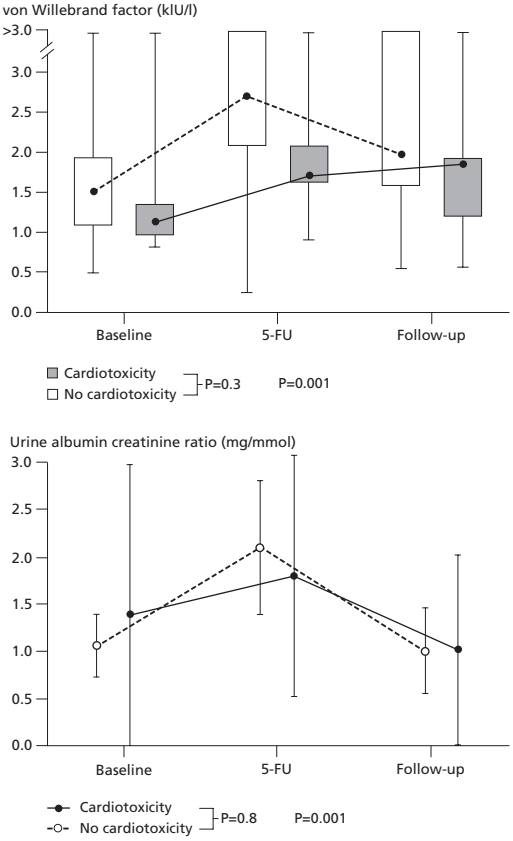


FIGURE 14

5-FU induced alteration of plasma von Willebrand factor antigen level (upper) in patients having cardiotoxicity or no such side effects. The median, the interquartile limits (box) and overall range (whiskers) are indicated. Alteration of Urine Albumin to Creatinine Ratio (lower) induced by chemotherapy in patients according to 5-FU cardiotoxicity. The means and 95% confidence intervals of the means (whiskers) are indicated. P-values relate to effect of 5-FU treatment between repeated measures and to difference in response between groups.[IX]



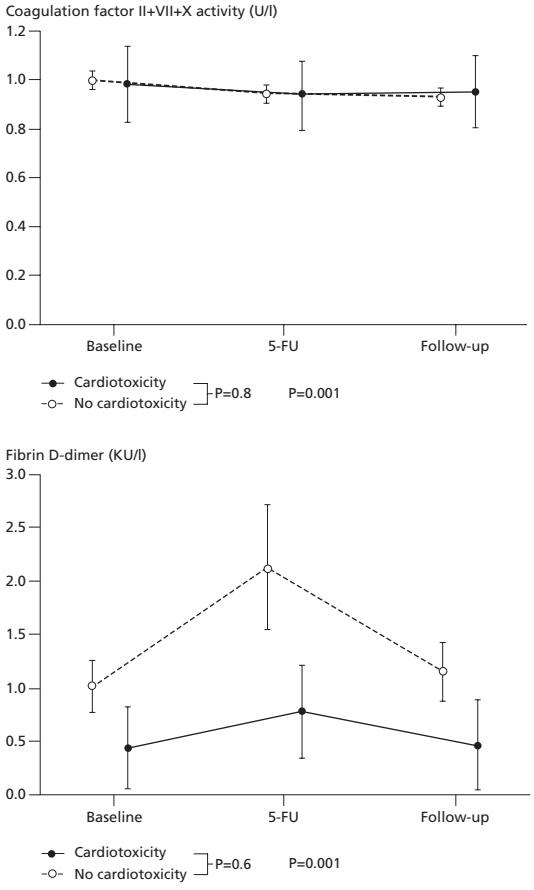
corroborated that 5-FU induces endothelial injury with disruption of the inner vascular lining and patchy exposure of the underlying matrix which may be a focus for platelet aggregation and fibrin formation.[179-181]

