

RESEARCH ARTICLE

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Increasing Immune Dysfunction is Associated with Increasing Matrix-Metalloproteinase-2 Expression and Predicts Biochemical Failure in Men with Bone Marrow Micro-Metastasis Positive Localized Prostate Cancer

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Abstract

Introduction: To determine if there was an association of the ALC (absolute lymphocyte count) and LCP (lymphocytopenia) with the expression of MMP-2 in bone marrow micro-metastasis, the changes occurring during follow-up and association with biochemical failure. **Methods and patients:** One month after surgery blood and bone marrow samples were taken to determine the presence of micro-metastasis, the presence of circulating prostate cells (CPCs) and ALC. CPCs and micro-metastasis were detected using immunocytochemistry and MMP-2 expression determined in micro-metastasis. Only men positive for micro-metastasis participated in the study. At end follow blood was taken for serum PSA, ALC and CPCs, if the ALC decreased by more than 10% bone marrow sampling was repeated and MMP-2 expression determined, similarly for men with BF. Men who had stable ALCs had an end of study evaluation of the bone marrow. **Results:** 402 men underwent radical prostatectomy, one month post surgery 79 men were positive for only bone marrow micro-metastasis and formed the study group; of whom 36/79 (45%) underwent BF. Clinical pathological findings were not significantly different between men with or without BF. In men with BF the ALC was significantly lower one-month post surgery. The 5 and 10 year Kaplan-Meier survival was 100% at 5-years and 65% at 10-years for the whole cohort. Men without BF had stable ALCs. A decrease of >10% in the ALC was associated with increasing MMP-2 expression in the micro-metastasis and surrounding stromal tissue, the appearance of CPCs 6-12 months later and BF. **Conclusions:** the immune host-tumour cell interaction in the microenvironment is dynamic and changes with time. A decreasing ALC may be a valuable marker in identifying men with high risk of BF and changes in immune mediated dormancy before the PSA rises.

Keywords: Minimal residual disease- immune function- lymphocytopenia- prostate cancer- biochemical failure

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Introduction

Radical prostatectomy is one of the treatment options for clinically localized prostate cancer, however some 10-15% of patients will undergo treatment failure within five years (Vassil et al, 2010). Treatment failure arises from the proliferation of tumour cells not eradication by curative therapy, these micro-metastasis not detected by conventional studies are termed minimal residual disease. We have recently described two sub-types of minimal residual disease (MRD), those patients with circulating prostate cells detected in blood (independent of whether there are micro-metastasis detected in the bone marrow or not) have a high risk of early treatment failure, while

patients only positive for bone marrow micro-metastasis are at risk for late failure and have a similar outcome to MRD negative patients for the first five years (Murray et al, 2019a; Murray et al, 2019b). Thereafter there is increasing late failure, with a ten-year biochemical free failure of 57% and restricted mean time to biochemical free failure of 9 years being reported (Murray et al, 2020a). This extended time between curative treatment and biochemical failure in an asymptomatic patient is due to dormancy. First proposed by Willis in 1934 (Willis, 1934) and defined as a state of temporary mitotic arrest by Hadfield in 1954 (Hadfield, 1954) it has been further studied and divided into three types, cellular dormancy, angiogenic dormancy and immune mediated dormancy

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(Aguirre-Ghiso., 2007). The dual role of the immune system in the progression or eradication of cancer has been described as immune editing (Schreiber et al., 2011). It has been suggested that immune editing can be explained by three mechanisms, firstly elimination; the action of the innate and acquired immune systems eliminate tumour cells before they become clinically apparent. Secondly equilibrium; whereby the tumour cells are maintained in a state of functional dormancy and finally that of escape; whereby tumour cell growth is no longer attenuated by the immune system resulting in the induction of an immune-suppressive micro-environment and clinically apparent disease (Mittal et al., 2014).

The presence of only bone marrow micro-metastasis after radical prostatectomy implies a state of equilibrium between the disseminated tumour cells and the immune system, which is maintained until biochemical failure occurs. An increasing serum PSA implies that this equilibrium is no longer maintained resulting in tumour growth and possible dissemination. Thus circulating prostate cells (CPCs) may be detected in the blood. It has been suggested that bone marrow micro-metastasis act as a reservoir for future metastatic growth (Pantel et al., 2014).

The use of peripheral biomarkers such as the neutrophil to lymphocyte ratio (NLR) as a measure of immune function has given contrasting results (Bahig et al., 2015; Minardi et al., 2015; Maeda et al., 2016; Cao et al., 2019). However, different cut-off values for the NLR have been used as well as pre-operative and post-operative values. In this report we used the absolute lymphocyte count (ALC) and the frequency of lymphocytopenia (LCP) as a marker of immune function, it has been reported that the absolute lymphocyte count shows less biological variability than neutrophil or platelet counts (Coskum et al., 2018).

