Medical Research Archives

ESMED
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OPEN ACCESS

Published: December 31, 2022

Citation: Murray NP, Aedo S, et al., 2022 Immune Dysfunction as Measured by Lymphocytopenia, Minimal Residual Disease and Outcome After Radical Prostatectomy For Localized Prostate Cancer, Medical Research Archives, [online] 10(12). https://doi.org/10.18103/mra.v10i12.3453

DOI
https://doi.org/10.18103/mra.v10i12.3453

ISSN: 2375-1924

RESEARCH ARTICLE

Immune Dysfunction as Measured by Lymphocytopenia, Minimal Residual Disease and Outcome after Radical Prostatectomy for Localized Prostate Cancer

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ABSTRACT

Background: An intact immune function may be important in eliminating or maintaining minimal residual disease latent.

Objective: Use the absolute lymphocyte count as an immune biomarker, determine its association with minimal residual disease and relation with biochemical failure.

Design, setting, and participants: prospective observational single centre study in men treated by radical prostatectomy for localized prostate cancer. One month after surgery blood and bone samples were taken to detect circulating prostate cells and micro-metastasis. A total of 404 men were enrolled, at each follow up time total PSA, ALC and CPC presence/absence were determined.

Outcome measurements and statistical analysis: observed times to biochemical failure (Kaplan-Meier) and restricted mean biochemical failure free survival times were assessed, changes in the absolute lymphocyte count and the presence/absence of circulating prostate cells were determined during follow-up and association with biochemical failure.

Results and Limitations: 404 men participated; 182 were minimal residual disease negative (Group A), 80 had only micro-metastasis (Group B) and 142 were circulating prostate cell positive (Group C); 175 men underwent biochemical failure, Group C were at high risk of early failure, Group B of late failure. One month post-surgery Group C men had lower absolute lymphocyte counts, compared to Groups A and B. During follow-up men with stable absolute lymphocyte counts did not relapse, a decreasing absolute lymphocyte count was associated with relapse within 18 months and the appearance of circulating prostate cells in the blood. After five years the absolute lymphocyte count decreased in Group B patients. A low absolute lymphocyte count one-month post-surgery or a decrease during follow-up was associated with an increased risk of treatment failure.

Conclusions: the absolute lymphocyte count is an important prognostic factor, it may change with time, a decrease is associated with pending biochemical failure and later appearance of circulating prostate cells.

Keywords: prostate cancer; biochemical faillure; immune dysfunction; lymphocytopenia; circulating tumour cells

Introduction:

Early in prostate cancer there are sub-populations of tumour cells, which may disseminate first to the neuro-vascular structures and then onto the circulation[1]. Those tumour cells, which survive and implant in distant tissues are outside the surgical field and thus not removed by radical prostatectomy. These surviving tumour cells are termed minimal residual disease (MRD) and not detected by conventional imaging or an elevated serum PSA post-surgery. MRD is the net result of the biological properties of subpopulations of primary tumour cells to disseminate, implant in distant tissues, survive and cause immunosuppression versus the effect of the immune response and treatment to eliminate them. Host immune systems both innate and acquired may act at differing stages in the metastatic process, eliminating tumour cells within the primary tumour, during their dissemination and in the metastatic niches[2]. This interaction between tumour cells and the immune system is dynamic and may change with time. 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[3,4] and as such the presence or absence of MRD and its biological properties. As a simple measure of immune function, various parameters of the full blood count, neutrophil, lymphocyte, platelet counts as well as the derivative, neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR) and the systemic immune inflammatory index (SII). The use of the NLR in localized prostate cancer has given conflicting results as a prognostic factor, nor is there a consensus on a cut-off value or if the NLR should be based on results obtained before or after surgery[5-8]. A meta-analysis of the use of the PLR as a prognostic factor also showed conflicting results[9]. Whereas the SII defined as the neutrophil count x platelet count/lymphocyte count was associated with an increased risk of biochemical failure but did not improve the clinical accuracy and clinical value beyond that of current predictive and prognostic models[10]. All these measures of immune function have a common denominator, lymphocytopenia. It has been proposed that peripheral lymphocytopenia (LCP) is a measure of the equilibrium between the anti-tumour immune response and pro-tumour inflammation[11]. Both neutrophil and platelet counts are subject to a higher biological variation in terms of age, sampling time and background inflammation, which is higher in older patients and as such the lymphocyte count may be a better marker of changes in immune function[12].

We present a prospective observational study of the outcome of radical prostatectomy as mono-therapy for prostate cancer, based on the three sub-types of MRD and immune dysfunction as determined by the ALC to determine if the sub-classification of MRD based on immune function improves the risk stratification for biochemical failure.

Methods and Patients: A single centre prospective observational study of men with prostate cancer recruited between January 2005 and December 2010 who underwent radical prostatectomy as the sole treatment for prostate cancer and followed up until biochemical failure or December 2020. The study was approved by the local ethics committee and complied with the Declaration of Helsinki, each patient providing written informed consent prior to starting the study. The following clinical data were registered; serum total PSA at diagnosis (ng/ml), surgical margins positive or negative, extra-capsular extension positive or negative, infiltration of seminal vesicles and lymph nodes and pathological Gleason score.

