Chapter 3

Circulating cell death products predict clinical outcome of colorectal cancer patients

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Abstract

Tumour cell death generates products that can be measured in the circulation of cancer patients. CK18-Asp396 (M30 antigen) is a caspase-degraded product of cytokeratin 18 (CK18), produced by apoptotic epithelial cells, and is elevated in breast and lung cancer patients. We determined the M30 antigen and total CK18 levels in plasma of 49 colorectal cancer (CRC) patients, before and after surgical resection of the tumor, by ELISA. Correlations with patient and tumour characteristics were determined by Kruskal-Wallis H and Mann Whitney U tests. Disease-free survival was determined using Kaplan-Meier methodology with Log Rank tests, and univariate and multivariate Cox proportional hazard analysis. Plasma M30 antigen and total CK18 levels in CRC patients were related to disease stage and tumour diameter, and were predictive of disease-free survival, independent of disease-stage, with hazard ratios (HR) of patients with high levels (> median) compared to those with low levels (≤ median) of 3.58 (95% CI: 1.17-11.02) and 3.58 (95% CI: 0.97-7.71), respectively. The M30/CK18 ratio, which decreased with tumour progression, was also predictive of disease-free survival, with a low ratio (≤ median) associated with worse disease-free survival: HR 2.78 (95% CI: 1.06-7.19). Remarkably, the plasma M30 antigen and total CK18 levels after surgical removal of the tumour were also predictive of disease-free survival, with patients with high levels having a HR of 3.78 (95% CI: 0.77-18.50) and 4.12 (95% CI: 0.84-20.34), respectively, indicating that these parameters also can be used to monitor patients after surgery. M30 antigen and total CK18 levels in the circulation of CRC patients are predictive of tumour progression and prognosis and might be helpful for treatment selection and monitoring of these patients.
Introduction

Death of tumour cells generates detectable protein products in the patient’s circulation, which may be used for cancer diagnostics and/or monitoring of therapy efficacy\(^1\). Apoptosis is a form of regulated cell death that is characterized by specific structural changes, mediated by proteases of the caspase family\(^2\). Caspase activity itself or the presence of specific degradation products can be used for the detection of tumour cell apoptosis.

The M30 antibody detects a caspase-degraded product, CK18-Asp396 (M30 antigen), of the important cytoskeletal protein cytokeratin 18 (CK18) of epithelial cells, which is expressed by most carcinomas, including those of breast, prostate, lung and colon\(^3\). Immunohistochemistry with the M30 antibody has been shown to be as specific as the morphological detection or TdT-mediated dUTP-biotin nick end-labelling (TUNEL) technique to establish of apoptosis in tissue\(^3\)\(^\text{-}^5\). Importantly, the levels of M30 antigen can also be determined in the circulation by a specific ELISA, allowing the detection of tumour cell apoptosis in the serum/plasma of cancer patients\(^6\)\(^,\)\(^7\). However, M30 antigen detection in the plasma is not tumour specific, healthy controls have background levels, due to apoptosis of normal epithelial cells. Circulating M30 antigen levels were found to be elevated in patients with lung and breast cancer, and were predictive to survival or recurrence outcome\(^8\)\(^,\)\(^9\). In addition, circulating M30 antigen levels increased shortly after chemotherapy in hormone-refractory prostate cancer and lung cancer, implying that this was a result of chemotherapy-induced tumour cell apoptosis\(^9\)\(^\text{-}^11\).

During non-programmed cell death, i.e., necrosis, intact CK18 of epithelial tumour cells is released into the circulation, which can be measured by a total CK18 ELISA, that also detects the M30 antigen\(^11\). Cytokeratins and their cleaved forms are secreted in aggregates into the circulation and both M30 antigen and total CK18 levels in human plasma samples have a long-term stability when stored at -80 °C\(^12\).