Matrix-metalloproteinase-2 (MMP-2) is a gelatinase and its expression in prostate cancer has been reported to be increased. It is associated with higher stage prostate cancer, higher Gleason scores, and as an independent prognostic factor for biochemical failure (Ross et al., 2003; Trudel et al., 2009). It is thought to be essential for the active dissemination of tumour cells into the circulation, permitting tumour cell extravasation through the basement membrane into the circulation (Murray et al., 2015). These circulating prostate cells (CPCs) continue to express MMP-2 and finally, home in on the bone marrow, implanting in the pre-metastatic niche. Here they interact with bone marrow stromal cells, which have an important role in determining tumour cell behaviour (Chung et al., 2005). The majority of bone marrow micro-metastasis do not express MMP-2, however those patients with MMP-2 positive micro-metastasis had a very high risk of early treatment failure and associated with the presence of CPCs (Murray et al., 2020b).

We report a single centre observational prospective study in men treated with radical prostatectomy as monotherapy for localized prostate cancer. To determine if there was an association of the ALC and LCP with the presence of bone marrow micro-metastasis only MRD; the expression of MMP-2; changes in these parameters at the time of biochemical failure or censored at the last control in those patients who remained free from

biochemical failure.

Materials and Methods

Patients and Methods

We conducted a prospective, observational single centre study of men who underwent radical prostatectomy as the sole treatment for prostate cancer between 2000 and 2012. The study was approved by the local ethics committee and complied with the Declaration of Helsinki. Consecutive patients undergoing radical prostatectomy for prostate cancer were invited to participate. Clinical details of age, pre-treatment serum total PSA was measured before digital rectal examination using the Siemens Advia CentaurXR[®] assay. The pathological study of the surgical piece was performed by a dedicated genitourinary pathologist according to the Gleason system. Pathological stage was defined according to the Partin criteria, organ confined, extra capsular extension, seminal vesicle invasion and lymph node invasion. EPE was defined as a specimen with cancer cells in contact with the prostatic capsule. All men had a nadir PSA post-surgery of < 0.01ng/ml.

Exclusion Criteria

- 1) Previous treatment or consideration for treatment with androgen blockade
- 2) Consideration for adjuvant radiotherapy
- 3) Infiltration of the seminal vesicles and/or regional lymph nodes with cancer.
- 4) Men with a positive bone scan.

Follow-up

Patients were followed up with serial total PSA levels, three monthly for the first year and six monthly thereafter. Biochemical failure was defined as a serum PSA >0.2 ng/ml on two separate occasions. Biochemical failure free survival time was defined as the time from surgery to the time of a post-surgery PSA of > 0.20 ng/ml or to the last follow up date.

One month after radical prostatectomy blood and bone marrow samples were taken to detect CPCs and bone marrow micro-metastasis. At each follow-up point, in addition to total PSA, blood samples were taken for a full blood count to determine the ALC and LCP and CPCs.

Detection of Secondary circulating prostate cells

One month after radical prostatectomy and at each follow-up time, an 8ml venous blood sample was collected in EDTA (Vacutainer[®]; Becton-Dickinson, Franklin Lakes, NJ, USA) and processed within 48 hours. CPCs detection was independently evaluated with the evaluators being blinded to the clinical details.

Collection of CPCs

Mononuclear cells were obtained by differential centrifugation using Histopaque 1,077 (Sigma-Aldrich, St Louis, MO, USA), washed, and re-suspended in a 100 µL aliquot of autologous plasma. 25 µL aliquots were used to make slides (silanized, DAKO, Carpinteria, CA,

USA), were dried in air for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde, and 25% phosphate buffered saline (PBS) pH 7.4 for five minutes and finally washed three times in PBS pH 7.4.

Immunocytochemistry

CPCs were detected using a monoclonal antibody directed against PSA, clone 28A4 (Novocastra Laboratory, Newcastle, UK), and identified using an alkaline phosphatase-anti alkaline phosphatase-based system (LSAB2, DAKO, USA), with new fuchsin as the chromogen. Positive samples underwent a second process with anti-MMP-2 clone 1B4 (Novocastra Laboratories, USA) and were identified with a peroxidase-based system (LSAB2, DAKO, UK) with DAB (3,3 diaminobenzidine tetrahydrochloride) as the chromogen. CPCs were defined according to the criteria of ISHAGE (International Society of Hemotherapy and Genetic Engineering) (Borgen et al., 1999). A CPC was defined as a cell that expressed PSA but not CD45; a leucocyte did not express PSA but expressed CD45 (Figures 1 and 2). A test was considered positive for CPCs when at least one cell/8mL of blood was detected; the number of CPCs detected/8ml blood sample was registered.