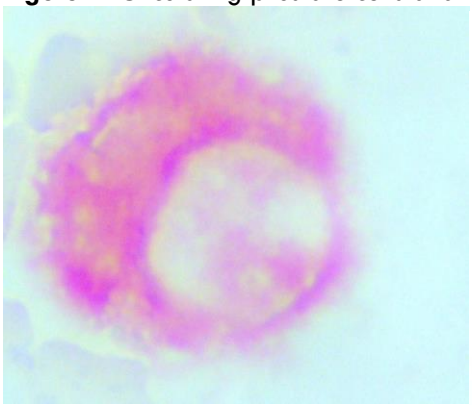
a) **Detection of secondary circulating prostate cells**: one-month post-surgery and every six months until biochemical failure or the end of the study an 8mL venous blood sample was taken and collected in a tube containing EDTA (Vacutainer®, USA)). Samples were maintained at 4° C and processed within 48 hours. CPC detection was independently evaluated with the evaluators being blinded to the clinical details.

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

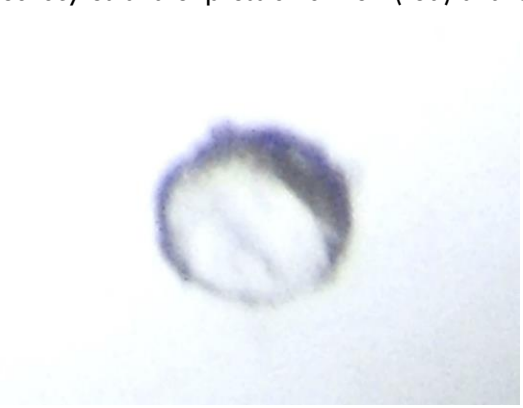
Immunocytochemistry: CPCs were detected using a monoclonal antibody directed against PSA, clone 28A4 (Novocastro Laboratory, UK), and identified using an alkaline phosphatase-anti alkaline phosphatase based system (LSAB2, DAKO, USA), with new fuchsin as the chromogen. Samples positive for PSA staining cells underwent a second process. The slides were incubated with anti-CD45 clone 2B11 + PD7/26 (DAKO, USA) and cells identified with a peroxidase-based system (LSAB2, DAKO, USA) with DAB (3,3 diaminobenzidine

tetrahydrochloride) as the chromogen. A secondary CPC was defined according to the criteria of ISHAGE (International Society of Hemotherapy and Genetic Engineering)[13]. A CPC was defined as expressing PSA but not CD45 and a leukocyte as expressing CD45 but not PSA (Figure 1). A test was considered positive for CPCs when at least 1 cell/8mL of blood was detected.

Figure 1: Circulating prostate cells and leukocytes and expression of PSA (red) and CD45 (brown).



Circulating prostate cell Expressing PSA (red) but not Membrane CD45 (brown)

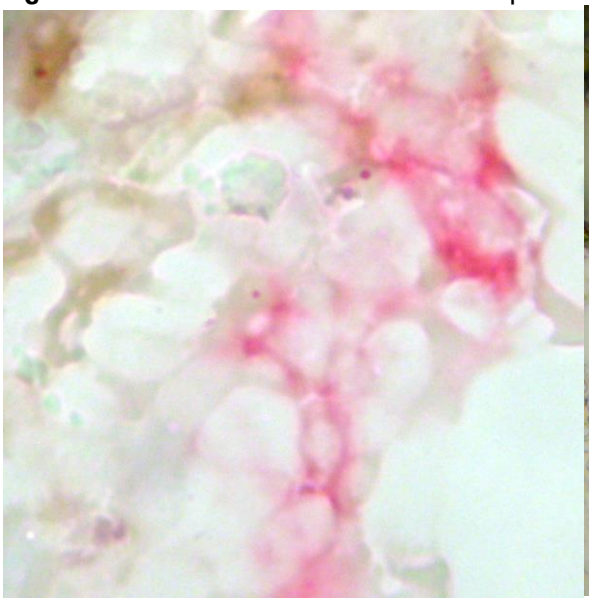


Leukocyte not expressing PSA (red) but expressing membrane CD45 (brown)