The ratio between M30 antigen and total CK18 levels in the circulation depends on the balance between caspase-mediated apoptosis and non-proteolytic necrosis. This balance might be an important factor and denominator to select patient for treatment that induces necrosis vs treatment that increases apoptosis. For instance, docetaxel treatment increased levels of M30 antigen in the serum of breast cancer patients, indicating apoptotic death of tumour cells, while cyclophosphamide/epirubicin/5-fluorouracil treatment increased total CK18 levels, indicating necrotic death of tumour cells\(^13\). The increase of total CK18 serum levels correlated to the clinical therapy response\(^13\). The M30/CK18 ratio was shown to be decreased, i.e., more necrosis over apoptosis, in endometrial cancer stage III/IV when
compared with stage II, indicating less apoptosis and/or more necrosis during tumour progression.\textsuperscript{11}

In the present study, we determined the M30 antigen and total CK18 levels in plasma of 49 colorectal cancer (CRC) patients and found these levels to predict clinical outcome of these patients.

**Material&Methods**

**Patients, plasma and tissue collection**

The study population consisted of 49 CRC patients that did not receive pre-operative treatment and had been admitted to the Leiden University Medical Centre for surgical resection. Citrate plasma samples were collected, with informed consent of the patients, before resection (P1, pre-operative), shortly after surgical resection (P2) and about 4.5 months after the operation (P3, post-operative), when feasible. Citrate plasma samples were collected before 9.00 a.m. under fasting conditions and stored at -70°C. Fresh tissue was collected from the surgical specimens immediately after resection, and attention was paid to collect material from the non-necrotic part of the tumour. Normal mucosa samples were obtained at a distance of approximately 10 cm from the tumour. Tissue samples were also frozen and stored at -70 °C until use. Macroscopic (diameter and localization of the tumor) as well as microscopic data were assessed, including classification according to the WHO. Colonic cancers were classified as being proximal or distal to, and including, the splenic flexure. Follow-up information, including post-operative adjuvant therapy, was available for a period up to 8 years. The study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre in compliance with the Helsinki Declaration.

**Tissue homogenization and protein determination**

Frozen tissue specimens were weighed and homogenized on ice for 2 minutes in 1 ml Tris-HCl, 0.1% Tween 80, pH 7.5 per 60 mg tissue using a Potter device (B Braun, Germany), and centrifuged twice at 8000 x g for 2.5 min at 4°C. Protein content was measured according to Lowry \textit{et al.} and standardized by bovine serum albumin.\textsuperscript{14}

**M30 antigen and total CK18 detection**

For the detection of M30 antigen and total CK18 in the plasma of CRC patients commercially available immunosorbent sandwich ELISAs were used, according to
manufacturer’s instructions (M30-apoptosense ELISA and M65 ELISA, Peviva, Sweden). For the determination of M30 antigen and total CK18 levels in tissues 1 µg/µl protein homogenates were diluted up to 1000 times, dependent on antigen levels. The antigen levels in the plasma were expressed as U/L and antigen levels in tumour tissue or normal mucosa were expressed as U/mg protein.

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). For relations between M30 antigen or total CK18 antigen levels in P1, P2 and P3 Wilcoxon signed-rank tests were used. For the relation with patient characteristics non-parametric Mann Whitney U tests were used, because the study parameters did not follow a normal distribution. Disease-free survival was estimated using Kaplan-Meier methodology with cancer-related death and local or distant recurrence as events, dichotomized for M30 antigen, total CK18 or M30/CK18 ratio levels, and Log Rank tests. Univariate and multivariate Cox proportional hazard models were used to explore the association of markers with disease-free survival. Kaplan Meier graphs were made with Graphpad Prism (version 4.0, Graphpad Prism Inc., La Jolla, CA, USA) software.

Results

M30 antigen and total CK18 levels in plasma of CRC patients

Overall the plasma M30 antigen and total CK18 levels correlated very well with each other, in plasma before, as well as shortly and longer after tumour resection (overall Spearman correlation coefficient Rho=0.64, P<0.0001).