The endothelium plays a central role in both the regulation of the arterial vasodilation, the hemostasis and the selective vascular permeability which is essential for proper vascular function.[173, 182] The increases of plasma vWf and of proteinuria following 5-FU based chemotherapy in a clinical setting indicated reversible global endothelial injury from such treatment (**Figure 14**).[IX, 183] The ensuing endothelial dysfunction caused by exposure to FU chemotherapy may reversibly impair the vasodilatory response of the arteries.[184] The endothelium plays a central role in the regulation of arterial vasomotor tone by releasing the physiologic transmitter nitric oxide that induces relaxation of the surrounding smooth muscle, thus leading to vasodilation.[182] Administration of nitroglycerine, which is converted into nitric oxide by alcohol dehydrogenase, immediately resolve the angina symptoms during episodes of 5-FU cardiotoxicity demonstrating that impaired vasodilation is concurrent to the pathogenesis.[173, 184] On the other hand antineoplastic agents such as anthracyclines cause sustained damage of the endothelium with impaired vasodilatory response of arteries though without leading to similar angina symptoms being characteristic of 5-FU induced cardiotoxicity,[182] which suggests that 5-FU also may directly interfere with the metabolism of the myocardium.[II, VIII, 56, 172, 177, 185]

Thromboembolic disorder is a recognized complication of cancer and chemotherapy that may be as-

FIGURE 15

Alterations of coagulation factor II+VII+X activity and level of fibrin D-dimer induced by 5-FU based chemotherapy. Mean levels are indicated at baseline, during 5-FU treatment and at follow-up. Whiskers denote 95% CI of the means. P-values relate to effect of 5-FU treatment between repeated measures and to difference in response between groups.[IX]



cribed to endothelial dysfunction as well.[186-190] In keeping with this report (**Figure 15**) recent experimental and clinical studies indicated that a procoagulant state associated with increased plasma fibrin D-dimer is common in colorectal cancer patients.[IX, 183, 189-193] The elevated level of plasma vWf, mediating adherence and aggregation of platelets to the subendothelium during primary hemostasis, may be a contributory factor to thrombosis in such situations (Figure 14).[IX, 183, 194-196]

The vascular endothelium in cancer patients is vulnerable to injury due to the influence of tumour cell-related factors together with the damage caused by chemotherapy.[197, 198] Accordingly a number of patients in the present study had baseline plasma levels of coagulation and fibrinolysis markers outside the reference interval indicating a procoagulant state ahead of chemotherapy.[190, 192]

Episodes of cardiotoxicity usually are transient and reversible without angiographic or electrocardiographic indications of sustained arterial occlusion, which contradicts that thromboembolism may be a mechanism involved in 5-FU induced cardiotoxicity.[II, 56]

The influence of 5-Fluorouracil on the cardiac function

Symptoms of 5-FU cardiotoxicity such as hypotension and fainting indicate acute influence on the circulation and heart pump function.[II, VIII, 56, 172] In addition, scattered necrosis with loss of cardiomyocytes or evident myocardial infarction following 5-FU treatment may permanently reduce the heart pump capacity eventually leading to heart insufficiency.[199]

