RESEARCH ARTICLE

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The Possible Role of Matrix Metalloprotienase-2 in the Relapse in Patients with Stage II Colon Cancer Treated by Curative Surgery

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Abstract

Aims: To determine the association of micro-metastatic matrix metalloproteinase-2 (MMP-2) expression, the absolute lymphocyte count (ALC)) and outcome in stage II colon cancer. **Materials and Methods:** A single centre, prospective observational study, one month post-surgery blood for ALC, circulating tumour cell (CTC) detection and a bone marrow biopsy for micro-metastasis detection were obtained. CTCs were detected using differential gel centrifugation and immunocytochemistry with anti-CEA and anti-MMP-2, the bone marrow biopsy for the detection of micro-metastasis was processed as for CTCs. At each follow-up ALC and CTC counts were determined. Bone marrow sampling was repeated if the ALC decreased by >10%, at relapse or at the end of the study period. Three MRD subgroups were defined, Group I MRD negative, Group II only positive for micro-metastasis and Group III in which CTCs were detected. **Results:** One hundred and eighty one patients participated; 105 (58%) patients formed Group 1, 36 (20%) formed Group II and 40 (22%) formed Group III for a median follow-up of 4 years. Of Group I 3/105 (3%), Group II 16/36 (44%) and Group III 34/40 (84%) patients relapsed. The ALC was significantly higher in Groups I and II, the expression of MMP-2 and MMP-2 score in Group II was significantly lower than in Group III patients. A low ALC was associated with a higher expression of MMP-2 in the micro-metastasis and presence of CTCs. **Conclusions:** Patients with stable ALCs did not relapse; decreasing ALCs were associated with increasing MMP-2 scores, the appearance of CTCs and relapse.

Keywords: Colon cancer- circulating tumour cells- micro-metastasis- immune function- lymphocytopenia- outcome

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Introduction

Solid tumours are complex structures, being formed not only of tumour cells but also fibroblasts, lymphocytes and myeloid cells. The structure formed is similar to that found in the process of wound healing, stimulating angiogenesis and causes changes in the extracellular matrix (ECM) which favours tumour cell dissemination (Coussons et al, 2002; Devell et al., 2021). Tumour cells undergo the epithelial-to-mesenchymal transition (EMT) which enables the tumour cell to produce matrix metalloproteinase-2 (MMP-2) which degrades the ECM and permits them to enter the circulation. These circulating tumour cells (CTCs) have also have been reported to express MMP-2 that permits them to invade distant tissues and form the pre-metastatic niche (Bhattacharya et al., 2018). In the pre-metastatic niche the interactions between the cellular components of the stromal tissue and the ECM is complex, involving growth factors, kinases, cytokines and cell adhesion molecules (Elbe et al., 2019; Niland et al., 2020).

Three subtypes of residual minimal disease (MRD) have been reported patients with colon cancer (Murray et al., 2021; Murray et al., 2021a). Each sub-type of MRD has differing relapse patterns; those patients who are negative for both bone marrow micro-metastasis and CTCs have a five year disease free progression (DFP) of 98%, while those patients who had CTCs detected one month after surgery suffered early treatment failure with only a 7% five year DFP (Murray et al., 2021). Patients who only had bone marrow micro-metastasis detected one month after curative surgery had for the first 2-3 years the same DFP as patients negative for MRD, but thereafter there is an increasing relapse rate, with a DFP of 68% at five years (Murray et al., 2021).

This latency period of 2-3 years in patients with only

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bone marrow micro-metastasis implies that the tumour cells are not able to proliferate, being kept in check by the host´s defence systems. However, after this time period there has been a change either in the tumour cells by clonal instability or a decrease in the host immune response or a combination of both.

It has been reported that primary tumours with a high MMP-2 expression detected in the tumour cells have a worse prognosis (Trudel et al., 2009; Gialeli et al., 2011; Jia et al., 2017). Higher levels of MMP-2 expression in bone marrow micro-metastasis (Murray et al., 2020) or in the blood (Zhang et al., 2012) have been associated with the presence of CTCs and a worse prognosis.

We report a single centre, prospective, observational study of the expression of MMP-2 in bone marrow micro-metastasis and the possible association with immune dysfunction as measured by the absolute lymphocyte count (ALC), the changes of the expression of MMP-2 and the ALC with time and possible association with the risk of relapse in patients with stage II colon cancer treated by curative surgery.