Detection of bone marrow micro-metastasis

Previous studies have used bone marrow aspirates to detect micro-metastasis, however prostate tumour cells detected in bone marrow aspirates are reported to be phenotypically different than those prostate cells detected in bone marrow biopsies and may not represent "true" micro-metastasis but rather cells circulating within the bone marrow (Murray et al., 2012). For this reason bone marrow biopsy "touch preps" were used as the sample to test for micro-metastasis. The biopsy was taken from the posterior superior iliac crest one month after surgery and the sample used to prepare four "touch preps" using sialinized slides (DAKO, USA) and the four slides processed as described for CPCs. A micro-metastasis was defined as cells staining positive for PSA and negative for CD45 (Figures 3 and 4).

Slides for both CPCs and micro-metastasis were analysed manually, stained cells were photographed using a digital camera and from the images it was determined if CPCs and/or micro-metastasis were present or absent by one trained observer.

Classification of Patients

a) Minimum residual disease: patients were divided into three MRD prognostic subgroups; Group A: negative for both CPCs and micro-metastasis, Group B: CPCs negative but micro-metastasis positive and Group C: CPCs positive with or without micro-metastasis detected.

b) Absolute lymphocyte count and lymphocytopenia: the absolute lymphocyte count was registered as lymphocytes/mm³ of venous blood, three groups were formed, >1,500 lymphocytes/mm³ (considered as normal), 1,000-1,500 lymphocytes/mm³ (mild lymphocytopenia) and <1,000 lymphocytes/mm³ (moderate to severe lymphocytopenia).

Expression of MMP-2: One month after surgery

samples positive for PSA expressing cells underwent further processing using anti-MMP-2, both CPCs and micro-metastasis. The criteria used for defining a cell expressing MMP-2 were that described by Trudel et al., (2009). A patient was considered positive for MMP-2 if >10% of cell expressing PSA co-expressed MMP-2. However, three groups of MMP-2 expression were defined; MMP-2 negative, >0%-<10% and >19% of pSA positive cells co-expressed MMP-2. These cells were additionally classified semi-quantitatively as having 0, +1, +2 and +3 intensity of immune staining for MMP-2. A mean MMP-2 score was calculated and defined as total MMP-2 expression/Number of PSA expressing cells. Additional bone samples were taken as follows: in men without biochemical failure at the end of the study, in men with decreasing lymphocyte counts at the second decrease of more than 10% of the post-surgical value, then six monthly and at biochemical failure, with the same method and scoring system.

Study endpoint

The primary study end-point was the presence or absence of biochemical recurrence and secondary endpoints were the mean time to failure after primary treatment, changes in the ALC, LCP, CPC status in the 12 months prior to failure or censure time and changes in MMP-2 expression in micrometastasis with relation to the ALC.

Statistical analysis

The analysis was performed using the program Stata/SE 16.0 for Windows (Stata Corp LLC). The quantitative and ordinal variables were described with respective central tendency and dispersion measurements, while nominal variables were described as proportions with their respective confidence intervals.

Results

A total of 538 subjects were recruited to the prospective database, of these 402 men underwent radical prostatectomy as mono-therapy. These 402 men had a median follow-up of 7.53 years (IQR 4.81 years). The mean age was 65.5 ± 8.28 years with a median serum PSA of 5.51 mg/dl (IQR 3.26 ng/ml).

183 men were MRD negative post surgery, 144 were positive for CPCs and 79 were positive for only bone marrow micro-metastasis, these later patients formed the study group.

Of the 79 patients 36 (45%) underwent biochemical failure within the study period, of which the clinical pathological findings are shown in Table 1. Between the group of patients who underwent biochemical failure and those who did not there were no significant differences with respect to age, PSA pre-surgery, Gleason score, staging or extra-capsular extension. With the respect to the median ALC this was significantly lower in the biochemical failure group, both post-surgery and at last follow up (p<0.0001 for both). In the biochemical failure group there was also a significant decrease in the median ALC from the post-surgery to the time of biochemical

Table 1. Clinical Pathological Findings in Patients with and without Biochemical Failure

