Surgical cytoreduction of the primary tumor reduces metastatic progression in a mouse model of prostate cancer

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Abstract. Metastatic prostate cancer (mPCa) is one of the most prevalent cancers in men worldwide. The main cause of death in these patients is androgen-resistant metastatic disease. Surgery of the primary tumor has been avoided in these patients as there is no strong evidence that supports a beneficial effect. From the biological point of view, it appears rational to hypothesize that the primary tumor may contribute to the establishment and growth of metastases. Considering this, we propose that cytoreductive surgery (CS) in advanced metastatic stage slows the progression of metastatic disease. To test this, we used a mouse model of resectable orthotopic prostate cancer (PCa) and performed CS. After surgery, metastases were smaller and less numerous in the treated mice; an effect that was observable until the end of the experiment. These results suggest that CS alone delays the progression of metastatic disease and that although this effect may be temporary, it may translate to prolonged survival, especially when used with adjuvant therapy.

Introduction

Prostate cancer (PCa) is the second leading cause of male tumor-related deaths in developed countries (1). The main treatment used in patients with metastatic prostate cancer (mPCa) is androgen deprivation therapy which has demonstrated an improved overall survival after prostate-specific antigen detection (2). However, these patients often enter in an androgen-independent stage associated with high mortality (3). There are several treatment options for these patients, but none includes surgical management of the primary tumor outside of a clinical trial setting (3,4). Surgeons are becoming more prone to surgical treatment in high risk and locally advanced disease as a part of a multimodality approach to the treatment of PCa, including the possibility to treat the primary tumor in M1 patients (4). There has arisen a question of whether removing the primary tumor in patients with metastatic disease is relevant, and during the last decade it has been addressed in several reports that analyze information available in retrospective studies (5,6). Currently, cytoreductive surgery (CS) is used rarely in mPCa patients but only to relieve the patient from local side-effects derived from tumor growth (7) and not in the context of PCa treatment (3). The effect of a reduction in tumor mass in a patient presenting with metastasis may be used as an additional therapeutic measure to prolong patient survival, but there are no current pre-clinical models that confirm or refuse this hypothesis (4). From the biological point of view, the primary tumor has an important role in initiating and maintaining the metastatic process. The tumor has the propensity for constantly delivering cells into circulation (8,9) while it also can prepare the distant pre-metastatic niche for successful implantation of disseminated cells (10,11). These processes can occur early in the disease, an observation that has clinical (12) and biological support (13). Treatment of the primary tumor may not only have a positive effect on the localized consequences of tumor growth but also in the distant tumors that grow as metastasis (14). Considering this clinical and biological background, we proposed that cytoreduction of the primary tumor reduces or slows the progression of metastatic disease in a mouse model of mPCa. To address this question we previously generated a murine model of CaP that is surgically resectable without significant side-effects (15). This model consistently generates metastasis in a time-dependent fashion and enables us to perform CS and study the behavior of tumors after treatment.

Materials and methods

Cells. PC3 cells were used for orthotopic injection. This cell line is derived from a bone metastasis of a human PCa patient and has a high tumorigenic and metastatic behavior when injected intravenously and orthotopically. These cells are androgen-independent and do not express PSA. In order to visualize the

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cells once injected in the mouse prostate, they were transduced with a gene that contains the sequence for firefly luciferase (GenTarget Inc.®) (PC3-LUC cells) using viral particles *pGreenFire1-LUC-CMV-EF1-Puro* (cat. TR011VA-P; System Biosciences, Mountain View, CA, USA) diluted in Dulbecco's modified Eagle's medium (DMEM)/F12 10% FBS with Polybrene (cat. H9268; Sigma-Aldrich) at a concentration of 5 μg/ml. Cells were left overnight for infection, and puromycin was added (1 μg/ml) to select cells that were stably transduced. Integration was verified using qPCR and fluorescence.

Animals and orthotopic injection. NOD-SCIDγ mice (NOD. Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ; Jackson Laboratory®, Sacramento, CA, USA) were obtained from our High Safety Animal Facility (Faculty of Medicine, University of Chile) and maintained in a laminar flow room under specific pathogen-free conditions. All food, water and litter were sterilized prior to use. Temperature (20-21°C) and humidity (50-60%) were controlled. Daily light cycles were 12-h light and 12-h dark. Cages were changed fully once or twice a week. Animals were manipulated under sterile conditions. Orthotopic injection was performed using the anterior lobe of the mouse prostate as previously described (primer paper). All experiments with animals were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (protocol CBA#0487 FMUCH).