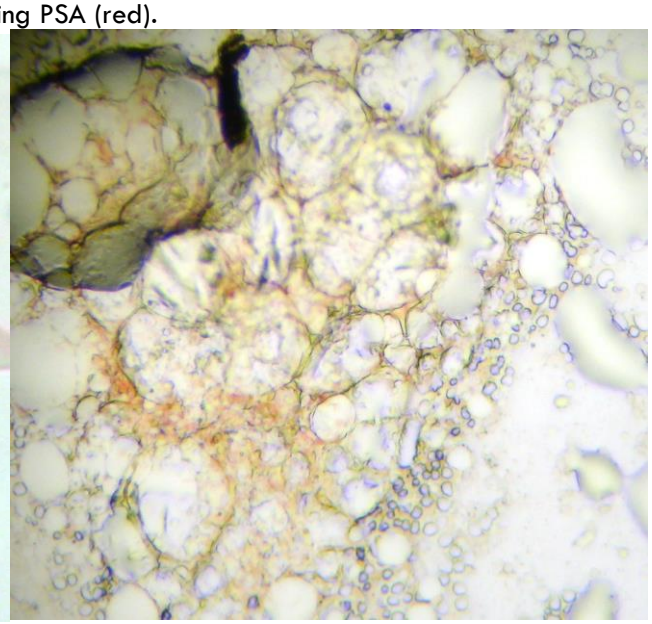
b) **Bone marrow biopsy**: Although previous studies have used bone marrow aspirates to detect micrometastasis (mM) we used biopsy specimens. We have previously reported that prostate tumor cells detected in bone marrow aspirates are phenotypically different than those prostate cells detected in bone marrow biopsies and may not represent "true" mM but rather cells circulating within the bone marrow[14]. For this reason bone marrow biopsy "touch preps" were used as the sample to test for mM.

A bone marrow biopsy was taken from the posterior superior iliac crest one month after surgery and the sample used to prepare four" touch preps" using salinized slides (DAKO, USA). The slides were air dried for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde and 25% phosphate buffered saline (PBS) for five minutes and then washed three times with PBS. All four slides were processed as described for CPCs, a micro-metastasis was defined as cells staining positive for PSA and negative for CD45 (Figure 2)

Figure 2: bone marrow micro-metastasis expressing PSA (red).



Micro-metastasis expressing PSA (red) but not CD45 (brown)



Bone marrow negative for micro-metastasis

The patients were divided into three groups; Group A negative for both CPCs and mM patients (without evidence of MRD); Group B CPC negative, mM positive; Group C CPC positive with or without bone marrow mM detected.

c) absolute lymphocyte count: absolute lymphocyte counts were determined, one month post-surgery and at each 6 month follow-up control until biochemical failure or the end of the study.

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) 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 >0.2ng/ml on two separate occasions. Six monthly blood samples were taken for CPC determination and absolute lymphocyte counts.

Study end point: The primary study end point was the presence of biochemical failure and secondary end point mean time to failure after primary treatment. Biochemical failure free survival time was defined as the time from surgery to the time of a post-surgery PSA of > 0.20ng/ml or the last follow up date. Patients were compared with respect the absolute lymphocyte count and subtype of MRD.

Statistical analysis

The statistical analysis was performed using the program Stata (Stata/SE 17.0 for Windows, Copyright 1985-2021 Stata Corp LLC). Descriptive statistics (measures of central tendency and dispersion) considered the measurement scale and the distribution of the variables.

Subtypes of Minimal Residual Disease (MRD) that included the following categories classified the subjects: CPC and mM negative, CPC negative and mM positive, and CPC positive). Also, The Absolute Lymphocyte Counts (ALC) were categorized according to follow-up time: at the beginning follow-up, average per subject during follow- up (includes repeated samples on the same patient during follow-up without including the ALC at the beginning and end of follow-up) and at the end of follow-up or biochemical failure. The subtypes of MRD were compared for age, total serum PSA, Gleason score, biochemical failure, ALC at the beginning follow-up, ALC average per subject during follow- up and ALC at the end of the follow-up. Pearson's chi-squared test was used to compare frequencies. Analysis of variance (ANOVA) with Bonferroni post-hoc tests was used to compare the means on data with an unbiased distribution. For data with a biased distribution, the Kruskal-Wallis with Dunn´s test was used to test whether samples originate from the same distribution. A p value <0.05 was considered to show statistical significance and all tests were two tailed.

On the whole cohort a nonparametric survival analysis was performed establishing the survival proportion of Kaplan-Meier, for all the subjects and for subtypes MRD. A non-parametric comparison (test Log-Rank) of survival between subtypes MRD was used to determine outcome.

From the predictors: dummy subtypes MRD (the groups: mM positive-CPC negative and CPC positive) and ALC (repeated samples for subjects over time from the beginning until of end follow-up) a mixed effects regression for linear and non-linear models (MERLIN) were perform[15,16]. In this manner the joint longitudinal data could be compared with the Weibull survival model. In brief, the MERLIN model was selected as the final model linking the expected value of ALC (repeatedly measured over time) , with the Weibull survival model incorporating the subtypes MRD.

The MERLIN final model was established with the prediction factors whose coefficients showed statistical significance (p value <0.05). For the final model, the adequate compliance of the following requirements was assessed: functional form, discrimination and calibration as well as the hazard ratios[17,18].

The functional form was used to preserve the quantitative nature of the covariates in the survival regression model. The correct functional form of the quantitative variables for the final model was checked assessing the association between the longitudinal response for the variable with repeated samples of ALC and the time before of censoring and event were performed with graph methods that includes locally weighted regression[17].