Materials and Methods

An observational, prospective study of consecutive stage II colon cancer patients referred between January 2007 and December 2014 to a single centre for MRD evaluation in accordance with the STROBE guidelines.

Inclusion criteria

Patients with negative surgical margins, pathological stage II colon cancer treated by curative surgery alone were included. After written informed consent the following clinic-pathological findings were registered, sex, age, the presence or absence of peri-neural and lympho-vascular infiltration, tumour differentiation was classified as good, moderate or poor, the depth of tumour invasion (T) and lymph node metastasis (N) using the TMN system to classify patients.

Detection of circulating tumour cells (CTC)

Three 4ml blood samples were drawn into EDTA (Becton-Vacutainer®) one month following curative surgery. To prevent possible epithelial cell contamination the first tube was discarded, the second and third tubes were kept at room temperature and processed within the same day. Mononuclear cells were separated from whole blood using Histopaque-1077® (Sigma- Aldrich) according to the manufacturer's instructions, the obtained cells were washed three times in PBS (phosphate buffered saline) pH7.4 and finally a suspension of cells in 100 µl of autologous plasma was obtained. This was used to prepare 4 slides (sialinized DAKO, USA) each with 25 µl of the cell suspension. The slides were dried in air and finally fixed using a solution of 70% ethanol, 5% formaldehyde and 25% phosphate buffered saline pH 7.4 (DAKO). The slides were incubated at room temperature for 60 minutes with monoclonal anti-carcinoembryonic antigen (anti-CEA) clone 11-7 (DAKO). An alkaline phosphatase-anti-alkaline phosphatase based system (LSAB2, DAKO) with neofuchsin as the chromogen was used to detect CTCs. Those samples testing positive for CEA staining cells were further processed using anti-MMP-2 clone 1B4 (Novacastra Laboratories, USA) and were identified with a peroxidase-based system (LSAB2, DAKO, UK) with DAB (3,3 diaminobenzidine tetrahydrochloride) as the chromogen.

The criteria of ISHAGE (International Society of Hemotherapy and Genetic Engineering) (Borgen et al, 1999) were used to define a CTC, as a nucleated cell expressing CEA. (Figure 1). When at least one cell/blood sample was detected a test was considered to be positive and the number of CTCs detected was recorded.

The criteria used by Trudel et al (2009) was used to classify the expression of MMP-2, if more than 10% of CEA positive cells expressed MMP-2 the sample was considered to be MMP-2 positive. We classified the MMP-2 expression into three groups >10 % CEA cells positive for MMP-2, 1-10% and 0%. The staining for MMP-2 was registered as 0, +1, +2 and +3 and as such a mean MMP-2 score could be calculated.

Bone marrow micro-metastasis detection

From the posterior superior iliac crest a bone marrow biopsy was taken one month after surgery and four touch preps were made; these slides were processed as described for CTCs. Micro-metastasis were defined as CEA expressing cells found in the bone marrow. Positive samples were stained for MMP-2 expression as described for CTCs (Figures 2, 3 and 4).

Patients were classified into three groups

Firstly Group I who were negative for both micro-metastasis and CTCs, Group II patients only positive for micro-metastasis and finally Group III those patients CTC positive, independent of whether they were positive for micro-metastasis.

Absolute lymphocyte count

Blood was taken to determine the ALC at the same time as CTC sampling. An ALC of less than 1,000 lymphocytes/mm3 of venous blood was defined as lymphocytopenia.

Follow-up

Three monthly check ups were conducted with blood taken to determine the ALC and presence or absence of CTCs for the first two years, then 6 monthly until relapse or after five years of follow up. Colonoscopy and CT scanning of the thorax, abdomen and pelvis were performed three months after curative surgery and then yearly. DFP was defined as the time from curative surgery to the date of relapse.

The indications for a repeat evaluation of the bone marrow was a >10% decrease in the ALC or at the time of relapse and at the end of the study period in those patients who did show evidence of metastatic disease.

Study end points

1) Determine the changes in the ALC in venous blood and MMP-2 expression in micro-metastasis during follow-up and their association with relapse.

Statistical analysis

The mean and median values were used to describe the clinico-pathological variables and the standard deviation and interquartile range (IQR) were used to measure their distribution. The Chi-squared test was used to compare frequencies of the differing variables and the Mann-Whitney test to compare the mean and median values.