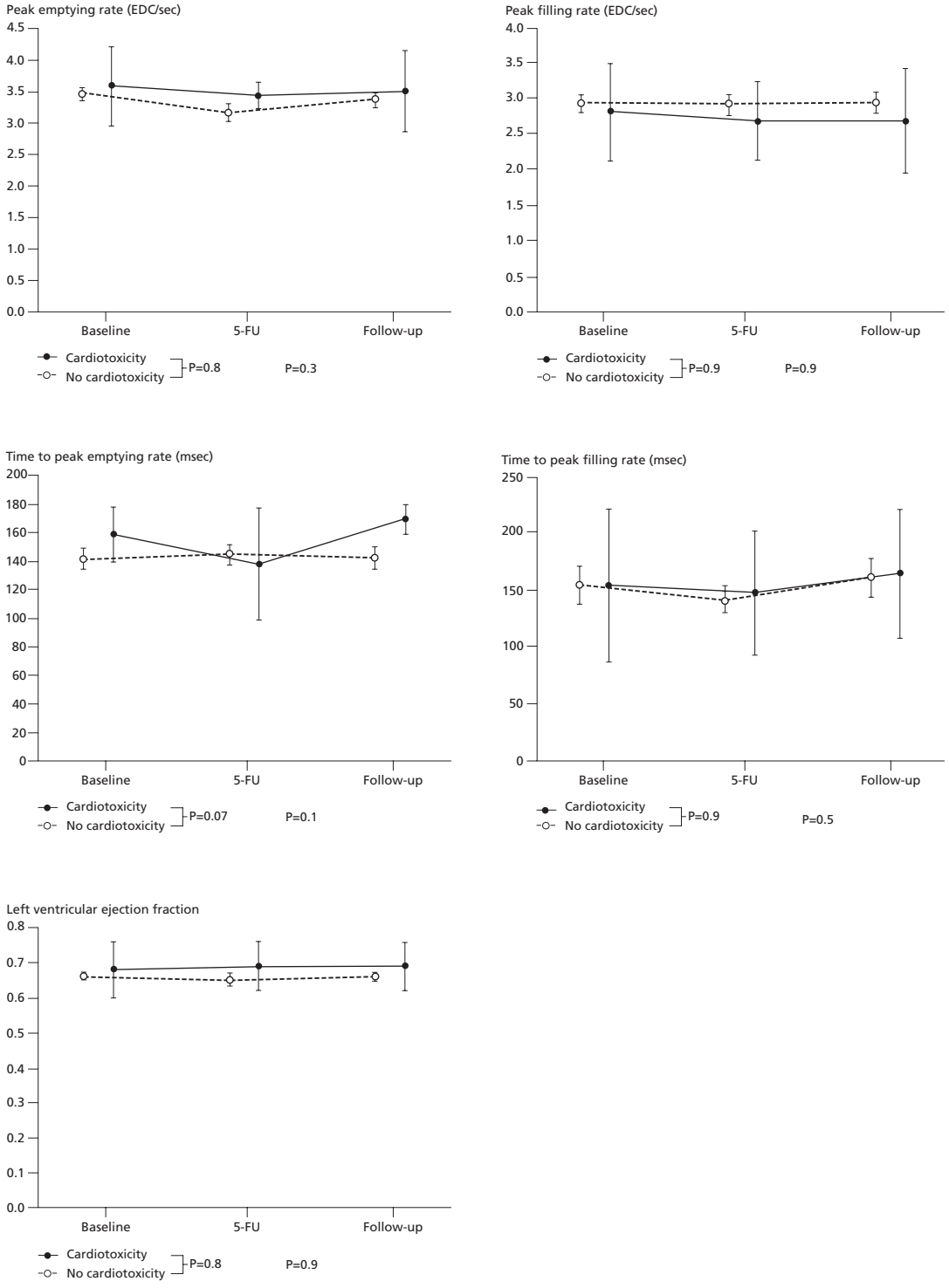
Left ventricular diastolic and systolic kinetics assessed using radionuclide ventriculography may be differentially affected in the acute and late phase of 5-FU cardiotoxicity depending on whether myocardial ischemia or cardiomyocyte loss may ensue, respectively. The atrioventricular pressure gradient for early diastolic filling is created by a suction effect from the release of the passive distension being stored in the elastic component of the ventricular wall during contraction. Myocardial relaxation however, requires energy to pump free myoplasmic calcium back into the sarcoplasmic reticulum. When energy availability is limited by ischemia, calcium may remain fixed to troponin for part of the diastole resulting in reduced distensibility of the myocardium and slower rate of ventricular filling.[200] On the other hand an inverse relationship have previously been established between histopathological evidence of cummulated myocardial damage and reduced left ventricular ejection fraction.[201, 202]

By serial assessments of left ventricular hemodynamics of patients being chemo naïve, during 5-FU therapy and at follow-up (**Figure 16**) the systolic parameters peak emptying rate (PER) 3.46±0.06 EDC/sec (P=0.3) and time to PER 143±3 msec (P=0.1) at baseline were not significantly altered by chemotherapy, neither in the subset having cardiotoxicity (P=0.07). Also the peak filling rate (PFR) 2.9±0.1 EDC/sec (P=0.9) and time to PFR 154±8 msec (P=0.5) at baseline were not significantly altered by chemotherapy irrespective of cardiotoxicity (P=0.9). Similarly the left ventricular ejection fraction at baseline 0.66±0.01 (mean±SE) did not change significantly during chemotherapy or at follow up in the entire cohort (P=0.4) or in any subset (P=0.2).[VIII, 172]

Although having high reproducibility and low inter-observer variability ventriculography has shortcomings too.[203] Due to the compensatory reserve of the myocardium that enables sufficient cardiac output in the presence of dysfunctional myocytes, ventriculography is somewhat insensitive for detecting subtle changes in the ejection fraction.