| | No biochemical failure N=43 | Biochemical Failure N= 36 | p value |
|-------------------------------------|-----------------------------|---------------------------|------------------------|
| Mean age (SD) | 66.7 ± 8.1 | 65.7 ± 8.8 | p=0.78 ^a |
| Median PSA ng/ml at diagnosis (IQR) | 5.50 (4.54-6.57) | 6.55 (5.10-6.42) | p=0.07 ^b |
| Stage | | | |
| T1 | 9 (20%) | 1 (2%) | p=0.051 |
| pT2 | 31 (72%) | 31 (86%) | |
| pT3 | 3 (8%) | 4 (12%) | |
| Gleason score | | | |
| ≤ 6 | 31 (72%) | 31 (86%) | p=0.46 ^c |
| 7 | 10 (23%) | 5 (14%) | |
| ≥ 8 | 2 (5%) | 0 (0%) | |
| ECE | | | |
| Yes | 30 (69%) | 19 (52%) | p=0.19 ^c |
| No | 13 (31%) | 17 (48%) | |
| Median ALC (IQR) | | | |
| post surgery | 2,400 (2,300-2,700) | 1,900 (1,300-2,400) | p< 0.0001 ^b |
| last value | 2,500 (2,200-2,750) | 1,550 (900-1,900) | p< 0.0001 ^b |
| | p=0.85 ^b | p=0.02 ^b | |
| Frequency LCP | | | |
| Post surgery | 38 (88%) | 24 (66%) | p=0.07 ^c |
| >1,500 | 5 (12%) | 7 (19%) | |
| 1,500-1,000 | 0 (0%) | 5 (13%) | |
| <1000 | | | |
| Last value | | | |
| >1,500 | 38 (88%) | 18 (50%) | p=0.0005 ^c |
| 1,500-1,000 | 4 (9%) | 8 (22%) | |
| <1,000 | 1 (2%) | 10 (28%) | |
| | p=0.57 ^c | p=0.27 ^c | |

ECE, extra-capsular extension; ALC, absolute lymphocyte count; LCP, lymphocytopenia; ^a, T-Test; ^b, Mann-Whitney test; ^c, Fisher's exact test; last test, taken at biochemical failure or censor date

failure. In the frequency of LCP, there was no significant difference between groups post-surgery, but at the censor time the biochemical failure group had a higher frequency of LCP (p=0.0005).

The frequency of patients who became CPC positive was significantly higher in the biochemical failure group 30/36 (83%) versus 5/43 (11%) (p<0.0001).

Decrease in absolute lymphocyte count is associated with biochemical failure

During follow-up, the median ALC significantly decreased in the patients who underwent biochemical failure and accompanied by the detection of CPCs in men previously CPC negative.. This event occurred approximately six months after the initial decrease in

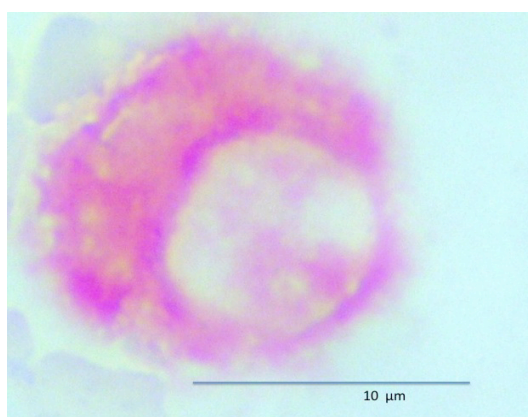


Figure 1. Circulating Prostate Cell Expressing PSA (red) and Negative for Membrane CD45 (brown).

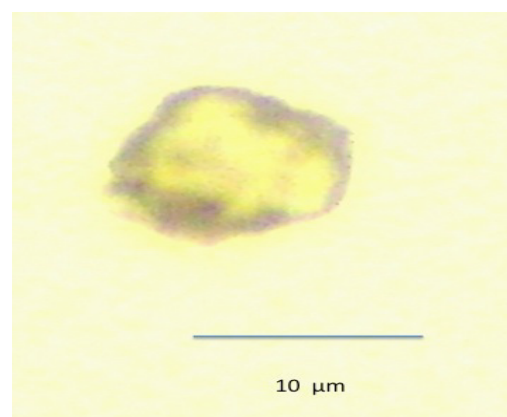


Figure 2. Leukocyte Negative for PSA (red) and Positive for Membrane CD45 (brown).

Table 2. Changes in the Median Absolute Lymphocyte Count and CPC Positivity Prior to Biochemical Failure

| CPC status (18 months) | CPC status (12 months) | CPC status (6 months) | CPC status (last follow up) |
|-------------------------|-------------------------|-------------------------|-----------------------------|
| CPC (-) N=79 | CPC (-) N=72 | CPC (-) N=55 | CPC 8-9 n036 |
| ALC 2,400 (2,000-2,600) | ALC 2,300 (1,900-2,600) | ALC 2,400 (2,200-2,550) | ALC 2,500 (2,300-2,650) |
| CPC (+) 0 | CPC (+) N= 7 | CPC (+) N=24 | CPC (+) |
| | ALC 800 (500-1,200) | ALC 1,300 (1,000-1,600) | ALC 1,400 (900-1,300) |
| | $p < 0.0001$ | $p < 0.0003$ | $p < 0.001$ |

ALC, absolute lymphocyte count; IQR, interquartile range; CPC, circulating prostate cell