The M30 antigen and total CK18 plasma values increased shortly after surgical resection of the tumour and dropped to about pre-operative values longer after surgery (Table I).
Table 1: M30 antigen and total CK18 levels in plasma of CRC patients.

|                          | P1 (n=49)       | P2 (n=20)       | P3 (n=28)       |
|--------------------------|-----------------|-----------------|-----------------|
| **Mean time to operation** | -13 (-50-0)     | +20 (7-60)      | +137 (82-364)   |
| (days, range)            |                 |                 |                 |
| **M30 antigen level (U/l)** |                 |                 |                 |
| Median (IQR)             | 59.1 (41.5-88.2)| 74.8 (39.7-107.9)| 55.6 (42.1-79.9)|
| P-value                  |                 | 0.02 vs P1      | 0.11 vs P2      |
| **Total CK18 level (U/l)** |                 |                 |                 |
| Median (IQR)             | 260.5 (181.6-378.3)| 308.1 (208.7-492.3)| 257.2 (183.8-457.2)|
| P-value                  | 0.08 vs P1      |                 | 0.05 vs P2      |

P1 = pre-operative plasma, P2 = post-operative plasma shortly after operation, and P3 = post-operative plasma longer after operation. Wilcoxon signed-rank tests were used to compare paired observations. P-values ≤ 0.05 were considered significant, shown in bold.

Correlation pre-operative plasma M30 antigen and CK18 levels with clinico-pathological patient parameters

The clinico-pathological parameters of the 49 CRC patients are shown in Table 2. M30 antigen and total CK18 plasma levels did not correlate with localization of the tumour, also not within the patients with only colonic tumours (not shown). Male and female CRC patients had similar plasma M30 antigen and total CK18 levels, and these were not correlated with patients’ age. The M30 antigen and total CK18 plasma values were higher in patients with more advanced tumour stages (Figure 1A and B, P=0.01 and P=0.05, respectively). M30 antigen levels correlated with the diameter of the tumour (Spearman correlation coefficient Rho=0.35, P=0.02). M30 antigen and total CK18 levels were significantly higher in the eight patients with a Dukes’ D tumour in which the tumour was not (or not curatively) resected.
Table 2: Clinico-pathological characteristics and pre-operative plasma M30 and total CK18 levels.

| Patient and tumour characteristics | No of patients (%) | Plasma M30 antigen (U/l) Median (IQR) | P-value | Total CK18 plasma level (U/l) Median (IQR) | P-value |
|-----------------------------------|--------------------|---------------------------------------|---------|--------------------------------------------|---------|
| Total n=49                        |                    |                                       |         |                                            |         |
| Gender                            |                    |                                       |         |                                            |         |
| Male                              | 31 (63)            | 61.1 (41.8-88.2)                     | 0.87    | 260.5 (174.1-382.8)                       | 0.92    |
| Female                            | 18 (37)            | 50.5 (37.6-110.2)                    | 0.32    | 252.9 (184.5-352.1)                       | 0.38    |
| Age #                             |                    |                                       |         |                                            |         |
| ≤ median                          | 25 (51)            | 59.1 (15.1-99.0)                     |         | 288.6 (181.6-403.5)                       |         |
| > median                          | 24 (49)            | 55.6 (34.2-74.0)                     |         | 232.4 (177.3-328.6)                       |         |
| Location                          |                    |                                       | 0.63    |                                            | 0.25    |
| Colon                             | 38 (78)            | 59.4 (40.5-102.8)                    |         | 268.6 (199.7-391.1)                       |         |
| Rectum                            | 11 (22)            | 56.4 (43.3-68.4)                     |         | 208.4 (174.1-377.9)                       |         |
| Dukes’ stage                      |                    |                                       | 0.01    |                                            | 0.05    |
| A/B                               | 27 (55)            | 49.3 (34.2-67.8)                     |         | 235.0 (170.5-303.5)                       |         |
| C/D                               | 22 (45)            | 69.2 (49.7-114.5)                    |         | 295.8 (202.3-706.3)                       |         |
| WHO classification                |                    |                                       | 0.43    |                                            | 0.56    |
| Adenocarcinoma                    | 45 (92)            | 59.1 (39.9-82.2)                     |         | 265.4 (186.7-378.3)                       |         |
| Mucinous carcinoma                | 4 (8)              | 77.8 (48.9-177.7)                    |         | 211.2 (172.0-363.1)                       |         |
| Tumour diameter *                 |                    |                                       | 0.03    |                                            | 0.07    |
| ≤ median                          | 24 (55)            | 49.1 (31.9-67.7)                     |         | 232.4 (174.5-187.7)                       |         |
| > median                          | 20 (45)            | 64.7 (43.7-133.3)                    |         | 287.3 (180.0-657.6)                       |         |
| Surgery                           |                    |                                       | 0.001   |                                            | <0.001  |
| Yes, curative resection           | 41 (84)            | 51.1 (36.5-73.7)                     |         | 235.0 (172.3-331.9)                       |         |
| No, or palliative                 | 8 (16)             | 162.9 (68.8-346.4)                   |         | 971.7 (306.1-2290.4)                      |         |