Yet these results were not indicative of clinically significant cummulative impairment of the left ventricular systolic function following 5-FU treatment, even if the

FIGURE 16



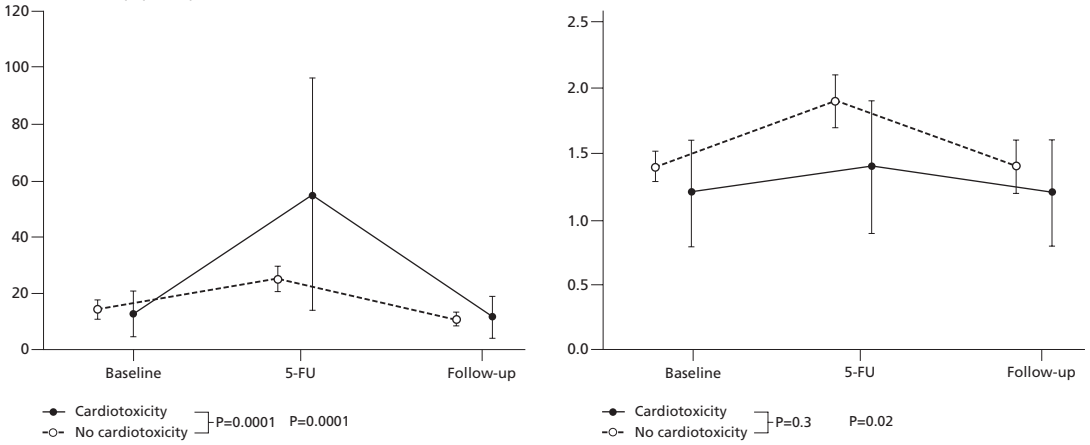
The influence of 5-FU on left ventricular systolic (left panel) and diastolic (right panel) kinetics in patients according to 5-FU cardiotoxicity. The assessments were done before chemotherapy (baseline), midway following one of the courses 5-7 and at follow-up spanning overall 6 months. Reevaluation following episodes of cardiotoxicity was done immediately or during a subsequent course of chemotherapy. The means and 95% CI of the means are indicated for peak emptying rate, time to peak emptying rate, left ventricular ejection fraction, peak filling rate and time to peak filling rate.[VIII]

actual cardiac damage is underestimated from the transient leak of CKMB being 3.0±0.1 µg/l (mean±SE) at baseline and 3.5±0.1 µg/l during 5-FU treatment (P=0.0002).[VIII, 172]

Furthermore while the effect of 5-FU on the heart is systemic by nature induced regional myocardial ishemia may be precipitated by local aterosclerotic narrowing of coronary arteries.[II, 56] Alteration of the distensibility for a region of the ventricular wall however may not be detectable on the global left ventricular kinetics.[204]

FIGURE 17

5-FU induced changes of plasma levels of NT-proBNP (left) and lactic acid (right) in patients having cardiotoxicity or no such side effects. The assessments were done before chemotherapy (baseline), midway following one of the courses 5-7 and at follow-up spanning overall 6 months. Reevaluation following episodes of cardiotoxicity was done immediately or during a subsequent course of chemotherapy. The mean levels are indicated. Whiskers denote 95% confidence intervals of the means. P-values relate to effect of 5-FU treatment between repeated measures and to difference in response between groups.[VIII]



The influence of 5-Fluorouracil on the myocardial metabolism

As left ventricular dysfunction develops, with myocardial ischemia and increasing ventricular wall stretch, counterregulatory mechanisms are up-regulated including activation of the neuroendocrine axis with stimulated proBNP secretion from cardiocytes eliciting increased natriuresis, diuresis and vasodilation.[57, 58]