The discrimination of a survival model reflects its ability to distinguish the outcomes between subjects. After 5 years, we compared the observed biochemical failure free survival with the predicted biochemical failure free survival predicted using the mean restricted biochemical failure free survival from the MERLIN final model. In this the Area Under the Curve (AUC) and the receiving operating characteristic (ROC) curve could be determined[15].

Besides, for MRD subtypes ("mM and CPC negative", "mM positive-CPC negative" and "CPC positive") and Absolute Lymphocyte Count Average (ALCA) per subject (from the beginning until the end of follow-up) ROC analysis non parametric[19] was performed comparing the AUC.
The calibration aspect of the survival model refers to agreements between the predicted outcome and observed outcome; and is shown comparing predicted survival and observed survival. For this, comparing Kaplan- Meier survival and survival predicted of MERLIN final model for marginal values of the subtypes MRD[20].

Results: Of a total of 641 men diagnosed with prostate cancer during the recruitment period, 404 men underwent radical prostatectomy as mono-therapy and were included in the study, whose biochemical failure free survival showed a biased distribution with a median of 6.9 years (interquartile range 1.6-12.3 years). The minimum and maximum biochemical failure free survival were 0.4 and 17.0 years respectively.
The age and PSA showed a biased distribution. The age (years) , PSA (ng/ml) and Gleason score showed a median (IQR) respectively 66 years (44-86 years), 5.54ng/ml (3.34-8.74 ng/dl) and Gleason 6. The median ALC pre-prostatectomy was 1,900 lymphocytes/mm^3 (IQR 1,600-2,500) and post surgery 2,100 lymphocytes/mm^3 (IQR 1,300-2,500), the median ALC was significantly higher comparing post versus pre-surgery ($p<0.001$ paired T-test), suggesting that immune function was improved after primary tumour removal.
For the 404 subjects; 182 (45.1%) subjects (group were CPC and mM negative, 80 (19.8%) were mM positive and CPC negative ad 142 (35.15%) were CPC positive. During the whole follow-up period 175 (45.3%) men had biochemical failure. Table 1 shows the clinical pathological findings for each MRD subgroup. Group C, CPC positive men, had a significantly worse prognosis in terms of biochemical failure and post prostatectomy has a significantly lower median ALC. At the start of follow up there was no significant difference in the median ALC between men MRD negative (Group A) and those only positive for micro-metastasis (Group B). However, with time, the median ALC remained stable in Group A, while in the other two groups the ALC significantly decreased with time, so that at the end of follow-up there was a significant difference between Groups A and B. This may be a reflection of the increasing biochemical failure seen in Groups B and C with time; in Group B patients the risk was of late biochemical failure, the immune status at the beginning of follow-up being the same of that of men MRD negative but in those who progressed to biochemical failure the immune function decreased with time.

Table 1 Clinical- Pathological Features according to presence of Micro-metastasis of the on 404 Men Treated by Radical Prostatectomy for Prostate Cancer and follow-up time of for Biochemical Failure.

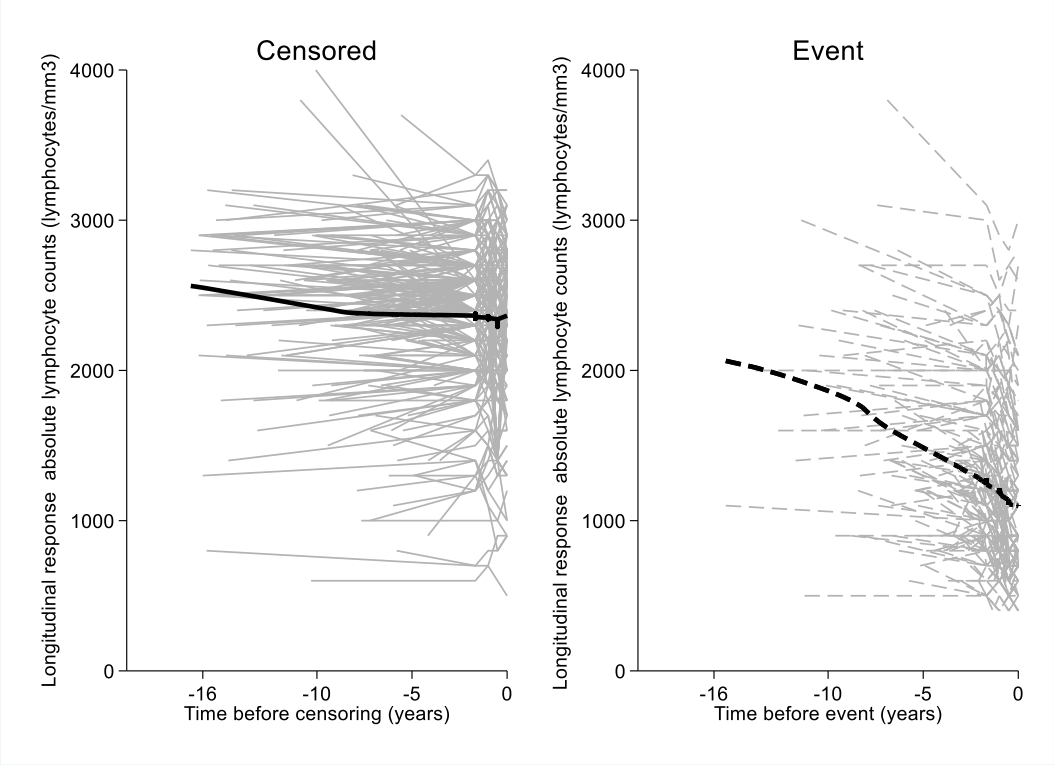
Characteristic	A CPC and mM negative n=182	B mM positive and CPC negative n=80	C CPC positive n=142	P value two tail
Age (years) Median; IQR	64; (54-74)	68; (56-80)	68; (55-81)	0.0176 [a]
PSA (ng/mL) Median; IQR	5.21; (3.36-7.06)	5.87; (3.42-8.32)	6.72; (4.13-9.31)	0.0001 [b]
Gleason Score Median; IQR	5; 1	6; 1	7; 2	<0.0001 [b]
Subject without free disease n (%)	8 (4.4%)	36(45.0%)	131 (92.3%)	< 0.001[c]
ALC at the beginning follow-up Mean ± SD	2349 ± 556	2126 ± 638	1302 ± 563	< 0.0001[d]
ALC average per subject during follow-up Mean ± SD	2350 ± 511	1959 ± 576	1140 ± 520	< 0.0001[d]