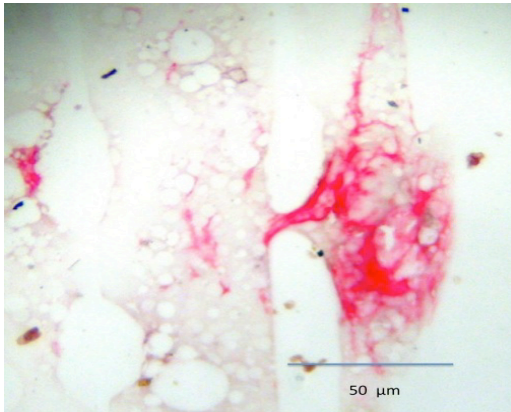


Figure 3. Bone Marrow Micro-Metastasis Expressing PSA (red) and Negative for Membrane CD45 (brown).

the ALC (Figure 5). The time to the decrease in the ALC was prolonged in this group of patients, occurring after five years of follow-up. This suggests that the biological properties of the tumour cells are different and have differing effects on immune function. The observed Kaplan-Meier survival in this group was 100% at five years and then an increasing rate of biochemical failure with a 10 year observed biochemical failure free survival of 65% with a mean restricted biochemical failure time

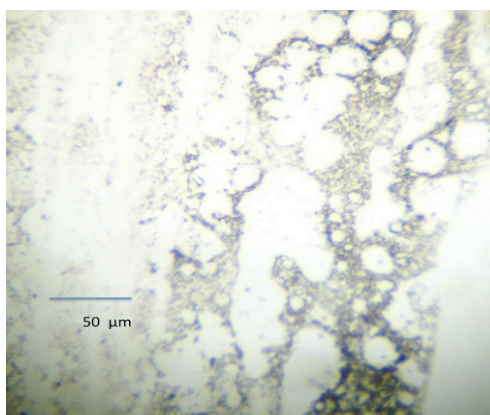


Figure 4. Bone Marrow Negative for Micro-Metastasis

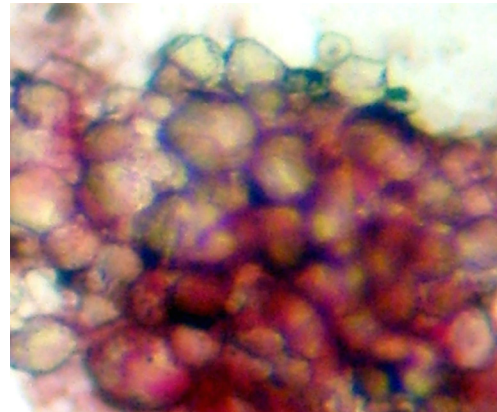


Figure 5. Bone Marrow Micro-Metastasis Expressing PSA (red) and Matrix Metalloproteinase 2 (brown). Adjacent stromal cells negative for PSA and positive for matrix metalloproteinase-2 (brown).

of 8,67 years.

Expression of MMP-2 and changes with time

One month after surgery, the expression of MMP-2 was registered for the three groups (Table 3).

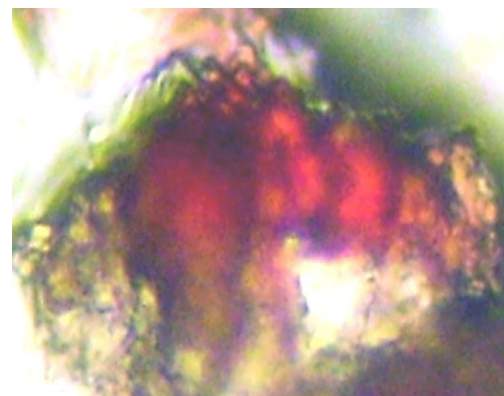


Figure 6. Bone Marrow Micro-Metastasis Expressing PSA (red) and Membrane Matrix Metalloproteinase- 2 (brown).

Table 3. Association of the Expression of MMP-2 in Bone Marrow Micro-Metastasis and the Absolute Lymphocyte Count

| One month after surgery | Group A | Group B N=79 | Group C N= 144 | p value |
|-------------------------|---------|-----------------|-----------------|---------------|
| MMP-2 \geq 10% | N/A | 2 (4%) | 18 (21%) | $p < 0.01^a$ |
| MMP-2 \geq 2% - < 10% | N/A | 3 (6%) | 38 (49%) | $p < 0.001^a$ |
| MMP-2 score | N/A | 0.36 ± 0.21 | 1.84 ± 0.37 | $p < 0.01^b$ |

N/A, not applicable; MMP-2, matrix metalloproteinase-2; ^a, 0 Chi squared; ^b, Mann Whitney test