Transient increases of plasma NT-proBNP indicated that most patients treated with 5-FU have reversible subclinical cardiac influence rather than being a discrete phenomenon confined to clinical symptoms only (**Figure 17**).[VIII, 172] The NT-proBNP level following 5-FU treatment exceeded the corresponding reference interval in 31 (29%) patients. In the entire cohort NT-ProBNP significantly increased from baseline 14.5±3.2 pmol/l (mean±SE) to 28.3±5.3 pmol/l during 5-FU therapy (P=0.0001). The 5-FU induced rise of NT-proBNP was significantly higher in females (P=0.0005). Nine (8.5%) patients with cardiotoxicity had significantly higher NT-proBNP of 55.3±40.8 pmol/l compared to 25.4±4.1 pmol/l in those without (P=0.0001).

Though the increase of NT-proBNP was significantly higher in patients with cardiotoxicity compared to those without, no clinically significant cut off level was evident from these data. The overlapping ranges of NT-proBNP however should be evaluated considering that five patients with previous symptoms of cardiotoxicity were assessed at reduced dose of 5-FU. Adjunctive antiangina treatment using diltiazem and isosorbide mononitrate also may have attenuated the 5-FU induced rise of NT-proBNP in these patients. [205]

The range of 5-FU induced NT-proBNP increases up to 184 pmol/l and median 19.2 pmol/l [VIII, 172] is consistent with other situations of transient myocardial heart ischemia such as unstable angina depending on extent and duration of ischemia[206-210] and exertion.[211] Of notion, females had significantly higher increment of the NT-proBNP level in response to ischemia.[208, 210, 212] Considerably greater rise of NT-proBNP may occur by the acute coronary syndrome, which however reflects the severity of underlying cardiovascular disease.[206-210] Extensive coronary multivessel disease and severe left ventricular dysfunction did not apply to the current study cohort.[VIII, 172]

Other anticancer agents have characteristic NT-proBNP profiles regarding onset, level of increase and duration reflecting diverse aspects of their mechanisms of cardiotoxicity, although the application of different assays may hamper comparison between studies. Cyclophosphamide causes endothelial damage leading to interstitial oedema and transiently decreased myocardial compliance.[213] Progressive heart failure results from prolonged exposure to anthracyclines due to accumulated dose dependent fibrosis and irreversible cardiomyopathy,[214, 215] whereas trastuzumab deprives the myocardium of a trophic stimulus by preventing ligand binding to the EGF receptor.[216] Cyclophosphamide treatment caused NT-proBNP to rise to a mean level of 624 pmol/l that reversed to baseline within weeks[213]. Increases of BNP above 43 pmol/l was significantly associated with symptoms of cardiac failure by cyclophosphamide treatment.[217] Progressive increases of NT-proBNP to median 407 ng/l by trastuzumab treatment[216] and to median 242 pmol/l or BNP to 8.8 pmol/l by anthracycline treat-

TABLE 13

Patient characteristics including heart and renal comorbidity and 5-FU cardiotoxicity.[II]