Bioluminescence. Primary and metastatic tumor growth was followed using the IVIS Lumina II® (Caliper Life Sciences, Hopkinton, MD, USA) system. Animals were anesthetized with a combination of ketamine and xylazine and injected intraperitoneally with 150 mg/kg of D-luciferin (potassium salt). Sixteen minutes after injection of D-luciferin, images were captured with the IVIS system. Images were taken in 5-7 day intervals until the end of the experiment.

Study design. Two groups of mice were used with 5 mice in each group. The first group (control) consisted of animals injected orthotopically in the prostate and then followed by luminescence imaging at regular intervals until completion of the experiments. These animals received no further manipulation. The second group (treatment) was injected orthotopically at time 0 and then subjected to CS of the primary tumor at day 30 in which metastatic growth had already occurred. These animals were followed by luminescence in the same manner as the first group until the end of the experiment.

CS. At day 30 post-injection of PC3-LUC cells, in the treatment group, animals were anesthetized and a midline incision was made on the skin and muscle, 1 cm caudal to the umbilicus. The prostate tumor and its associated seminal vesicle was identified, externalized and isolated. Using 6/0 absorbable suture, a ligature was made at the base of the anterior lobe, above the deferent duct. The prostate lobe was then cut over the ligature and removed with attention not to rupture the seminal vesicle. The same procedure was repeated for the left anterior prostatic lobe, along with its associated seminal vesicle. After checking for hemorrhages, the abdominal and skin incisions were closed in two planes. The tissue obtained from the CS was submerged in 10% neutral buffered formaldehyde. A

luminescence image was taken after the procedure to ensure that no more than 5% of the tumor luminescence remained detectable. This percentage was considered a successful treatment.

Histology and immunohistochemistry. Tissues were fixed by immersion in neutral buffered formalin for 24 h and then they were trimmed and placed in histologic cassettes for dehydration, inclusion in paraffin and staining with hematoxylin and eosin (H&E). Using the same tissues, an indirect immunoperoxidase method was performed as follows. Antigen retrieval was achieved by exposing the samples to 90°C for 30 min in citrate buffer (10 mM, pH 6.0). Then the samples were incubated with the primary antibody [anti-human monoclonal anti-mitochondria (ab3298; Abcam, Cambridge, MA, USA), mouse monoclonal anti-CD-24 (CBL561; Chemicon, Temecula, CA, USA), rabbit monoclonal anti-CD-44 (ab51037; Abcam), mouse monoclonal anti-CD-133 (17A6.1; Millipore, Billerica, MA, USA) and mouse monoclonal anti-KI-67 (clone MIB-1; DakoCytomation, Glostrup, Denmark)] at 4°C overnight. The samples were treated with a streptavidin-biotin detection method (Histostain®-Plus Bulk kit, Zymed®, LAB-SA detection system and DAB-Plus Substrate kit; all from Invitrogen, Camarillo, CA, USA) followed by hematoxylin counterstaining. Images were obtained with a Leica® microscope, model DM3000. For semi-quantititive analysis of immunolabeling, a hybrid score (H-score) was calculated for each marker. This score resulted from determining the percentage of positive cells (from 0 to 100%) and the intensity of staining (from 0-3 with 0, 1, 2, 3 grades corresponding to no staining, weak, medium and high intensity, respectively). These two numbers were then multiplied to obtain a score that ranged from 0 to 300. A proliferative index (PI) was calculated counting the number of KI-67 positive cells in three images obtained at x400 for each of three samples per group. The number of total and positive cells was obtained using the ImageJ software (ver. 1.48).

Statistics. Data were compiled and analyzed using Prism 6.0 software. Results were considered significantly different if $p < 0.05$, according to the specific statistical analysis used, as described in each figure legend.