ALC at the end of the follow-up time Mean ± SD	2347 ± 509	1942 ± 692	1068 ± 514	< 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"

Figure 5 shows the association of repeated ALC counts with time, in men who did not undergo biochemical failure the ALCs were stable, whereas in those who underwent biochemical failure the ALC decreased in the 18 months prior to biochemical failure.

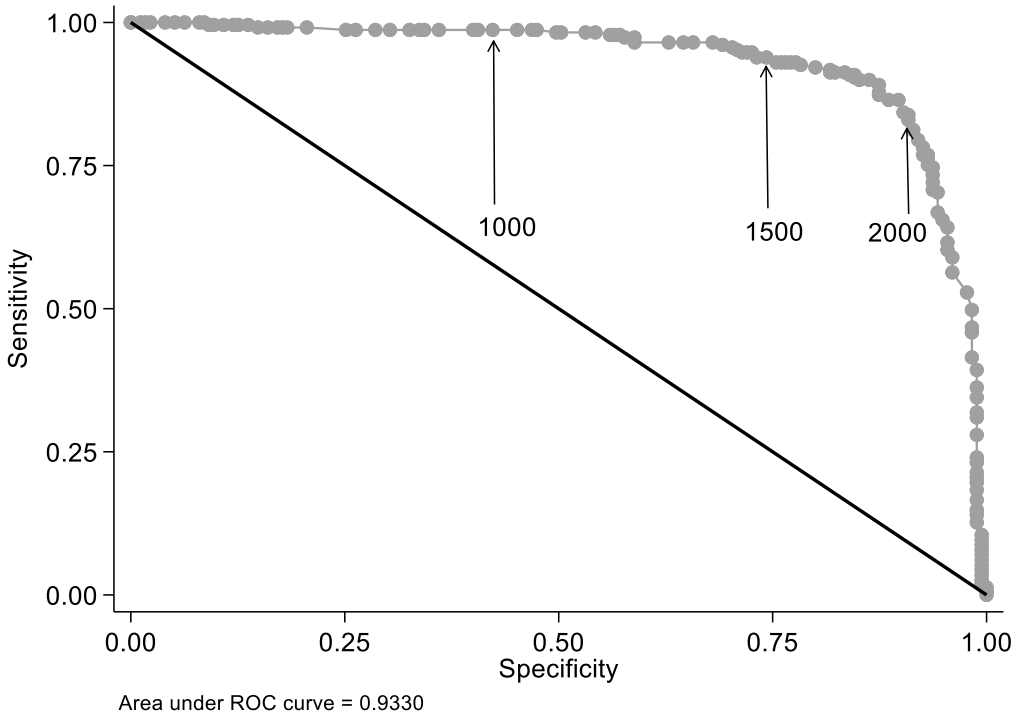
Figure 5 Association between the longitudinal response of repeated samples of absolute lymphocyte counts and the time before of censoring and event with locally weighted regression showed on black line on 404 Men Treated by Radical Prostatectomy for Prostate Cancer



This decrease in the ALC was accompanied by the detection of CPCs in men previously CPC negative and an increase in the numbers of CPCs detected in those initially positive. This event occurred approximately six months after the initial decrease in the ALC.

To evaluate the optimum cut off point for the ALC, three values were analyzed; using a cut-off value of 1000 lymphocytes/mm^3, which divided patients into those with severe and moderately severe LCP, and the rest showed a sensitivity and specificity respectively of 0.987 and 0.423. Using a cut-off value of 1500 lymphocytes/mm^3, which divided patients into LCP and the rest showed a sensitivity and specificity respectively of 0.93 and 0.754. Finally using a value of 2000 lymphocytes/mm^3 , dividing patients into normal and the rest, the sensitivity and specificity were respectively 0.834 and 0.909. The Receiving operating characteristics using three cut-off points are shown in Figure 6.