# Median 68 years, range 31-84. * Median 4.5 cm, range 2-13.5 (some values missing). P-values were calculated with Mann Whitney and Kruskall-Wallis tests and were considered significant when ≤0.05, shown in bold.

Pre-operative plasma M30 antigen and CK18 levels and disease-free survival

The disease-free survival of the patients with low M30 antigen plasma levels before resection (≤ median) was significantly better compared with patients with high M30 antigen plasma levels (Figure 1C). This was also the case for total CK18 plasma levels (Figure 1D). When the patients were subdivided in “early” and “advanced” tumours, Dukes’ A/B stage carcinoma (n=27) and C/D stage carcinoma (n=22), the plasma M30 antigen level was also prognostic within these subgroups (Figure 1E and F).

Calculation of hazard ratios as estimates of relative risk of death or disease recurrence is shown in Table 3. Tumour progression, M30 antigen and total CK18 levels in plasma before surgery were predictive of recurrence or death.
Figure 1: Pre-operative plasma M30 antigen and total CK18 levels, and survival.

M30 antigen and total CK18 levels in pre-operative plasma of all CRC patients with a Dukes’ A/B vs Dukes’ C/D stage carcinoma (P1, A and B). Box plots with line indicating median value, box indicating IQR and bars indicating the range. Kaplan Meier disease-free survival curves of all CRC patients, groups divided upon median values of M30 antigen (C) and total CK18 levels (D) in pre-operative plasma (P1). Patients were also subdivided in Dukes’ A/B (E, n=22) and Dukes’ C/D (F, n=27) stage carcinoma.
Table 3: Univariate and multivariate Cox regression analysis of disease-free survival.