Results

Primary tumor growth. After injection of PC3-Luc cells to both the control and treatment groups, we followed tumor growth by bioluminescence (Fig. 1A, Table I). The IVIS system has a level of background signal that is shown with a green line. This is the minimum signal that can be measured when an animal injected with cells but not with luciferin is photographed. CS was performed in the treatment group at day 30 (arrow). In all cases, the luminescence signal of the remnant tumors was <5% of the original signal. On days 42, 51, 58, 63 and 70, luminescence of the primary tumors in the treatment group was lower ($p < 0.01$) than that of the control group demonstrating the effect of surgery on the tumor mass. The tumor mass was significantly reduced by the procedure but it was not eliminated, simulating the case of an advanced tumor which is not amenable to complete resection. At the end
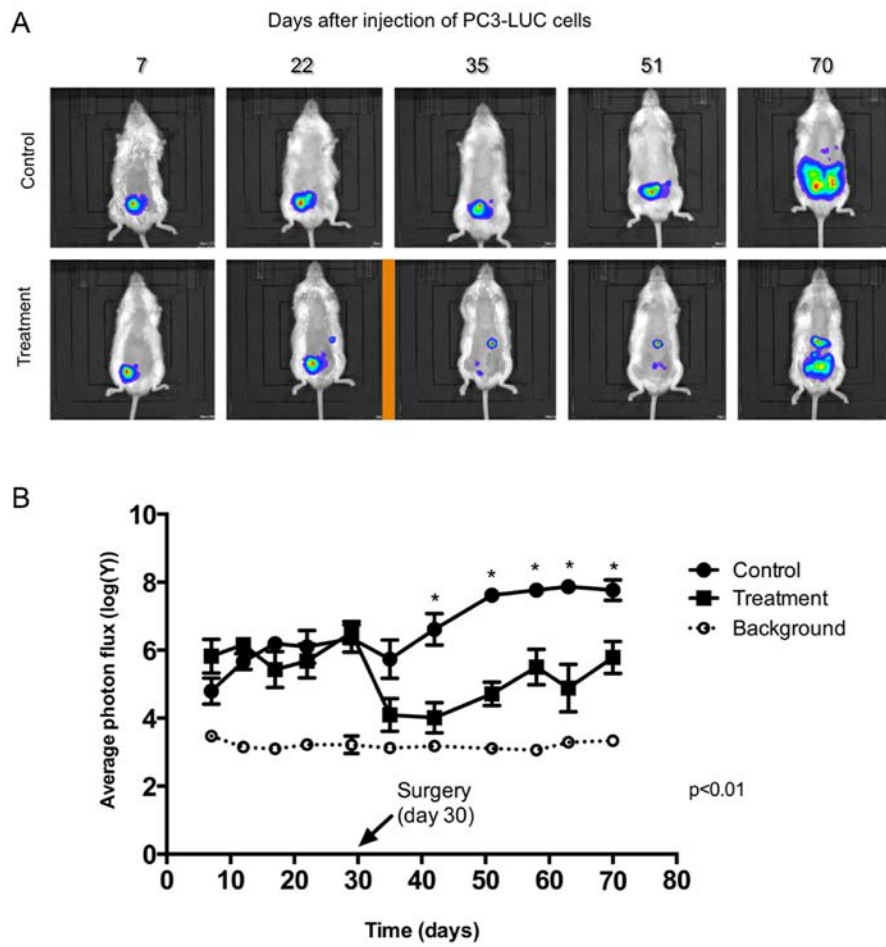
Figure 1. (A) Representative images of the mice in the control and treatment groups at 7, 22, 35, 51 and 70 days post-injection of PC3-LUC cells. Orange bar represents time of CS in mice from the treatment group. (B) Photon flux of the primary tumors in animals without treatment (control) and animals that were subjected to CS at day 30 post-injection of PC3-LUC cells (treatment). The dotted line shows the basal level of luminescence (background) which is the minimum signal obtained when measuring a mouse injected with PC3-LUC cells but without luciferin injection. Means were calculated based on a logaritmic transformation of the original data. Multiple Student's t-test; *p<0.01, significant differences between control and treatment groups. Table I is a summary of these results.

Table I. Photon flux of the primary tumors.

Day	p-value	Control Mean [log(photon flux)]	SD (±)	Treatment Mean [log(photon flux)]	SD (±)
7	0.140	4.798	0.851	5.821	1.108
12	0.118	5.667	0.522	6.161	0.355
17	0.217	6.193	0.494	5.429	1.173
22	0.563	6.099	1.083	5.680	1.109
29	0.743	6.346	0.899	6.522	0.729
35	0.058	5.736	1.265	4.095	1.078
42	0.004[a]	6.612	1.036	4.012	1.001
51	0.00003[a]	7.619	0.169	4.715	0.766
58	0.003[a]	7.766	0.320	5.503	1.169
63	0.003[a]	7.866	0.408	4.884	1.560
70	0.007[a]	7.768	0.675	5.785	1.050

Photon flux of the primary tumors [means and standard deviation (SD)] in animals without treatment (control) and animals that were subjected to cytoreductive surgery at day 30 post-injection of PC3-LUC cells (treatment). Means were calculated based on a logarithmic transformation of original data. Multiple Student's t-test; [a]p<0.01, significant differences.

of the experiment (day 70) a rise in the level of luminescence was observed in the treatment group. This was still significantly lower than the level in the tumors in the control group and lower than the level in the tumors before surgery. In addition, in the treatment group, tumor size was less homogenous as demonstrated by their dispersion (SD, standard deviation; Table I).