Figure 6 Receiving Operating Characteristic (ROC) analysis for Absolute Lymphocyte Count Average (ALCA) per subject (from beginning until end follow-up) for absence of failure biochemical on 404 Men Treated by Radical Prostatectomy for Prostate Cancer



Arrows indicate: ALCA of 1000 showed sensitivity and specificity respectively of 0.987 and 0.423. ALCA of 1500 showed sensitivity and specificity respectively of 0.93 and 0.754. ALA of 2000 showed sensitivity and specificity respectively of 0.834 and 0.909

Kaplan Meier survival analysis of biochemical failure free survival according to Minimal Residual Disease subtypes.

At the end of the study, the Kaplan-Meier for biochemical failure free survival of the whole group was 40.7% (95% CI: 32.6% to 48.7%). The Log-Rank Test showed a p-value less than 0.01 at comparing the survival for biochemical failure between the subtypes of MRD (Table 2).

When compared to Group A patients (MRD negative), the MERLIN final model showed for measures repeated of ALC a HR of 0.20 (p-value than less to 0.001; 95% CI 0.15 to 0.28); the MRD subtype Group B (mM positive and CPC negative) showed a HR of 6.69 (p value <0.001; 95% CI: 3.08 to 14.56) and MRD subtype Group C (CPC positive) showed a HR of 16.17 (p-value<0.01 95%CI: 7.66 to 34.13)

Table 2 Comparing observed survival (Kaplan-Meier) versus predicted with mixed effects regression for linear and non-linear model for biochemical failure free progression at 5 and 10 years minimal residual disease on 404 Men Treated by Radical Prostatectomy for Prostate Cancer

Variable predictor		Survival to 5 years Percentage		Survival to 10 years Percentage	
		Observed [a]	Predicted [b] (95%CI)	Observed [a] (95% CI)	Predicted [b] (95% CI)
Minimal Residual Disease	mM and CPC negative n=182	96.87	98.04 (95.99 to 99.05)	92.40	92.07 (84.55 to 96.01)
	mM positive and CPC negative n=80	95.64	90.58 (81.92 to 96.01)	46.27	57.53 (44.70 to 68.37)
	CPC positive n=142	71.38	72.57 (64.09 to 79.37)	38.54	26.28 (16.41 to 37.22
All subject n=404		85.99	87.02 (85.91 to 88.12)	61.92	60.58 (58.24 to 62.91)

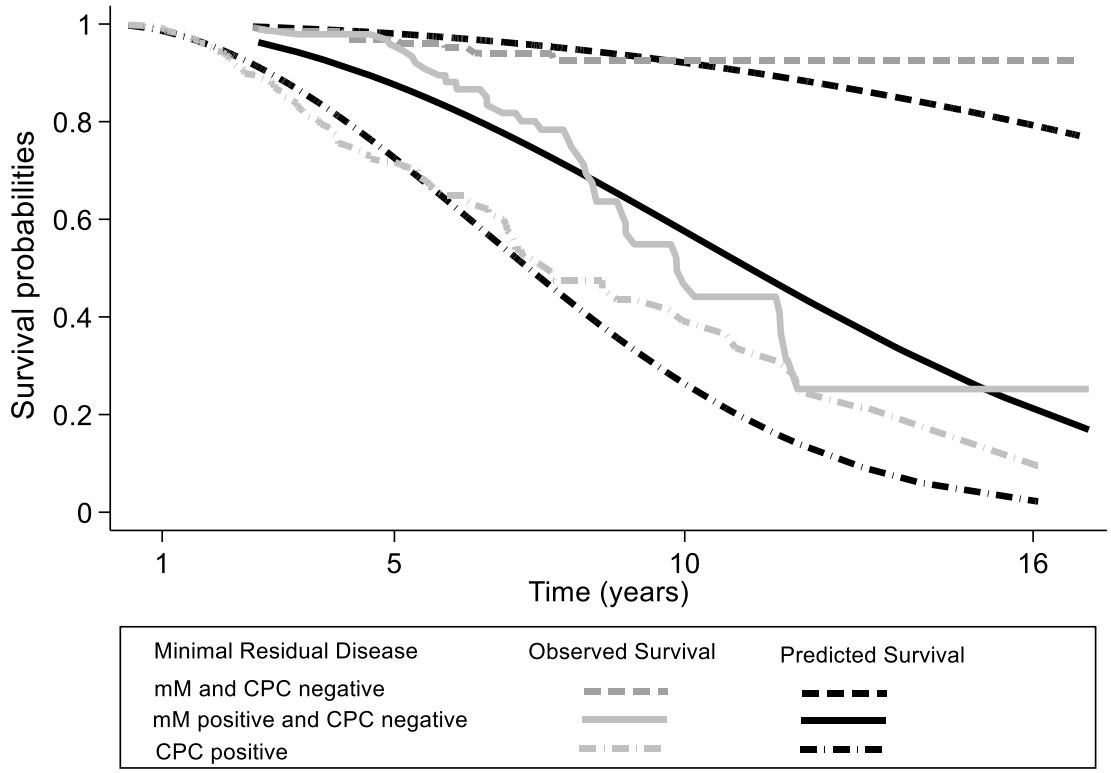
CPC= Circulating Prostate Cells; mM= micrometastasis; CI= confidence interval; Observed survival= Kaplan-Meier Survival adjust for mean Absolute lymphocyte counts for each subject; [b] Predicted Survival Model from mixed effects regression for linear and non-linear model.