| Patient and tumour characteristics | Univariate Hazard Ratio (95% confidence interval) | P-value | Multivariate Hazard Ratio (95% confidence interval) | P-value |
|-----------------------------------|-------------------------------------------------|--------|-------------------------------------------------|--------|
| Gender                            | 1 (ref)                                          | 0.27   | 1 (ref)                                          | <0.001 |
| Male                              | 1.79 (0.64-4.97)                                 |        |                                                 |        |
| Age (median 68, range 31-84)     | 0.83                                            |        |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        |                                                 |        |
| > median                          | 1.10 (0.45-2.73)                                 |        |                                                 |        |
| Location                          | 0.32                                            |        |                                                 |        |
| Colon                             | 1 (ref)                                          |        |                                                 |        |
| Rectum                            | 0.54 (0.16-1.85)                                 |        |                                                 |        |
| Dukes’ stage                      | <0.001                                          | <0.001-0.021 |                                                 |        |
| A/B                               | 1 (ref)                                          |        | 1 (ref)                                          |        |
| C/D                               | 8.21 (2.70-24.97)                                | 6.01-9.60 (1.32-33.31) |        |        |
| Tumour diameter                   | 0.32                                            |        |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        |                                                 |        |
| > median                          | 1.68 (0.61-4.65)                                 |        |                                                 |        |
| WHO classification                | 0.82                                            |        |                                                 |        |
| Adenocarcinoma                    | 1 (ref)                                          |        |                                                 |        |
| Mucinous carcinoom                | 0.79 (0.11-5.90)                                 |        |                                                 |        |
| M30 antigen plasma level P1       | 0.003                                           | 0.03   |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |
| > median                          | 5.28 (1.75-15.92)                                | 3.58 (1.17-11.02) |        |        |
| Total CK18 plasma level P1        | 0.02                                            | 0.055  |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |
| > median                          | 3.30 (1.19-9.16)                                 | 3.58 (0.97-7.71) |        |        |
| M30/CK18 ratio P1                 | 0.12                                            | 0.04   |                                                 |        |
| ≤ median                          | 2.09 (0.82-5.31)                                 | 2.78 (1.06-7.19) |        |        |
| > median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |
| M30 antigen plasma level P3       | 0.054                                           | 0.10   |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |
| > median                          | 4.71 (0.97-22.85)                                | 3.78 (0.77-18.50) |        |        |
| Total CK18 plasma level P3        | 0.055                                           | 0.08   |                                                 |        |
| ≤ median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |
| > median                          | 4.69 (0.97-22.72)                                | 4.12 (0.84-20.34) |        |        |
| M30/CK18 ratio P3                 | 0.04                                            | 0.10   |                                                 |        |
| ≤ median                          | 5.28 (1.08-25.72)                                | 3.96 (0.78-20.08) |        |        |
| > median                          | 1 (ref)                                          |        | 1 (ref)                                          |        |

Multivariate Cox hazards analysis of pre-operative or post-operative M30 antigen, total CK18 antigen level or M30/CK18 ratios combined with Dukes’ stage. P-values ≤0.05 were considered significant, shown in bold.

The M30/CK18 ratio indicates the balance between caspase-mediated apoptosis and non-proteolytic necrosis. M30/CK18 ratios of pre-operative plasmas of CRC patients were calculated and a large variety was observed in different patients with a median ratio of 0.20
(IQR: 0.15-0.26). The M30/CK18 ratio tended to decrease with increasing Dukes’ stage (Figure 2A), indicating more necrosis over apoptosis, during tumour progression. The disease-free survival of patients with high ratios tended to be better when compared with those with lower ratios (Figure 2B). This relation was particularly present in patients with Dukes’ C/D stage carcinomas (Figure 2C), in contrast to patients with Dukes’ A/B stage carcinomas where no such relation was found (Figure 2D).

**Figure 2: Pre-operative plasma M30/CK18 ratios and survival.**
Pre-operative plasma M30/CK18 ratios decrease with increasing Dukes’ stage (P1, A). Box plots with line indicating median value, box indicating IQR and bars indicating the range. Kaplan Meier disease-free survival curves of all CRC patients, groups divided upon median pre-operative plasma M30/CK18 ratios (B). Patients were also subdivided in Dukes’ C/D (C, n=22) and Dukes’ A/B stage carcinoma (D, n=27)
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M30 antigen, CK18 levels and M30 antigen/CK18 ratios in post-operative plasma

Post-operative plasma M30 antigen levels of patients about 4.5 months after resection of Dukes’ C/D stage carcinomas were also somewhat, but not significantly, higher compared with patients with Dukes’ A/B stage carcinomas (Figure 3A). The total CK18 plasma levels, however, were significantly higher in Dukes’ C/D tumour patients compared with Dukes’ A/B tumour patients (Figure 3B). High M30 antigen levels as well as total CK18 plasma levels after tumour resection were associated with worse disease-free survival (Figure 3C and D), also found in the univariate Cox hazard analysis (Table 3). M30/CK18 ratios in post-operative patients’ plasma were in a similar range as in the patients’ plasma before surgery, and also tended to decrease with increasing Dukes’ stage, i.e., significantly higher in Dukes’ A/B compared with Dukes’ C/D patients (Figure 3E). The disease-free survival was again better in patients with high post-operative plasma M30/CK18 ratios (Figure 3F).