Table 4. Changes in the Absolute Lymphocyte Count and MMP-2 Expression in Bone Marrow Micro-Metastasis with Time, Comparing Men with and without Biochemical Failure

| Time of sampling | ALC decreasing (N=37) | ALC stable (N=43) | p value |
|-------------------------------|-----------------------|-------------------|-----------------------|
| One month post surgery | | | |
| Median ALC (IQR) | 2.00 (1.35-2.47) | 2.4 (2.26-2.70) | p=0.99 ^a |
| MMP-2 | | | |
| > 10% | 2 (5%) | 0 | |
| 1-10% | 4 (11%) | 0 | p=0.54 ^b |
| 0% | 31 (84%) | 43 (100%) | |
| MMP-2 score | 0.37 ± 0.24 | 0.36 ± 0.21 | p=0.97 ^a |
| 18 months prior to BF/censure | | | |
| median ALC (IQR) | 1.80 (1.20-2.12) | 2.50 (2.30-2.60) | p<0.0001 ^a |
| MMP-2 | | | |
| >10% | 5 ((14%) | | |
| 1-10% | 8 (22%) | | |
| 0% | 24 (64%) | | |
| MMP-2 score | 0.98 ± 0.47 | | |
| 12 months prior to BF/censure | | | |
| median ALC (IQR) | 1.50 (1.10-2.12) | 2.50 (2.40-2.60) | p<0.0001 ^a |
| MMP-2 | | | |
| > 10% | 11 ((30%) | | |
| 1-10% | 15 (40%) | | |
| 0% | 11 (30%) | | |
| MMP-2 score | 1.37 ± 0.85 | | |
| 6 months prior to BF/censure | | | |
| median ALC (IQR) | 1.30 (1.00-1.50) | 2.50 (2.20-2.70) | p<0.0001 ^a |
| MMP-2 | | | |
| > 10% | 12 (32%) | | |
| 1-10% | 20 ((54%) | | |
| 0% | 5 (14%) | | |
| MMP-2 score | 1.83 ± 0.74 | | |
| BF/censure | | | |
| median ALC (IQR) | 1.10 (0.80-1.30) | 2.50 (2.20-2.80) | p<0.0001 ^a |
| MMP-2 | | | |
| >10% | 12 (32%) | 0 (0%) | |
| 1-10% | 20 (54%) | 1 (2%) | |
| 0% | 5 (14%) | 42 (98%) | p<0.001 ^b |
| MMP-2 score | 1.97 ± 1.05 | 0.38 ± 0.15 | p<0.001 ^a |

ALC, absolute lymphocyte count; IQR, interquartile range; BF, biochemical failure; a, Mann Whitney test; b, Fisher's exact test

During follow-up we monitored the changes in Group B

Patients with respect to the ALC, biochemical failure and expression of MMP-2 in the micro-metastasis. Group B were divided into patients who had stable ALCs and those who had decreasing ALCs (Table 4). Men with decreasing ALCs overtime had increasing MMP-2 expression in the micro-metastasis within the study period all underwent biochemical failure. Those men with stable ALCs did experience biochemical failure and the end of study MMP-2 score was no significantly different when compared one month post surgery.

Of the patients with a decreasing ALC all underwent biochemical failure, whereas none of those men with an ALC stable underwent biochemical failure. The ALC

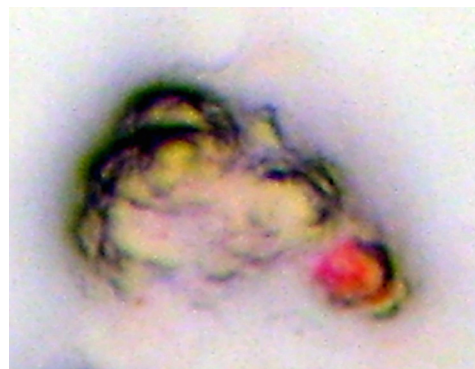


Figure 7. Bone Marrow Negative for Micro-Metastasis with CPC MMP-2 Positive Circulating in the Inter-Trabecular Space