Metastasis. Fig. 2 and Tables II and III show the general results for the metastases measured (Fig. 2A, Table II) and counted (Fig. 2B, Table III) by bioluminescence from day 42 after injection of the PC3-LUC cells until the end of the experiment at day 70. The size of the metastases (Fig. 2A), estimated by bioluminescence, was lower in the treatment group from day 51 until the end of the experiment. This size increased at the end of the experiment but failed to reach that of the control group. The effect on size was constant from day 42 until the end of the experiment showing that the main bulk of the primary tumor is needed to allow for metastasis to grow. The percentage of animals (Fig. 2B) that presented metastasis was similar in both cases, confirmed by contingency tables that showed no difference (p>0.05) in the frequency of animals affected. This responds to the fact that

Table II. Photon flux of metastatic tumors.

		Control			Treatment		
Day	p-value	Mean [log(photon flux)]	SD (±)	N	Mean [log(photon flux)]	SD (±)	N
42	0.135	5.894	1.252	3	4.803	1.015	3
51	0.002[a]	6.816	0.501	3	4.608	0.469	4
58	0.018[a]	6.232	0.946	5	4.770	0.462	4
63	0.003[a]	6.692	1.109	5	4.801	0.439	4
70	0.005[a]	7.120	0.960	5	5.441	0.966	5

Photon flux of metastatic tumors in animals without treatment (control) and animals that were subjected to cytoreductive surgery at day 30 post-injection of PC3-LUC cells (treatment). Means were calculated based on a logarithmic transformation of original data. Multiple Student's t-test. [a]$p<0.05$, significant differences between control and treatment groups.

Table III. Number of metastatic tumors.

		Control			Treatment		
Day	p-value	Mean	SD (±)	N (%)	Mean	SD (±)	N (%)
42	0.600	1.33	0.58	3 (60)	2.00	1.00	3 (60)
51	0.726	2.67	2.08	3 (60)	2.25	1.50	4 (80)
58	0.296	3.60	1.34	5 (100)	2.50	1.91	4 (80)
63	0.003[a]	5.80	1.30	5 (100)	2.50	1.91	4 (80)
70	0.012[a]	5.80	1.30	5 (100)	3.20	1.79	5 (100)

Number of metastatic tumors in animals without treatment (control) and animals that were subjected to cytoreductive surgery at day 30 post-injection of PC3-LUC cells (treatment). Multiple Student's t-test. [a]$p<0.05$, significant differences between control and treatment groups.

surgery was performed in animals that already had metastatic dissemination and therefore, surgery did not revert the process but only delayed it. Although the number of metastases was similar on days 42, 51 and 58, animals that received surgery (treatment group) had a lower number of metastases at the end of the experiment (days 63 and 70). This suggests that surgery of the primary tumor prevents the growth of new metastases. This result was also observed when counting metastases by microscopy (Fig. 3).

Necropsy and histology. The most frequent site affected by metastasis was the pancreas. It was followed by the hilum of the liver, spleen and stomach, the hilum of the kidney, mesentery, liver (parenchyma) and kidney (parenchyma). All mesenteric tumors were associated with a mesenteric vessel. None of the animals had tumors in the abdominal wall muscles or diaphragm, nor on the antimesenteric surface of the intestine, all of which were considered signs of carcinomatosis. Hence, all observed metastatic tumors were the result of circulatory dissemination. No apparent difference was noted in the histology of metastasis or the primary tumor when comparing both groups. This morphology was described in a previous study (15). Smaller tumors had a solid growth with scant amounts of stroma. Larger tumors had more stroma and
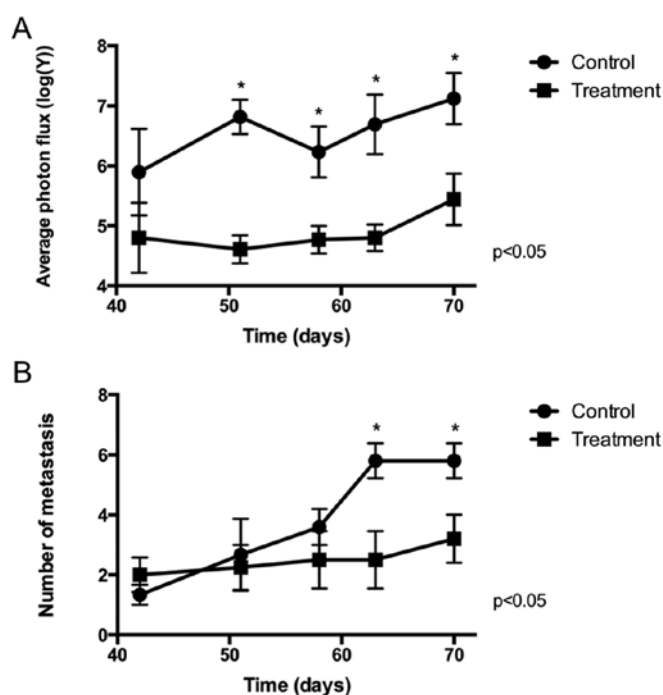