In 8 of the 28 patients post-operative treatment with radiotherapy and in only one patient treatment with combined radio-chemotherapy was started before collection of the 4.5 months plasma samples. There were no significant differences in post-operative plasma M30 antigen and total CK18 levels, and in M30/CK18 ratios in patients receiving post-operative adjuvant therapy compared to the patients without adjuvant therapy (Figure 4).

Multivariate Cox regression analysis

A multivariate Cox proportional hazards model of disease-free survival was used to evaluate whether the plasma M30 antigen and total CK18 levels showed independent prognostic significance from tumour staging. These analyses showed that patients with high pre-operative M30 antigen plasma levels have a 3.6 times increased relative risk of CRC-related-death or disease recurrence (Table 3), independent of Dukes’ staging. For total CK18 levels, this was quite similar although just not significant. Patients with low pre-operative plasma M30/CK18 ratios had a 2.8 times increased risk to develop recurrence or death. Post-operative M30 antigen, total CK18 and M30/CK18 ratio levels all had similar prognostic significance, although not significant (Table 3).
Figure 3: Post-operative M30 antigen, total CK18 and M30/CK18 ratios, and survival.

Post-operative (P3) M30 antigen and total CK18 levels of CRC patients with a Dukes’ A/B vs Dukes’ C/D stage carcinoma (A and B). Box plots with line indicating median value, box indicating IQR and bars indicating the range. Kaplan Meier disease-free survival curves of all CRC patients, groups divided upon median post-operative M30 antigen (C, n=28) and total CK18 levels (D, n=28). Plasma M30/CK18 ratios were decreased in patients Dukes’ C/D vs Dukes’ A/B (E). Kaplan Meier disease-free survival curves of CRC patients, groups divided upon median post-operative plasma M30/CK18 ratios (F, n=28).
Correlation tumour and plasma M30 antigen

Corresponding CRC tissue was obtained in 40 cases. M30 antigen levels were significantly (P=0.05) higher in tumor tissue when compared with normal adjacent tissue with a median value of 2.1 (IQR: 0.3-7.7, n=40) vs 1.8 (IQR: 0.1-4.2, n=36) U/mg, respectively. Tumour M30 antigen, total CK18 levels and M30/CK18 ratios were only found to correlate with tumour location, with rectal tumours having higher levels (Table 4). Surprisingly, M30 antigen plasma levels showed a tendency to inversely correlate with the M30 antigen level of the tumour (Rho=-0.307, P=0.054). Tumour M30 antigen, total CK18 levels and M30/CK18 ratios were found to be not prognostic for disease-free survival (Figure 5).

Table 4: Clinico-pathological characteristics and tumour M30, total CK18 and M30/CK18 ratios.