The three prognostic groups were compared for age, sex, primary tumour differentiation, lymphovascular infiltration and peri-neural infiltration and micrometastatic MMP-2 expression. The Kruskal–Wallis test was used to test whether samples originated from the same distribution and Pearson's chi-squared test was used to compare frequencies between MRD sub-groups. A $p < 0.05$ was taken to signify statistical significance.

Ethical Considerations

The study was approved by the local ethics committee and performed in complete conformity with the Declaration of Helsinki.

Results

One hundred and eighty one subjects, 82 (46%) male, with a median age of 68 years (IQR 52-84) were enrolled in the study. Group I was comprised of 105 (58%) patients, Group II comprised 36 (20%) patients and 40 (22%) patients formed Group III. Patients were followed-up until relapse or the end of study period in October 2018, with a median follow-up of 4.0 years (IQR3.1-4.9 years). A total of 53 (29%) of all the patients relapsed, 3/105 (3%) relapsed in Group I, 16/36 (44%) relapsed in Group II and 34/40 (84%) relapsed in Group III.

Patients in Group I were significantly older than the subjects in Group II and III (p=0.0176), had a higher frequency of T3 tumours (p<0.001) and a higher frequency of well differentiated tumours (p<0.0001) compared with the other two groups (Table 1).

The relapse rate significantly increased from Group I to Group III(p<0.001). The number of patients with micro-metastasis expressing MMP-2 and the MMP-2 score was significantly higher in Group III compared to Group II (p<0.001) (Table 1). The ALC one month after surgery was significantly lower in Group III as compared with the other two groups (p< 0.001), with no significant difference found between Groups I and II. A lower ALC one month after surgery was associated with a higher expression of MMP-2, both as the number of cells expressing MMP-2 and the MMP-2 score. The expression of MMP-2 in bone marrow micro-metastasis was significantly higher in Group III patients as compared with patients in Group II (p<0.001) as was for the MMP-2 score (p<0.01).

For all three groups we determined the expression of

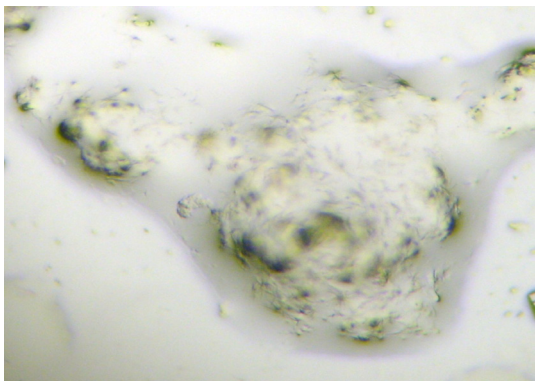

Figure 2. Bone Marrow Staining Negative for Carcino-Embryonic Antigen.

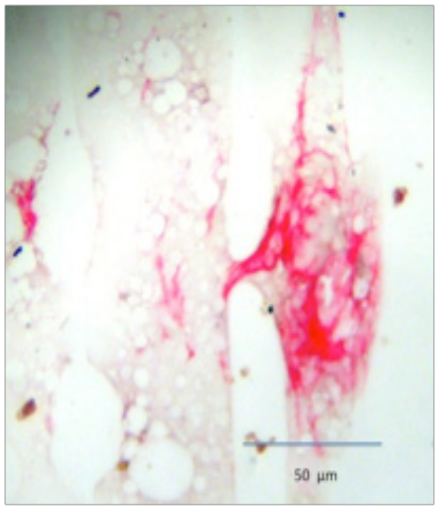

Figure 3. Bone Marrow Micro-Metastasis Staining Red for Carcino-Embryonic Antigen and Negative for Membrane MMP-2.

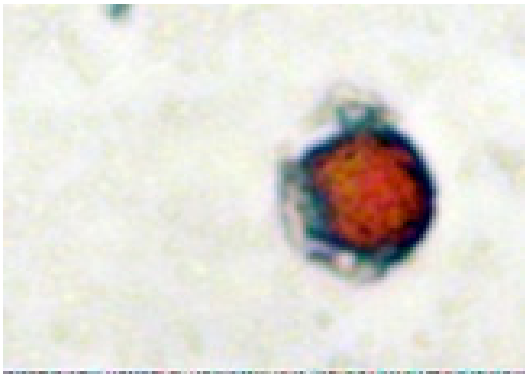

Figure 1. Circulating Tumour Cell Expressing Carcino-Embryonic Antigen (Red) and Membrane MMP-2 (Brown).