After 10 years of follow-up, the observed versus predicted failures biochemical for MERLIN final model according to the restricted mean biochemical failure free survival time showed an area ROC of 95.35 (95%CI:93.71 to 96.99). The ROC analysis showed: a) AUC 85.71 (95% CI 82,52 to 88.88) for biochemical failure free survival for MRD Group A; b) AUC of 50.68 (95% CI: 46.7 to 54.62) for biochemical failure free survival for MRD Group B; c) AUC of 85.03 (CI 81.52 to 88.55) for biochemical failure free survival for MRD Group C; finally an AUC of 93.30 (CI 95%: 90.72 to 95.87) for biochemical failure free survival for the ALC (Figure 2).

Comparing the AUCs between the MRD subtypes and the ALC showed a p-value than less 0.001.

There was agreement when comparing the predicted MERLIN final model with the observed survival (Kaplan-Meier Survival) (Figure 7 and Table 2).

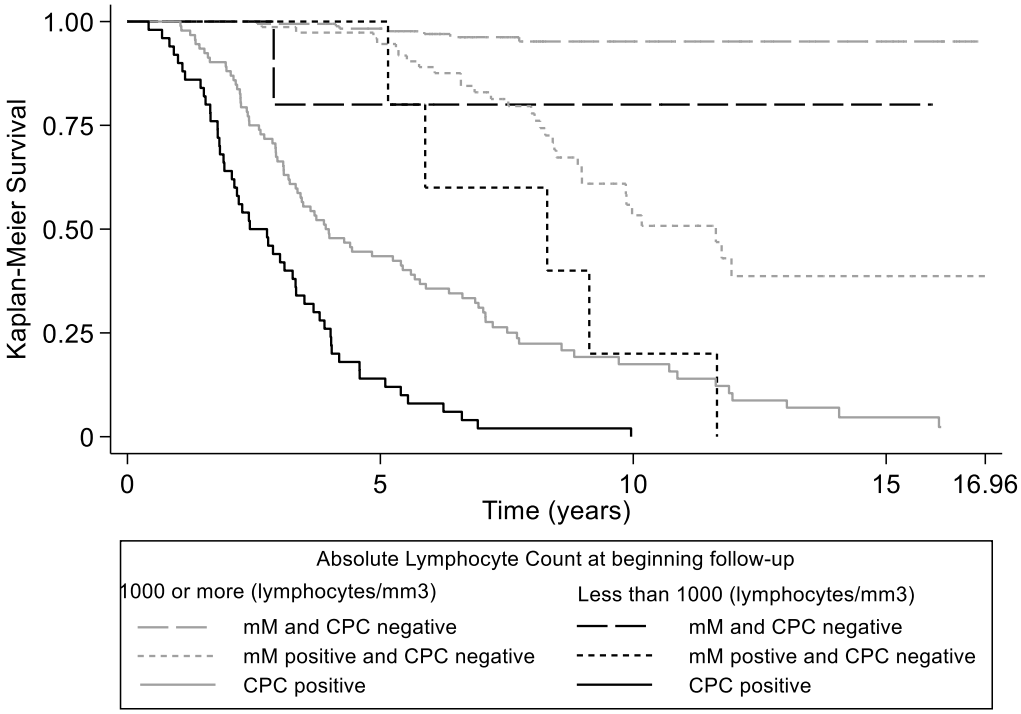
Figure 7: Comparing predict survival with MERLIN (mixed effects regression for linear and non-linear model) versus Kaplan-Meier survival model (observed survival) for biochemical failure free progression at 16.96 years by Minimal Residual Disease Subtypes in 404 Men Treated by Radical Prostatectomy for Prostate.



CPC= Circulating Prostate Cells; mM=micrometastasis**Kaplan Meier biochemical failure free survival combining Minimal Residual Disease subtypes with a cut-off value of 1,000 lymphocytes/mm³ blood.**

Each MRD was divided into two groups, those with an ALC >1,000/mm³ and those with an ALC <1,000/mm³ determined one month after radical prostatectomy. For each group the Kaplan Meier survival curves were determined; men with lymphocytopenia had a worse outcome for each subgroup of MRD (log rank test < 0.001) (Figure 8).

Figure 8: Kaplan- Meier estimates by Absolute Lymphocyte Count at beginning follow-up) and Minimal Residual Disease subtype for failure biochemical in 404 Men Treated by Radical Prostatectomy for Prostate Cancer.