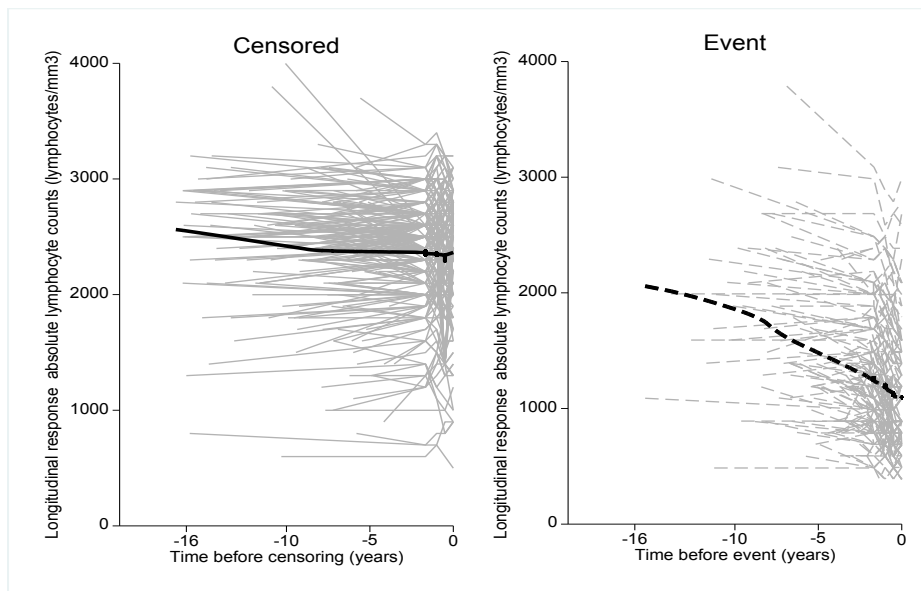


Figure 8. Association between the Longitudinal Responses of Repeated Samples of Absolute Lymphocyte Counts and the Time before of Censoring and Event with Locally Weighted Regression Shown on Black Line in 80 Men Treated by Radical Prostatectomy for Prostate Cancer.

was significantly lower at the time of failure in those patients who relapsed as compared with the ALC post surgery in the same group. This decrease in the ALC was associated with an increasing expression of MMP-2 in the micro-metastasis. Not only did prostate cancers express MMP-2 but surrounding bone marrow stromal cells also expressed MMP-2 (Figure 5). There was the appearance of CPCs expressing MMP-2 some 6-12 months after immune dysfunction commenced. In contrast in men who did not undergo treatment failure there were no significant differences between the ALC and MMP-2 score one month after surgery and at the end of the study. CPCs were not detected in these patients.

Discussion

The results of the study suggest that deterioration in the immune function as measured by the ALC is association with the appearance of CPCs and subsequent biochemical failure. The clinical pathological findings at radical prostatectomy were not significantly different between patients who underwent biochemical failure and those who did not. In men who underwent biochemical failure there was a significant deterioration in the ALC with time, in contrast those who did not undergo biochemical failure did not show a significant change in the ALC. CPCs appeared up to 12 months pre-biochemical failure, with increasing numbers of CPC positive patients closer to the time of biochemical failure.

This suggests that the change in immune function is due to a change in the biological properties of the micro-metastasis that permits the dissemination of secondary CPCs. This interaction between tumour cells and the immune system is dynamic and may change with time. Cancer cells in the bone are under constant selective pressure and equilibrium between the immune response and the tumour cells results in long-term latency or

dormancy (Teng et al., 2008). Clonal instability of cancer cells, selection of resistant cancer cells or the reduction of tumour load as a result of treatment may all modulate the immune response leading to disease progression.

These changes result in a minimal residual disease that may have different biological properties when compared with the primary tumour. Thus detection and characterization of minimal residual disease and its immune modulating effects may help in the design of personalized treatment protocols to improve patient outcome in non-metastatic disease.

The dual role of the immune system in cancer progression has been well documented; this so-called immunoediting was proposed to describe this balance (Schreiber et al., 2011). The equilibrium between tumour cell dormancy ends whereby the growth of tumour cells is no longer attenuated by the immune system, resulting in the induction of an immunosuppressive tumour microenvironment and biochemical failure (Mittal et al., 2014). This equilibrium lasted up to nine years in some patients, the rate of late failure increasing after five years.

Expressions of MMP-2 in tumour cells has been previously reported, and by their nature as gelatinases are able to open the basement membrane to permit tumour cells to disseminate into the circulation (Ross et al., 2003; Trudel et al., 2009). This was thought to be their primary role in tumour dissemination. However the results presented here suggested that there might be an association with immune dysfunction and MMP-2 expression.

It has been suggested that there is an intimate relationship between the immune systems and tumour cells within the microenvironment. Escape from immune-surveillance prefigures the rapid progression of human cancers. Various immune escape mechanisms have been proposed; cancers may secrete immunosuppressive factors to modify host immune responses. There is increasing evidence that the metalloproteinase are important in this

cross-talk. Metalloproteinases 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). MMPs are produced directly by cancer cells or through induction of MMP synthesis by surrounding stromal cells (Figure 5). This cancer derived MMPs trigger the proteolysis cleavage of cytokines and their receptor's, including tumour necrosis factor (TNF) receptor R, interleukin (IL) 6R and IL -2R. This suggests that MMPs play an additional role in the immune escape of the tumour by disturbing anti-tumour immune defence mechanisms, reducing NK cell cytotoxic function (Lee et al., 2008).