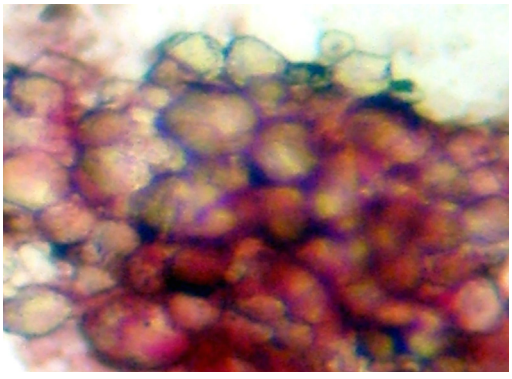

Figure 4. Bone Marrow Micrometastasis Staining Red for Carcino-Embryonic Antigen and Brown for Membrane MMP-2

Table 1. Clinical- Pathological Features According to Presence of Micro-Metastasis of the on 181 Patients Treated by Curative Surgery for Colon Cancer and Follow-up Time to Relapse.

Characteristic	Group I N=105 CTC and mM negative	Group II N=36 mM positive CTC negative	Group III N=40 CTC positive	p value two tailed
Age (years)				
Median (IQR)	71 (57-85)	68 (53-83)	68 (46-90)	0.0176[a]
Sex (male)	44 (42%)	16 (44%)	22 (55%)	0.48[b]
Tumour size				
T3	92	21	19	0.0001[b]
T4	13	15	21	II vs III p=0.5
Differentation				
Well	70 (67%)	17 (47%)	5 (12%)	<0.0001[b]
Moderate	23 (22%)	13 (36%)	24 (60%)	
Poorly	12 (11%)	6 (17%)	11 (28%)	
Relapse n (%)	3 (3%)	16 (44%)	34 (85%)	< 0.001[c]
ALC at the beginning of follow-up				
Mean ± SD	2349 ± 556	2126 ± 638	1302 ± 563	< 0.0001[d]
MMP-2	N/A			
MMP-2 > 10%	N/A	1 (3%)	7 (18%)	< 0.001[d]
MMP-2 1-10%	N/A	2 (6%)	21 (53%)	
MMP-2 0%	N/A	33 (91%)	12 (29%)	
MMP-2 score mean ± SD	N/A	0.29 ± 0.21	1.95 ± 0.41	< 0.001[d]
ALC average per subject during follow-up				
mean ± SD	2350 ± 511	1953 ± 546	1069 ± 510	< 0.001[d]
ALC at the end of follow up				
mean ± SD	2347 ± 509	1881 ± 692	987 ±314	< 0.0001[d]

CPC, Circulating Prostate Cell; mM, micrometastasis; IQR, interquartile range; SD, standard deviation; ALC, absolute lymphocyte counts on lymphocytes/mm^3; [a], Kruskal-Wallis´s test with Dunn´s test with p-values than less to 0.05 for differences between groups "A versus B" and "A versus C"; [b], Kruskal-Wallis´s test with Dunn´s test with p-values than less to 0.05 for differences between groups "A versus B", "A versus C" and "B versus C"; [c], Pearson's chi-squared test with Marascuillo procedure showed test with p-values than less to 0.01 for differences between groups "A versus B"; and " B versus C" and " A versus C"; [d], Anova one way (or single-factor) with post-hoc tests with the Bonferroni correction with p-values than less to 0.01 for differences between groups "A versus B", "A versus C" and "B versus C"

MMP-2 in bone marrow micro-metastasis when there was a 10% or more decrease in the ALC and the timing of the samples in relation to relapse or the end of the study and if CTCs were present or not. As Table 2 shows, patients with stable ALCs did not relapse; patients who relapsed had decreasing ALCs starting approximately 12 months before a relapse was detected. This was associated with increasing MMP-2 scores in the bone marrow micro-metastasis and