It can be seen from the results shown in Table 3, that men with lymphocytopenia one month after radical prostatectomy had a significantñly worse biochemical failure free survival in each MRD sub-type (log rank test p<0.001). Importantly, men in Group C had a significantly higher frequency of men with lymphocytopenia (35%) compared with Groups A (3%) and Group B (6%) respectively (p<0.0001 Chi squared for both), with no significant difference between Groups A and B (p=0.31 Chi squared).

Table 3: Observed Survival Kaplan-Meier by Absolute Lymphocyte Count at beginning follow-up) and Minimal Residual Disease subtype for failure biochemical in 404 Men Treated by Radical Prostatectomy for Prostate Cancer

Minimal residual disease subtype	Absolute lymphocyte at beginning follow-up (lymphpcyte / mm^3) n	Kaplan- Meier Survival (%)		
		5 years	10 years	15 years
mM and CPC negative Group A N=182	1000 or more n=177	98.29 (94.80 to 99.45	95.19 (89.97 to 97.73	95.19 (89.97 to 97.73
	Less than 1000 n=5	80.00 (20.38 to 96.92)	80.00 (20.38 to 96.92)	80.00 (20.38 to 96.92)
mM positive CPC negative Group B	1000 or more n=75	94.59 (86.23 to 97.94)	53.33 (38.88 to 65.81)	38.68 (23.03 to 54.09)

N=80	Less than 1000 n=5	100	20 (0.84 to 58019=	
CPC positive Group C N=142	1000 or more n=92	43.48 (33.23 to 53.28)	17.46 (9.9 to 26.6)	4.66 (1.06 to 12.61)
	Less than 1000 n= 50	14 (6.15 to 25.00)		

Discussion: Reports of the association between immune function and prostate cancer have been limited in that they use different cut off values, pre-prostatectomy or post-prostatectomy values, which makes comparisons difficult. We used the ALC taken one month after prostatectomy as the baseline value. We used the post-prostatectomy value, as removal of the primary tumour has been reported to improve the immune function. Removal of the primary tumour in prostate cancer increases cytotoxic T-cell activity against prostate cancer during the first four weeks post-surgery and thereafter decreases in high-risk prostate cancer[21]. Inversely regulatory T-cells (Tregs), which cause immune-tolerance of cancer cells, decrease after surgery, the decreases lasting up to eight weeks and thereafter increasing[22, 23]. Although we did not measure lymphocyte sub-sets the median ALC increased after prostatectomy suggesting an improvement in immune function as a result of tumour removal and thus implying that baseline values taken after surgery maybe a more accurate reflection of immune status.

What this study suggests is that a stable immune function is associated with a better outcome, and that before treatment failure there is a decrease in immune function, these dynamic changes have been reported in patients treated with eribulin for breast cancer and improvements in the ALC are associated with a better prognosis[24,25]. Previously it has been reported that there is an association between lymphocytopenia and the presence of circulating tumour cells in metastatic breast cancer[26] and in gastrointestinal cancer[27]. In patients with metastatic castration-resistant prostate cancer the association of the presence of circulating prostate cell and immune dysfunction was associated with a worse prognosis[28]. This is the first report of the association of immune dysfunction with minimal residual disease in non-metastatic prostate cancer patients. The frequency of immune dysfunction was significantly higher post-prostatectomy in patients CPC positive, in those patients who underwent biochemical failure the immune dysfunction worsened and was associated with an increased number of CPCs detected. Similarly in Group B patients, deterioration in the immune function was associated with the appearance of CPCs and biochemical failure. 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[29]. 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[30]. 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[31]. 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[32]. 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[33,34]. 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[35].

The study has several limitations; firstly it was a single-centre study; 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[36].

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[14]. 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%[37,38]. The advantages are that samples do not have 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[39].

For CPC detection we used differential gel centrifugation and immunocytochemistry, we acknowledge the method dependent detection of CPCs which has been previously reviewed[40]. The method we used is based on CPC cell size and will not have detected cells not expressing PSA, however the method has the advantage of being cheap and could be carried out in the routine laboratory of a general hospital without the need for high cost technology. In an internal validation of the method the inter and intra-observer reliability of the CPC determination in 30 patients determined in duplicate by three independent pathologists the observed inter-operator agreement was 89% and inter-operator agreement was 90%, for a kappa statistic of 0.77 and 0.79 respectively, considered to be a good agreement[41]. However, despite these limitations the results imply that the determining factor for biochemical failure is related to the presence of MRD (biological characteristics of tumour cells) and their interaction with the immune system, which is dynamic and changes with time.

Conclusions: 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. These preliminary results warrant further multi-centric studies to confirm the results.

Conflicts of Interest: The authors report no conflicts of interest.

Funding: The study was funded by a Hospital de Carabineros de Chile Research Grant.

Acknowledgements: The authors wish to thank Mrs. Ana Maria Palazuelos for her help in the writing of this manuscript.

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