| Patient and tumour characteristics | No of patients (%) | M30 antigen level (U/mg) Median (IQR) | Total CK18 level (U/mg) Median (IQR) | M30/CK18 x 100 Median (IQR) |
|-----------------------------------|--------------------|--------------------------------------|---------------------------------------|-----------------------------|
| Gender                            |                    |                                      |                                       |                             |
| Male                              | 26                 | 2.1 (0.2-7.7)                        | 203.0 (64.0-318.2)                    | 1.9 (0.3-3.7)               |
| Female                            | 14                 | 1.3 (0.2-10.3)                       | 159.0 (33.2-273.5)                    | 1.7 (0.2-3.6)               |
| Age #                             |                    |                                      |                                       |                             |
| ≤ median                          | 20                 | 1.4 (0.3-6.1)                        | 165.0 (53.0-331.7)                    | 1.8 (0.5-3.4)               |
| > median                          | 20                 | 2.2 (0.2-9.7)                        | 219.1 (57.0-248.9)                    | 2.2 (0.2-3.8)               |
| Location                          |                    |                                       |                                       |                             |
| Colon                             | 32                 | 0.8 (0.2-5.7)                        | 147.0 (47.1-243.3)                    | 1.4 (0.2-2.4)               |
| Rectum                            | 8                  | 12.7 (3.5-19.5)                      | 291.5 (214.6-369.7)                   | 3.7 (2.8-6.3)               |
| Dukes’ stage                      |                    |                                       |                                       |                             |
| A/B                               | 25                 | 1.6 (0.3-9.7)                        | 177.1 (71.8-310.2)                    | 1.8 (0.2-3.4)               |
| C/D                               | 15                 | 1.3 (0.1-5.9)                        | 222.4 (32.7-252.1)                    | 2.0 (0.4-3.8)               |
| WHO classification                |                    |                                       |                                       |                             |
| Adenocarcinoma                    | 37                 | 1.6 (0.3-7.7)                        | 190.3 (56.9-281.9)                    | 1.9 (0.3-3.4)               |
| Mucinous carcinoma                | 3                  | 1.1                                 | 65.4                                 | 1.7                        |
| Tumour diameter *                 |                    |                                       |                                       |                             |
| ≤ median                          | 20                 | 4.3 (0.3-10.3)                       | 203.0 (56.9-310.2)                    | 2.3 (0.3-3.9)               |
| > median                          | 18                 | 1.3 (0.1-5.7)                        | 154.0 (36.7-281.9)                    | 1.6 (0.2-2.3)               |

Clinico-pathological characteristics and tumour M30 antigen, total CK18 and M30/CK18 ratios. P-values were calculated with Mann Whitney and Kruskall-Wallis tests and were considered significant when ≤0.05, shown in bold. # median 68, range 31-84), * median 4.5, range 2.3-13.0 (some values missing).
Figure 4: Post-operative therapy and post-operative plasma M30, total CK18 and M30/CK18 ratios.

Post-operative plasma M30 antigen (A), total CK18 (B) and M30/CK18 ratios (C) in plasma of the CRC patients treated with adjuvant therapy (n=9) vs non-treated patients (n=19). Box plots with line indicating median value, box indicating IQR and bars indicating the range.
Discussion

In the present study we found M30 antigen and total CK18 levels in plasma from CRC patients to be related to patient and tumour characteristics, to change in relation to tumour resection, and to be a predictor for disease-free survival. The observation that the death of tumour cells generates detectable products in the circulation of cancer patients is interesting for diagnostics purposes and monitoring therapy that induces tumour cell death. Cytokeratins are abundantly present in epithelial cells and their expression is usually retained or even increased after oncogenic transformation. CK18 is cleaved by caspase-3 during apoptosis, resulting in the release of the degraded CK18-Asp396 product, i.e., the M30 antigen, into the circulation. It has previously been shown that circulating M30 antigen levels are elevated in patients with various epithelial cancer types and to be increased during chemotherapy.

The M30 antigen and total CK18 levels in the plasma of CRC patients, before and after surgical resection of the tumour, correlated very well with each other, as expected,
because the CK18 ELISA recognizes the soluble fragments of CK18 that are detected in the M30 ELISA, as well as other soluble non-caspase cleaved CK18 fragments. Both M30 antigen and total CK18 plasma values increased shortly after surgical resection of the tumour, likely due to the surgical procedure. Because the apoptotic cells are randomly distributed throughout colorectal carcinomas, it is evident that products of apoptotic tumour cells do not all enter the circulation of these patients but are released into the lumen and leave the body via stool, especially in tumours that have not invaded and spread to adjacent lymph nodes. Our results showed a correlation between M30 antigen and total CK18 plasma levels and CRC stage, confirming that patients with advanced disease have higher M30 antigen and total CK18 levels. The positive correlation of M30 antigen plasma levels with tumour diameter further supports that the plasma levels are indeed elevated due to the presence of the tumour, with larger tumors responsible for more antigen “secretion”. Because we found no correlation between tumour diameter or Dukes’ stage with M30 antigen level within the tumour, these parameters might be the cause of the inverse correlation between M30 antigen plasma levels and M30 antigen tumour levels. Taken together these results strongly indicate that M30 antigen and total CK18 levels are reflected in the plasma of CRC patients due through the presence of the tumour.