Figure 2. (A) Photon flux of metastatic tumors in animals without treatment (control) and animals that were subjected to cytoreductive surgery (CS) at day 30 post-injection of PC3-LUC cells (treatment). Means were calculated based on a logaritmic transformation of original data. (B) Number of metastatic tumors in animals without treatment (control) and animals that were subjected to CS at day 30 post-injection of PC3-LUC cells (treatment). Multiple Student's t-test. [*]$p<0.05$, significant differences between control and treatment groups.

some had the formation of pseudoacinar structures. Treatment had no effect on the microscopic structure of the tumors.

CD-24, CD-44, CD-133 immunoreactivity and PI. Fig. 4 contains a summary of these results. A hybrid score (H-score) was used as a combined parameter of staining intensity and percentage of stained cells. In this analysis, tumors resected from animals at day 30 (treatment group) were included and represent the marker status of the primary tumors before surgery. CD-24 immunolabeling was high on all primary tumors, including the tumors resected on day 30 in the treat-
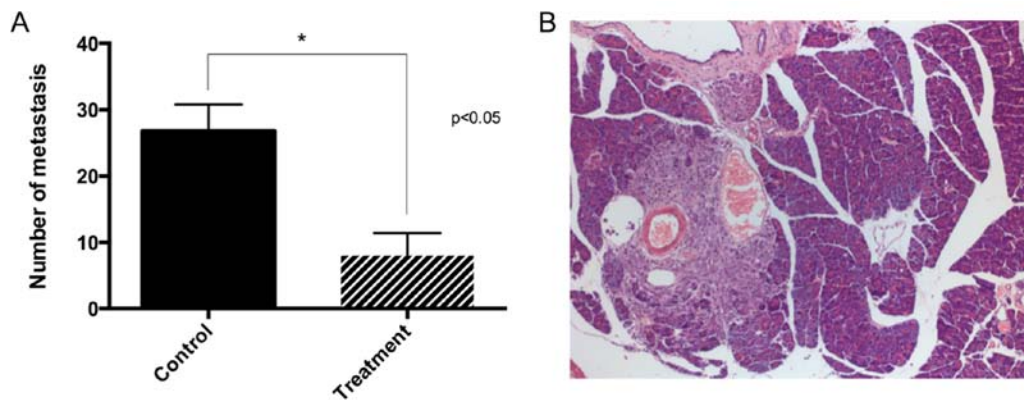
Figure 3. (A) Number of metastases counted by microscopy at the end of the experiment. Paired Student's t-test. p<0.05. (B) Representative image of a pancreatic metastasis.

ment group. In contrast, in both groups, CD-24 H-score was lower in the metastatic tumors. There was no difference between H-score in metastasis from the control and treatment group. CD-44 immunolabeling was high in all tumors and no differences were observed between them. CD-133 immunolabeling was higher in all tumors of the treatment group compared to the tumors in the control group. Interestingly, CD-133 H-score in the resected tumors (treatment group) was similar to the one observed in tumors in the control group.

The PI (Fig. 5) was calculated as a percentage of KI-67 positive cells in x400 fields. We compared the PI between primary and metastatic tumors in both groups. When comparing PI in the primary tumors, a higher percentage of KI-67-positive cells was found in the treatment group. These tumors arose from the remaining cells after CS suggesting that this is an effect of surgical treatment. Similarly, PI was higher in metastatic tumors of the treatment group versus metastases of the control group. These results suggest that proliferation was activated in the metastatic tumors from mice that received surgery. The effect of surgery on proliferation was, therefore, observed in all tumors confirming a systemic effect of treatment. When we compared PI between the primary tumors of the control group (day 70) with those resected at day 30 from mice in the treatment group (before any effect of surgery was possible) no difference was found, further indicating that a higher proliferative activity was a consequence of the surgical procedure.