Comorbidity	No.	Gender	Age	BMI	Smoking	Atherosclerosis risk factors	Heart disease	Renal function: P-creatinine mM	Renal function: clearance ml/ min	Cardiotoxicity: CTC	Cardiotoxicity: symptoms
Heart	1	M	45	Normal	Previous	Hypercholesterolemia	AMI, angina	0.069	126*	3	Angina
	2	F	55	Moderate	Never	Diabetes, hypertension	AMI, angina	0.083	84*	4	AMI
	3	F	56	Moderate	Previous	Hypertension	Heart insufficiency	0.089	90*	3	Angina
	4	F	57	Normal	Never	Hypertension	Angina	0.089	63*	3	Angina
	5	M	65	Moderate	Current	Claudicatio	AMI, angina Heart insufficiency	0.087	96	4	AMI
	6	M	66	Severe	Current	Hypertension	Angina, arrhythmia Heart insufficiency	0.105	105*	3	Angina
	7	F	66	Normal	Previous		AMI, angina	–	–	3	Angina
	8	F	67	Normal	Current		Angina	0.075	60*	3	Angina
	9	F	72	Normal	–		Angina, heart insufficiency	0.095	54*	3	Angina
	10	M	73	Normal	Never		Angina	0.099	60*	3	Angina
	11	F	75	Normal	–	Diabetes, hypertension	Angina, heart insufficiency	0.108	42*	3	Incompensatio dyspnoea, angina
	12	M	77	Moderate	Previous	Hypertension Cerebral ischaemia	Angina Heart insufficiency	0.097	66*	3	Angina
Renal	13	M	46	Normal	Never			0.398	<30	3	Angina, arrhythmia
	14	M	54	Normal	Never			0.123	75*	3	Angina
	15	M	57	Normal	Previous		Transient arrhythmia	0.089	101-71	3	Incompensatio, dyspnoea, angina
	16	F	69	Normal	Never			0.084	48	3	Angina
None	17	M	24	Normal	Current			0.112	84*	2	Angina
	18	M	33	Normal	Current			0.112	84*	3	Angina, arrhythmia
	19	F	34	Normal	Never			0.055	106	3	Angina
	20	M	38	Moderate	Current			0.067	150*	3	Angina
	21	F	44	Normal	Previous			0.081	75*	3	Angina
	22	F	58	Normal	–			0.067	66*	2	Angina
	23	F	59	Moderate	Never			0.084	66*	3	Angina
	24	M	59	Normal	Previous			0.102	78*	3	Angina
	25	M	59	Moderate	Current			0.059	144*	3	Angina
	26	M	62	Normal	Never			0.085	78*	2	Angina
	27	F	65	Moderate	Never			0.090	72*	3	Angina
	28	M	70	Normal	Never			0.065	85	3	Angina, arrhythmia
	29	F	75	Normal	Never			0.079	63*	3	Angina

* clearance estimated from P-creatinine.

ment[214, 215] continued for months after cessation of treatment. No tresholds for NT-proBNP were identified being associated with heart failure by anthracycline or trastuzumab treatment.[214-216]

While the NT-proBNP increases following anthracycline and trastuzumab treatment correlate with cumulative structural changes of the myocardium, 5-FU mainly causes transient and fully reversible interference with the metabolism. The accumulation of NT-proBNP in the periferal circulation lag behind the 5-FU cardiotoxicity due to delay in proBNP production[58] and release into the coronary circulation.[209, 218]

Accordingly the NT-proBNP level was lower following the initial 5-FU bolus than the level during the subsequent continuous infusion of de Gramont's and FOLFIRI regimens.[219]

Experimental studies on animal heart perfusion with 5-FU demonstrated induction of myocardial production of lactic acid indicating a shift to anaerobic metabolism.[177, 185] Likewise following the dose and schedule of 5-FU treatment that apply in a clinical setting the plasma lactic acid exceeded the reference interval in 30 (28%) patients. The lactic acid level significantly (P=0.0001) increased from baseline 1.3±0.1 mmol/l (mean±SE) to 1.8±0.1 during 5-FU therapy, which did not significantly differ between subsets having symptoms of cardiotoxicity or not (P=0.4) (Figure 17).[VIII, 172] However the ratio of plasma lactic acid deriving from cardio-specific processes remains un-clarified. Hence the influence of 5-FU on the metabolism of other tissues may contribute to the severe lactacidosis occasionally reported.[220, 221]

Cardiovascular comorbidity as risk factor

Cardiovascular disease is assumed to be a risk factor for development of 5-FU cardiotoxicity based on the relationship with endothelial dysfunction and the interference with vasoactivity and with myocardial blood perfusion.[167, 168] Though significant atherosclerosis is no prerequisite for 5-FU cardiotoxicity to occur[164, 169] cardiovascular disease may pose a risk in terms of higher inci-

TABLE 14

Clinical characteristics, cardiovascular status and risk factors in 106 completely resected colorectal cancer patients according to 5-FU induced cardiotoxicity during adjuvant chemotherapy.[VIII]