Furthermore MMP-2 causes TH2 polarization further restricting the anti-tumour immune response and in addition cleaves the IL-2R α suppressing the proliferative of cytotoxic T-cells and causing apoptosis (Cross, 1998). What triggers these changes is unknown, possibly clonal instability in the tumour cells. What the results suggest is that is there an association between decreasing immune function and increasing MMP-2 expression, leading to the escape of the micro-metastasis from its dormant state and disease progression. The use of the ALC is a simple marker widely available that could identify these high-risk patients. The study highlights the dynamic changes during follow-up and the PSA levels may not be the only useful biomarker to assess the possibility of disease progression. It was previously thought that the main role of MMP-2 was to permit the dissemination of tumour cells, the co-expression of MMP-2 in bone marrow micro-metastasis was associated with the presence of CPC and a high risk of early treatment failure (Murray et al., 2020).

The increase in MMP-2 expression may be related to androgen independence, in cell culture studies androgens stimulate MMP-2 expression, which occurs at the gene transcription level via the androgen receptor transactivation and dependent on P13K activity, although these men were not treated with androgen blockade, clonal selection of Arv7 variants may in an autocrine fashion increase intracellular androgen levels causing an increase in MMP-2 expression. It was beyond the bounds of this study to test this hypothesis.

The results imply that the immune function has an important role in maintaining micro-metastasis dormant, that with time because of clonal changes in the micro-metastasis and/or decreased immune function due to aging changes the dynamics of the micro-metastasis-host interaction. The net result is that the period of dormancy ends and micro-metastatic growth and dissemination occurs, in other words a more biologically aggressive disease. This implies that the regulation of the micro-metastasis by the host immune system is lost permitting the re-activation of tumour cells and disease progression or biochemical failure. This delicate balance between the immunological factors in the microenvironment and the phenotypic characteristic of the tumour cells is dynamic and will determine patient outcome. As implied by this study these characteristics change with time, the bone marrow microenvironment is

not passive and can attract and react to infiltrating tumour cells. Similarly tumour cells are heterogeneous, are highly plastic in their phenotypic characteristics and may change from a latent/quiescent state to one of reactivation and proliferation, which is clinically seen as a relapse many years after primary curative treatment, as seen in this study.

The study has its limitations, firstly being a single centre study, secondly the low number of patients and CPCs were detected using differential gel centrifugation and immunocytochemistry. Secondly the detection of bone marrow micro-metastasis has been reported using monoclonal antibodies against pan-cytokeratin, anti-PSA and anti-PMSA (prostate specific membrane antigen) and immunocytochemistry. Using reverse-transcriptase polymerase reaction with for PSA and PMSA is reportedly up to ten times more sensitive it may not be important to detect all tumour cells. Patients transplanted for chronic myeloid leukaemia may have very small numbers of leukemic cells detected in bone marrow samples post-transplant but remain in remission for many years, the leukemic cells surviving for prolonged periods before being eliminated by the immune system (Cross, 1998).

We used a biopsy specimen because it possible that prostate cells detected en bone marrow aspirates are similar to CPCs and not true micro-metastasis (Murray et al., 2012). Although it maybe considered an invasion procedure the adverse effects are ten times less than a prostate biopsy with a risk of adverse events of less than 0.08% (Bain, 2005). The advantages are that samples do not have to be decalcified or need an antigen recuperation process and as such epitopes are not destroyed, secondly the diagnostic accuracy between touch-preps and biopsy samples is reported to be 84% with a positive correlation of 85% with the biopsy specimen. On the other hand, the strength of the study is that it was a homogenous population treated in a general hospital without the need for high cost equipment, important in the public health service.

In conclusion, immune dysfunction plays an important role in the outcome of patients post radical prostatectomy, even using a simple absolute lymphocyte count it is possible to stratify patients, with time there are dynamic changes, a decreasing absolute lymphocyte count is associated with impending biochemical failure and could be used as a simple marker to predict treatment failure. This decrease in immune function is associated with an increasing expression of MMP-2 in bone marrow micro-metastasis and the later appearance of CPCs. The results imply that MMP-2 plays an important role in immune dysfunction and not only as a mechanism for tumour cell dissemination. These results warrant further investigation with multi-centric studies to confirm the results.

Author Contribution Statement

Concept: NPM; design: NPM; supervision: NPM, ER and CF; resources: NPM Materials: NPM, CF, AS and ER; data collection and/or processing: CF, AS, and ER; analysis and/or interpretation: NPM, SO, AS, CF, and ER; literature search: CF and ER; writing manuscript: NPM and SA; Critical review: CF, AS, ER and Final

approval: all.

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Ethical Considerations

The Hospital Ethical Committee approved the study.

Availability of date

Owing to the Chilean Law on patients' rights data are not publically available.

Conflicts of Interests

The authors report no conflicts of interest.

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