Discussion

Surgical removal of the primary tumor as a treatment for metastatic cancer is an old idea that has been revisited throughout the history of oncology (16). Several studies in the 70's and 80's suggested that this treatment results in an increased number of metastases (17,18). More recently, some evidence suggests that treatment of the primary tumor may activate angiogenesis, which in turn, may activate dormant metastatic cells (19). In the present study the observed effect of CS was a reduction in size and number of metastatic foci when compared with the non-treated mice. This effect suggests that surgery not only slows down the development of already established metastases but also reduces the

number of new metastases, probably arising from dormant sites. Escaping dormancy depends on several factors, including angiogenesis, appropriate interaction with the local microenvironment, response to immune surveillance, and cancer stem cell presence (20,21). There is ample evidence showing a promotive role of the primary tumor in preparing the microenvironment or pre-metastatic niche and facilitating survival and proliferation at distant sites (10,11). This effect can be related to the mobilization of bone marrow-derived hematopoietic progenitor cells (11), the upregulation of extracellular matrix ligands and the expression of chemoattractants (22). All these factors may regulate the angiogenic-dependent dormancy status (23). Our results are in accordance with these observations. Another mechanism proposed for the primary tumor to distantly regulate the pre-metastatic niche is the delivery of microvesicles that can modify distant cells by transferring several classes of molecules, such as microRNAs (24). microRNAs have been proposed as regulators of several steps involved in the metastatic cascade, particularly regulating the occurrence of oligometastatic disease (25). In this context, the removal of the bulk mass of the primary tumor may alter delivery of these molecules, resulting in a less aggressive presentation. This hypothesis must be tested in an appropriate setting. Another important mechanism regarding tumor development is the role of cancer stem cells in initiating primary and metastatic tumors. To address this issue we evaluated expression of CD-24, CD-44 and CD-133 markers by immunolabeling. We found that the primary and metastatic tumors in the treated group had low CD-24 and high CD-44 and CD-133 expression compared to levels in the tumors from the non-treated animals. This combination is suggestive of a higher presence of less differentiated cells with high expression of stem cell markers. This denotes a possible switch in differentiation markers. Potential roles for CSCs are initiation of tumors, resistance to therapy and origination of metastasis (26,27). The presence of CSC markers suggests that after surgical trauma more of these cells are able to induce the growth of the remaining tumor. This may be happening in metastasis as well, suggesting that after surgery, CSCs may have a role in repopulating the tumors (either metastatic or primary) rather than generating new metastatic foci.
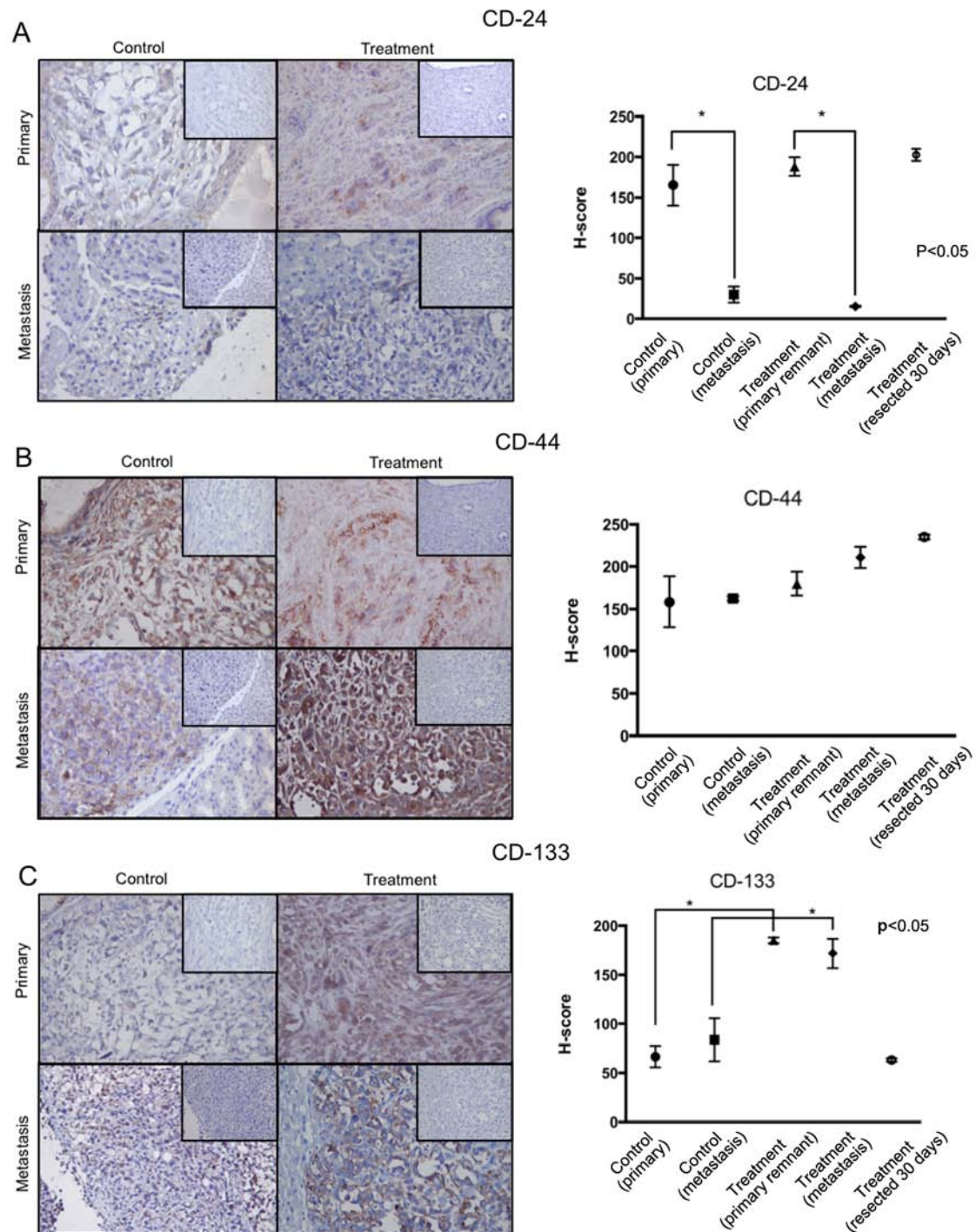
Figure 4. Representative images of (A) CD-24, (B) CD-44 and (C) CD-133 expression along with immunolabeling by determining H-score (original magnification, x400). In the images, boxes represent negative controls for each marker (original magnification, x100). Graphs show H-score in tumors from the control group (primary and metastatic, respectively) and from tumors in the treatment group (primary, metastatic and the resected tumors at surgery). Original magnification in all photomicrographs, x400. ANOVA and multiple Student's t-tests. *p<0.05.