	Cardiotoxicity		
	no n=97 no. (%)	yes n=9 no. (%)	P
Gender			
Female	53 (55)	6 (67)	0.6
Male	44 (45)	3 (33)	
Age			
<70 years	72 (74)	6 (67)	0.7
≥70 years	25 (26)	3 (33)	
Primary tumour			
Colonic	69 (71)	5 (56)	0.4
Rectal	28 (29)	4 (44)	
Stage			
II	21 (22)	1 (11)	0.3
III	58 (60)	5 (56)	
IV	18 (18)	3 (33)	
Cardiovascular disease	7 (7)	1 (11)	0.8
Hypercholesterolemia	26 (27)	5 (56)	0.2
Hypertension	24 (25)	2 (22)	0.9
Diabetes mellitus	16 (16)	0 (0)	0.4
Body mass index			
Normal	52 (54)	4 (45)	0.6
Overweight	30 (31)	3 (33)	
Obese	15 (15)	2 (22)	
Smoking			
Never	40 (41)	4 (45)	0.6
Previously	34 (35)	4 (45)	
Currently	23 (24)	1 (10)	
Renal clearance			
≤ 60ml/min	14 (14)	0 (0)	0.5

TABLE 15

Multivariate regression analysis associating clinical characteristics with changes in plasma NT-proBNP following 5-FU treatment.[VIII]

	Regression coefficients		
Predictor variables	**mean**	**95% CI**	**P**
Gender (female)	0.69	0.31-1.08	0.0005
Age (≥70 years)	0.17	–0.25-0.58	0.4
Cardiovascular disease	0.56	–0.19-1.30	0.1
Hypercholesterolemia	0.06	–0.36-0.47	0.8
Hypertension	–0.22	–0.69-0.24	0.3
Diabetes mellitus	–0.32	–1.01-0.37	0.4
BMI (≥30 obese)	0.24	–0.04-0.51	0.09
Smoking (ever)	0.06	–0.31-0.44	0.7
Renal clearance (≤60 ml/min)	0.27	–0.31-0.84	0.4

dence [167, 168] and a trend for severity (**Table 13**) of cardiac adverse reactions from 5-FU.[II, 56] Using NT-proBNP and vWf as markers for cardiac and endothelial function, respectively, no evidence was found indicating that cardiovascular disease may imply greater risk of cardiotoxicity and vulnerability of the endothelium from 5-FU treatment (**Table 14** and **Table 15**).[VIII, IX, 172, 183] This conclusion however is made with the reservation that the degree of atherosclerosis is estimated indirectly from the cardiovascular history.

Renal comorbidity as risk factor

Markedly augmented susceptibility to 5-FU induced cardiotoxicity along with intercurrently impaired renal function has previously been reported.[II, 56] Other case reports have noticed reduced renal function and simultaneous markedly increased cardiotoxicity without ascribing this coincidence any causality.[166, 169, 222]

Theoretically the renal function is of significance to the NT-proBNP plasma level by 5-FU treatment for more reasons. Excretion of potentially cardiotoxic fluorometabolites by the kidneys accounts for up to 90% of an intravenous dose within 24 hours.[223] Secondly as NT-proBNP normally is cleared by the kidney the level correlate inversely with renal function independently of cardiac disease.[224]

While no association between renal function and the 5-FU induced NT-proBNP increment appeared by multivariate analysis (Table 15),[VIII, 172] this result may be compromised by the inaccurate estimate of the renal function being calculated from the plasma creatinine level. Direct assessment of the glomerular filtration rate is required to clarify this matter.

Conclusion

Cancer derived factors and 5-FU based chemotherapy in a clinical setting induces global reversible endothelial injury. The ensuing endothel dysfunction with impaired vasoactivity may be a concurrent cause of development of 5-FU cardiotoxicity. 5-FU therapy generally does not lead to long-term impairment of the left ventricular pump function.

Transient increases of plasma NT-proBNP indicated that most patients treated with 5-FU have reversible influence on myocardial metabolism. The increase was significantly higher in patients with cardiotoxicity compared to those without. The usability of NT-proBNP as predictive marker for 5-FU cardiotoxicity remains to be clarified. Validation of NT-proBNP as a predictor for cardiotoxicity presupposes that the increase following the first full dose of 5-FU correlates with cardiac adverse reactions during the subsequent chemotherapy. Furthermore uniform study conditions using ergometry is required to elucidate the potentially reduced exertion treshold related to angina and the corresponding level of NT-proBNP. The prospect is that NT-proBNP, as a validated biomarker in this setting, may facilitate safer application of 5-FU and may serve as a tool to clarify the pathophysiology and risk factors of cardiotoxicity.