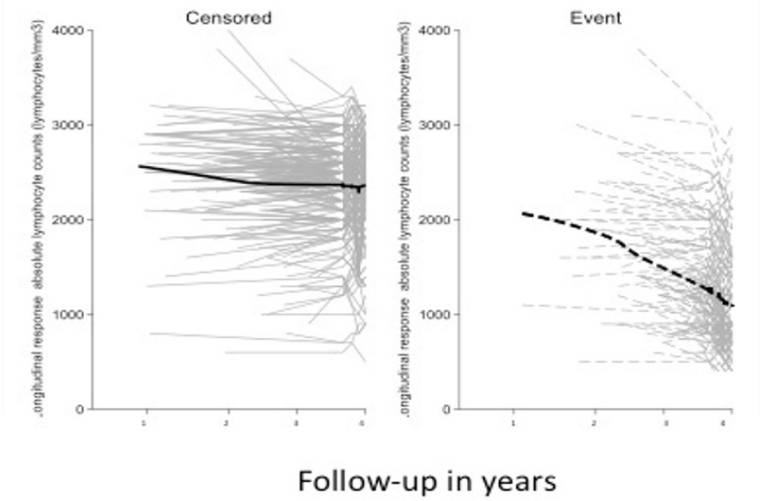

Figure 5. Changes in the Absolute Lymphocyte Count during Follow up and Risk of Relapse in Stage II Colon Cancer Patients

Table 2. Changes in the Absolute Lymphocyte Count and *MMP-2* Expression in Bone Marrow Micro-Metastasis with Time, Comparing Men with and without Relapse for the Three MRD Subtypes.

	Group I ALC decreasing	stable	Group II ALC decreasing	stable	Group III ALC decreasing	stable	p value two tailed
Nº patients	105		36		40		
T = 0							
ALC median	2349		2126		1302		p < 0.0001[a]
MMP-2							
MMP-2 ≥10	0		1 (3%)		7 (18%)		p < 0.001[a]
MMP-2 1-10%	0		2 (6%)		21 (53%)		
MMP-2 0	105 (100%)		33 (91%)		12 (29%)		
MMP-2 score	0.05		0.29		1.95		p <0.001[a]
CTCs present	0		1 (3%)		40 (100%)		p <0.0001[a]
Decrease in ALC > 10%	4	101	18	18	33	7	p <0.001[a]
ALC median	2098	2416	1902	2207	1053	1299	p < 0.01[a]
MMP-2							
MMP-2 ≥ 10%	1	0	7	0	17	2	p < 0.001[a]
MMP-2 1-10%	2	0	9	1	15	4	
MMP-2 0%	1	101	2	17	1	1	
MMP-2 score	0.21	0.04	0.72	0.09	2.15	1.05	p < 0.01[a]
CTCs present	1	0	9	1	33	7	p < 0.01[a]
At relapse	4	101	16	18	34	6	p < 0.001[a]
ALC median	1453	2402	1376	2348	943	1382	p < 0.01[a]
MMP-2							
MMP-2 ≥ 10%	4	0	14	1	29	4	p <0.001[a]
MMP-2 1-10%	1	0	2	1	5	2	
MMP-2 0%	0	100	0	16	0	0	
MMP-2 score	0.54	0	1,24	0.13	2.31	1.31	p < 0.001[a]
CTCs present	5	0	14	1	34	6	p < 0.001[a]
relapse	3	0	16	0	34	0	p <0.001[a]

CTC, circulating tumour cell; MMP-2, matrix metalloproteinase-2; ALC, absolute lymphocyte count

in Group II the appearance of CTCs. In the following twelve months the median ALC continued to decrease and the expression of MMP-2 increased. As Figure 4 shows not only tumour cells expressed MMP-2 but also that the stromal cells adjacent to the tumour cells also expressed MMP-2 at the time of relapse.

Discussion

The results of this study suggest that a deterioration in the ALC as a measure of immune function is associated with the appearance of CTCs and future relapse. One month after curative resection, those patients who had CTCs detected had a worse prognosis, with a higher frequency of and a shorter time to relapse. Independent of the MRD subtype, there was a significant deterioration in the ALC over time in those patients who relapsed. In contrast patients a stable ALC appeared to have a lower relapse rate, even in those patients initially CTC positive i.e. Group III. CTCs appeared approximately 2-8 months prior to relapse. After curative surgery, the results suggest that the change in immune function with time is due to the effect of undetected MRD. Changes in the properties of the micro-metastasis, in this study those found in bone marrow, but could also imply in those patients with visceral micro-metastasis affects the immune system. This permits the dissemination of CTCs from the micro-metastasis and the end of the latency or dormant period, where although the patient has no clinical evidence of metastasis using conventional monitoring there are undetected microscopic foci of tumour. The biological properties of the disseminated tumour and the host's immunological response will determine the dormancy or latent period and this may change with time. There is constant selective pressure on the micro-metastatic tumour cells, instability of clonal sub-types, the effect of treatment reducing the tumour load or the selection of resistant tumour cells may all modulate the immunological response leading to disease progression (Teng et al., 2008). As such tumour cells found in MRD may have different biological characteristics when compared to the original primary tumour. Thus in designing personalized treatment protocols the detection and biological properties of MRD in conjunction with its immune modulating effects may improve the design of therapy to maintain tumour cells dormant in non-metastatic colon cancer.