The role of immunity has been proposed to explain a potential negative effect of surgery. Some authors have suggested that surgery-related stress results in suppression of cellular mediated immunity, allowing metastatic development (28,29). These authors propose that measures must be taken to avoid a perioperative immune depressive state, which may lead to uncontrolled metastatic development. However, they highlight that this effect probably occurs mainly in early stages of tumor development, before the immune system is co-opted by the tumor to develop tolerance (28). In this study we used NOD-SCID-γ mice, which have severely dysfunctional innate and adaptive immunity (30) and hence have no immune barrier for tumor development. This is confirmed by the rapid development of primary and metastatic tumors in non-treated animals. However, in this experiment the effect of surgery on metastatic development was readily observable, suggesting that maximum metastatic growth in these mice depended not only on the lack of immune response but also on the presence
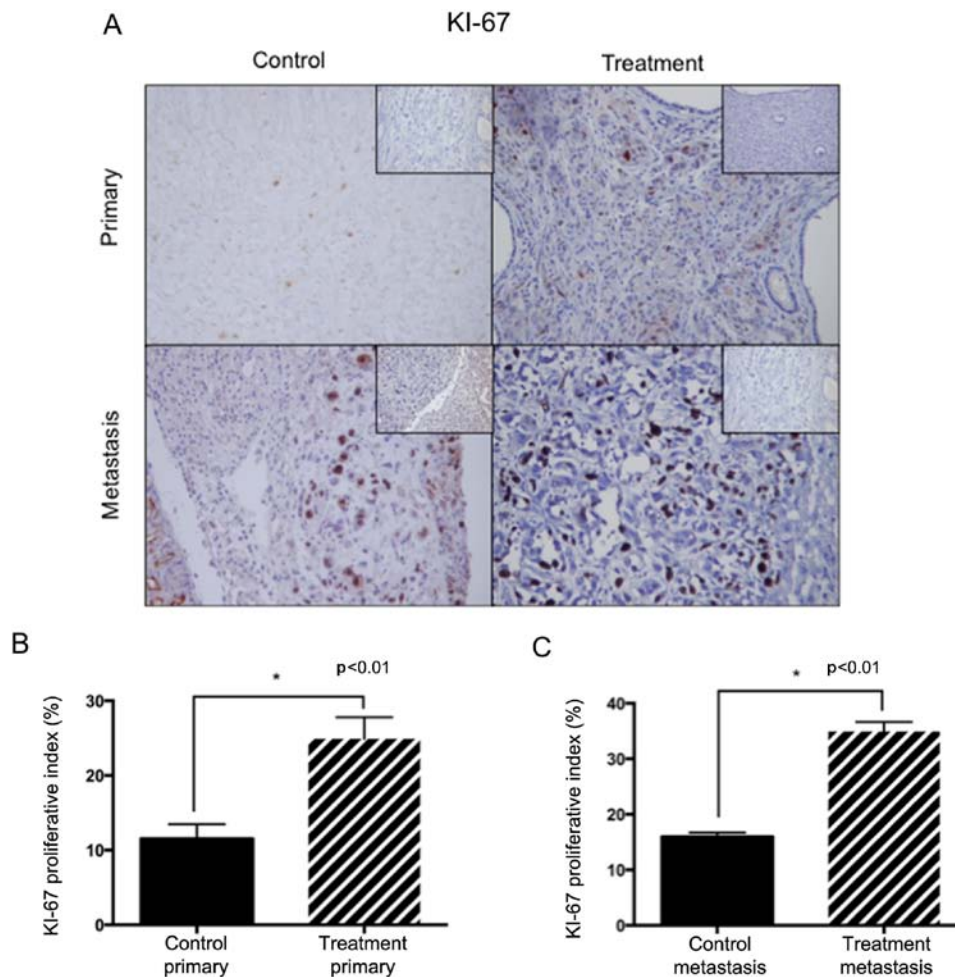
Figure 5. (A) Representative images of KI-67 labeling in primary and metastatic tumors from mice injected with PC3-LUC cells without treatment (control) and with cytoreductive surgery (CS) at day 30 (treatment). (B) The proliferative index (PI) (%) in the same animals, comparing primary tumors between groups. (C) PI in the same animals comparing metastatic tumors. Unpaired t-test. *p<0.01.