M30 antigen plasma levels were related to patient’s outcome, independent from Dukes’ stage, with patients with higher levels having worse disease-free survival. These observations are comparable to the reports about M30 antigen levels in sera of patients with breast and lung cancer. Patients with recurrent breast cancer also had highest M30 antigen levels in their circulation and there was a correlation with the number of organs affected, suggesting increased M30 antigen levels in the circulation associated with cancer progression, which concurs with our observation of a relation with Dukes’ stage. Patients with lung cancer also had increased serum levels of M30 antigen, and patients with the lowest basal M30 antigen levels showed the best survival. Moreover, M30 antigen and/or total CK18 levels were found to be increased due to chemotherapy in lung, prostate and breast cancer patients, showing the induction of tumour cell death. In addition, the increase in total CK18 levels in breast cancer patients correlated with clinical response to therapy and survival. Thus, circulating M30 antigen and total CK18 levels could potentially be used to monitor treatment efficacy in cancer patients. However, we did not find a relation between post-operative plasma M30 antigen and/or total CK18 levels, in the plasmas obtained about 4.5 months after surgical intervention, and the post-operative adjuvant treatment the patients received, that started already within 1 month after surgery. Apparently, plasma M30 antigen
and/or total CK18 levels in CRC patients are intrinsically related to the tumour and less indicative for treatment response.

The ratio between plasma M30 antigen and total CK18 levels reflects differences in apoptosis and necrosis, and might reflect tumour-related differences in those two cell death modes. Necrosis is believed to be a major process in hypoxic tumours as it does not need ATP to be executed, in contrast to apoptosis. Furthermore, hypoxia blocks apoptosis and contributes to treatment resistance\(^\text{17, 18}\). Therefore, plasma M30/CK18 ratios can potentially be used to predict the response and determine which patients should be treated aggressively. In the present study, the M30/CK18 ratio tended to lower with increasing Dukes’ stage, indicating that necrosis increases more than apoptosis during tumour progression, similar as reported for endometrial cancer\(^\text{11}\). High levels of necrosis in more advanced tumour stages fits with the idea that hypoxia forces the tumour to form new blood-vessels and to invade the muscularis mucosa to reach the circulation for oxygen supply, finally resulting in more advanced tumor stages\(^\text{19, 20}\). Decreased plasma M30/CK18 ratios during tumour progression, furthermore, fit with the idea that there is a decrease in apoptotic sensitivity of tumour cells during colorectal tumour progression\(^\text{16}\). Interestingly, the patients M30 antigen plasma levels and M30/CK18 ratios after tumour resection are also of prognostic relevance for the patient’s disease-free survival. Both post-operative plasma M30 antigen and M30/CK18 ratios were independent of the post-operative treatment the patients received. Thus, determination of plasma M30 antigen and M30/CK18 ratios might also be a powerful independent tool to monitor patients after resection. In order to be conclusive, however, these interesting preliminary observations, due to the limited power of our study with only 49 CRC patients, merit further evaluation in larger patient groups.

**Conclusions**

M30 antigen and total CK18 levels in the circulation of colorectal cancer are prognostic for disease-free survival independent of disease-stage, and might be helpful to select patient’s treatment and in monitoring the patient after surgery, which should be confirmed in larger prospective studies.

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Chapter 3

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