Expression of MMP-2 in tumour cells has been previously reported (Murray et al., 2020; Murray et al., 2021a), by their nature as gelatinases they are able to open the basement membrane and extracellular matrix allowing tumour cells to escape into the circulation (Ross et al., 2003). This was thought to be their primary role in tumour dissemination. However, the results presented here suggest that there might be an association with immune dysfunction. It has been proposed that MMPs modulate the immune system by regulating the bioavailability and activity of cytokines, chemokines and growth factors, playing a critical role in the overall regulation of the pattern, type and duration of the immune response (Nissinen et al., 2014). As seen in this report MMPs are produced directly by cancer cells or through induction of MMP synthesis by surrounding stromal cells, which may also express MMP-2. This triggers the proteolytic cleavage of cytokines and their receptors, including tumour necrosis factor receptor R, interleukin (IL) 6R and IL 2R and reducing NK cell cytotoxic functions (Lee et al., 2008). MMP-2 also induces TH2 polarization of lymphocytes further restricting the anti-tumour immune response and in addition cleaves the IL-2Ralpha receptor suppressing the proliferation of cytotoxic T-cells and causing apoptosis (Cross, 1998). The events that trigger these changes are unknown. The results of thus cohort study suggest that decreasing immune function is associated with increased MMP-2 expression, the appearance of CTCs and the end of the latency period.

The results also imply that the immune function has an important role in maintaining micro-metastasis in a dormant state, patients who did not relapse had stable ALCs. As implied by this study the delicate balance between the immunological system in the microenvironment and the phenotypic characteristics of tumour cells is dynamic and may change with time. The microenvironment of the bone marrow is active, attracting and reacting to invading tumour cells. Equally tumour cells are heterogeneous; their highly plastic nature permits changes in their biological characteristics from being latent to one of reactivation and proliferation. As seen in this report these changes are seen in clinical situation as treatment failure many years after primary curative resection.

The study has its limitations; it was a single centre study with a relatively low number of patients and that differential gel centrifugation and immunocytochemistrywas used to detect CTCs.. By using a single antibody only those tumour cells expressing CEA will be detected. Thirdly we used bone marrow biopsies rather than aspirates, because it is possible that tumour cells detected in aspirates are CTCs circulating in the bone marrow and not true micro-metastasis (Murray et al., 2012). Although it may be considered an invasive procedure the risk of adverse events is less than 0.08% (Bain, 2005), which is similar to the risk of perforation using colonoscopy (Navarro et al., 2017).

The use of bone marrow touch-preps is that they do not require decalcification or a process of antigen recuperation, which limits the destruction or damage to epitopes. The use of touch preps had a diagnostic accuracy of approximately 84% and correlates positively with regards to standard bone marrow biopsy in up to 85% of cases. The strength of the study is that the cohort was a homogenous population treated in a typical general hospital using equipment and immunocytochemical methods that are found in a typical immunocytochemistry laboratory, thus avoiding the need for high cost equipment or testing.

In conclusion, the results suggest that the outcome of patients with stage II colon cancer treated by curative surgery in part depends on the immune system. Even using the ALC as a simple measure of immune function it is possible to identify subjects with a higher risk of treatment failure. This is a dynamic process which may change with time, changes in the micro-metastasis with increasing MMP-2 expression is associated with worsening immune function and the appearance of CTCs and relapse. Larger multi-centre trials are needed to confirm these findings.

Author Contribution Statement

Design of the study: NPM, SA; Funding: NPM; Collection of data: RV, PR, DH; Analysis of data: SA; Immunocytochemistry: NPM; Writing of manuscript: NPM, SA; Revision of manuscript: RV, PR, DH.

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Conflicts of Interest

The authors report no conflicts of interest.

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