of a fully developed primary tumor. Therefore, we conclude that the effect of surgery is, at least in part, independent of the immune status.

We chose to perform CS in contrast to complete removal of the tumor as this is more realistic in the context of a PCa T3 tumor and it is similar to other cancers, notably ovarian cancer (31). Our results show an important reduction in the luminescence signal in primary tumors as a consequence of treatment. After this reduction, a slow relapse occurred. The relapsed tumors in the treatment group did not reach the size of the primary tumors of the non-treated animals during the time of the experiment. Furthermore, they did not reach the size of the tumors before surgery. Although we saw a higher PI in the relapsed tumors, this was not sufficient to overcome the overall effect of surgery. A similar tendency was observed in the metastatic tumors, where a higher PI was observed in metastases from the treated animals. Apparently this is a late effect from surgery and may translate to future tumor growth. The significant reduction in primary and metastatic tumor size and numbers and the delay of their development, suggest that this treatment may translate to a survival benefit for mPCa patients, supporting the observations made in clinical trials (32).

CS is an accepted treatment for metastatic kidney (33), breast (34) and ovarian (31) cancer. This treatment depends on anatomical presentation and feasibility of surgery. This is clear in ovarian carcinoma in which patients with suboptimal tumor debulking derive no benefit from the surgical procedure and are only exposed to its complications (31). Furthermore, in PCa patients with locally advanced disease (T3 and/or N1), treatment of the primary tumor only achieves significant results when they are at high risk to die but not in those with a slow progression of the disease (35). If CS is to be used in mPCa, pre-clinical and clinical evidence is necessary. There are several reports of retrospective studies that suggest this treatment may result in a survival benefit (6,32). Recently, a large retrospective clinical study analyzed data from PCa patients in stage IV (M1) that had definitive treatment of the prostate tumor (5). The conclusion of this study suggests that there is a survival benefit for this treatment option. However, an editorial comment about this article points out that this treatment modality is not supported by any published guidelines and that more evidence is necessary to recommend the treatment of the primary tumor in mPCa patients outside of a clinical trial (4); moreover, concludes that well-thought-out clinical trials need proper patient selection (4). In agreement with this, here we showed in a pre-clinical model, that CS of the primary tumor alone can reduce metastatic burden and may translate to a survival benefit for cancer patients. Current

modalities for treatment of mPCa patients include mainly androgen blockage and, when resistance develops, variable application of chemotherapy and radiotherapy (3). Surgery of the primary tumor may be a useful addition to these sequential approaches with the main goal of prolonging survival. Here we show evidence that may promote the design of prospective studies that address this subject.

Acknowledgements

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