GENERAL CONCLUSIONS AND FUTURE ASPECTS

Tumour biomarkers

Tumour enzymes related to the pyrimidine homeostasis and pharmacokinetic of 5-FU play key roles in the biology and 5-FU sensitivity of colorectal cancer. Hence the

current data indicate that TS and DPD expression in colorectal cancer are associated to prognosis independent of chemotherapy, while also being predictive for efficacy of adjuvant 5-FU therapy.

Tumour enzyme activity varies individually due to inherited traits or as a result of gene deregulation from microsatellite instability or chromosomal aberration.

Microsatellite instability due to mismatch repair deficiency is one of the main biomarkers in colorectal cancer as it not only indicates the pathogenesis but also provides information on prognosis and prediction of response to chemotherapy. While there is no evidence that TS or DPD expression differ between microsatellite instable and stable colorectal cancers, future investigations into the genes that are deregulated by microsatellite instability may clarify the molecular foundation for the resistance to 5-FU therapy and distinct clinicopathological characteristics of microsatellite instable carcinomas.

Evidence was found that chromosomal aberration is correlated to the TS immunoreactivity in cancer cells. However there are shortcomings to the FISH technology. Though the analysis provides a quantitative estimate of gene specific copy number changes in the cancer cells it cannot account for the loss or amplification of either a dominant or a recessive allele. Such analyses may complement the FISH technology in order to understand the relationship between a genotype and the tumour biology.

The MMP-9 and TIMP-1 expression in carcinoma cells and peritumoural stromal cells have diverse roles in cancer cell invasion and host versus tumour immune response. This recognition is essential for their application as prognostic markers and for development of rational anti-cancer therapies based on specific targeting of the matrix proteinase system.

Age

As life expectancy is projected to increase both the incidence and the relative proportion of elderly patients with colorectal cancer is expected to rise. Available data suggests that elderly patients aged ≥75 years being in good performance and without significant comorbidity have a reasonable life expectancy to benefit from 5-FU based chemotherapy without excess toxicity. Future research should not impose upper age limits and consideration should be given to stratification by age at trial entry in order to identify those regimens associated with favourable toxicity profile and improved survival in elderly patients.

Carditoxicity of 5-Fluorouracil

5-FU based chemotherapy induces global reversible endothelial injury leading to activation of the coagulation. The significance of the endothelial damage for the pathogenesis of 5-FU induced cardiotoxicity remains unclarified. 5-FU therapy generally does not lead to long-term impairment of the left ventricular pump function.

Transient increases of plasma NT-proBNP, which was significantly higher in patients with cardiotoxicity compared to those without, indicated that most patients treated with 5-FU have reversible influence on the myocardial metabolism. The usability of NT-proBNP as predictive marker for 5-FU cardiotoxicity remains to be clarified. Validation of NT-proBNP as a predictor for cardiotoxicity entails that the increase following the first full dose of 5-FU correlates with cardiac adverse reactions during the subsequent chemotherapy. Furthermore uniform study conditions using ergometry is required to elucidate the potentially reduced exertion treshold related to angina and the corresponding level of NT-proBNP. The prospect is that NT-proBNP, as a validated biomarker in this setting, may facilitate safer application of 5-FU and may serve as a tool to clarify the pathophysiology and risk factors of cardiotoxicity.

Future aspects

Currently the choice of 5-FU based chemotherapy of colorectal cancer is based on the spread of disease and the clinical characteristics including comorbidity of the patient. The individually adjusted (tailored) chemotherapy is another principle also taking account of the biologic characteristics of the tumour and the pharmacogenetic profile of the patient in order to improve the efficacy and reduce the adverse reactions associated with the treatment. The outlook is that additional biomarkers may provide the foundation for tailoring of chemotherapy regarding selection of active agents and regimens as novel antineoplastic drugs are introduced. In addition prognostic tumour biomarkers may be utilized to select patients for different follow-up programs.

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