Original Research

Human periprostatic white adipose tissue is rich in stromal progenitor cells and a potential source of prostate tumor stroma

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Abstract

A body of growing evidence now implicates white adipose tissue as a relevant source of stromal progenitor cells recruited to the tumor microenvironment to form supportive tumor stroma. While the role of periprostatic (PP) adipose tissue in prostate cancer progression has been barely appreciated, we sought to determine the progenitor cell population in PP adipose tissue and the association with prostate cancer. We isolated and characterized $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ progenitor cells (adipose-derived stem cells [ASC]) in paired samples of PP and preperitoneal visceral adipose tissue from prostate tissue and peripheral blood mononuclear cells of prostate cancer and nodular prostatic hyperplasia patients. ASC were quantified by flow cytometry and confirmed through target gene expression. Here we show a significantly higher amount of ASC in PP than in visceral adipose tissue, independent of body mass index and prostatic disease. In the prostate, ASC are increased in cancer compared with prostatic nodular hyperplasia patients. Concordantly, adipsin gene (*CFD*) expression, which is known to be up-regulated in adipose stem cells, was overexpressed in PP adipose tissue, in the prostate of cancer patients and in prostate $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ sorted cells. ASC were found at higher levels in the blood of prostate cancer patients simultaneously overweight/obese. Present findings indicate that PP adipose tissue is a reservoir of progenitor cells with the potential to migrate towards prostate tumors, although its clinical significance merits further evaluation.

Keywords: adipose tissue, obesity, prostate cancer, progenitor cell, tumor microenvironment

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Introduction

Adipose tissue enveloping the prostate has been the focus of recent research in the context of obesity and prostate cancer. Previous work showed that periprostatic (PP) adipose tissue has the potential to modulate prostate cancer aggressiveness through effects in tumor cell metastatic behavior, both directly and in response to tumor stimulation.[1–3] Moreover, recent clinical reports evidenced an association between PP adipose tissue thickness and prostate cancer severity.[4,5]

Accumulating evidence over the last few years demonstrated that interactions between non-tumor cells in the

Table 1 Clinicopathological characteristics of participants

	BPH	PCa	*P* value
Age (years)	68.0 ± 3.1	58.7 ± 2.6	0.045
PSA at diagnosis (ng/mL)	5.7 ± 1.4	5.7 ± 0.8	0.977
Body mass index (kg/m^2)	28.7 ± 1.3	27.3 ± 1.4	0.498
Prostate weight (g)*	127.5 (120–135)	42.3 (34–55)	0.044
Clinical stage†			
Localized (T1–T2)	–	83.3%	
Advanced (T3–T4)	–	16.7%	
Gleason score†			
≤7 (3+4)	–	33.3%	
≥7 (4+3)	–	66.7%	

Data presented as mean ± standard error of mean; differences among disease groups were calculated using the *t*-test

*Data represents median (min–max), and differences were tested with the Mann–Whitney test

†Data presented as percentage of cases

BPH, benign nodular hyperplasia patients; PCa, prostate cancer patients; PSA, prostate-specific antigen

microenvironment and tumor cells influence their phenotypic behavior.[6,7] In demand for tumor microenvironment origins, it was found that most tumor vascular and fibrovascular stroma originates from neighboring adipose tissue,[8] as during rapid tumor development, local cells may not be capable to meet the expanding growth needs. In fact, white adipose tissue is a rich reservoir of $CD34^+/CD45^-$ cells containing above 200-fold more progenitor cells/mL than bone marrow.[9] These adipose tissue-derived stem cells have been described as $CD31^-CD34^+CD45^-CD146^-$ (adipose-derived stem cells [ASC]),[10] with a high proliferative potential and the ability for migration and capillary formation.[11] Their activity and amounts are modulated by the degree of adiposity and microenvironment (adipokines, hypoxia and oxidative stress).[12,13]

Several studies strongly support that ASC home to tumor sites and promote tumor growth, either directly or after differentiation to supportive stroma cells (e.g. endothelial cells and myofibroblasts).[14–18] Furthermore, data specifically from murine prostate cancer models demonstrated that the presence of ASC on prostate tumors was mediated by stromal-derived factor-1/C-X-C chemokine receptor type 4 axis.[15,16,19,20]

Taken together, these findings suggest a role for ASC in prostate cancer pathophysiology, albeit no studies in humans have reported the eventual depots of origin or their deposition in the prostate. Thus, we sought to investigate human PP and visceral adipose tissue depots as reservoirs of ASC and if they could be found in the prostate of cancer or benign nodular hyperplasia (BPH) patients.

Materials and methods

Patients and sample collection

Men eligible for retropubic radical prostatectomy ($n = 6$) or prostate surgery of BPH ($n = 6$), without other major co-morbidities, were included in this study after informed consent agreement. Clinicopathological characteristics are described in Table 1. This study was approved by the ethics committees of D Pedro V Military Hospital and Porto Hospital Centre.

Samples of PP (anterolateral fat pad) and visceral adipose tissue (preperitoneal visceral fat from the lower abdominal incision), and prostate tissue were collected during surgery. Adipose tissue was washed in phosphate-buffered saline (PBS) (Gibco, Invitrogen, Barcelona, Spain) and divided into two pieces, one immerged in RNAlater (Ambion, Applied Biosystems, Carlsbad, CA, USA); the other maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 medium (Gibco). Immediately after prostate/nodular hyperplasia removal, a fragment of prostate from tumor-bearing regions or from nodular hyperplasia was collected and maintained in prewarmed RPMI 1640 (Gibco) media supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin and streptomycin (Gibco). Mirror sections of the prostate fragments were stained with hematoxylin–eosin and analyzed by uropathologists to ensure the presence or absence of malignancy. Peripheral blood was collected before anesthetic induction.

Adipose tissue samples and prostate fragments were weighted, minced and incubated with collagenase A (2 and 3 mg/mL, respectively) (Roche Applied Science, Mannheim, Germany) at 30°C with shaking (120 rpm) (1 and 2 hours, respectively). Pellets were re-suspended in supplemented (with biotin 16 μmol/L, panthotenate 18 μmol/L and ascorbate 100 μmol/L, all from Sigma-Aldrich, St Louis, MO, USA) DMEM/F-12 medium with 10% newborn calf serum (NCS) (Sigma-Aldrich) (adipose tissue) or RPMI 1640 with L-glutamine and HEPES (25 mmol/L) (Gibco) with 10% FBS (prostate) and filtered through 100 μm followed by 40 μm mesh size cell strainers (BD Falcon, BD Biosciences, Franklin Lakes, NJ, USA). Cells were cryopreserved in the respective media with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

Mononuclear cells from peripheral blood were isolated through a density gradient centrifugation (Histopaque 1083; Sigma-Aldrich). Cells were cryopreserved in RPMI 1640 supplemented with L-glutamine and HEPES (25 mmol/L) with 10% DMSO.

Flow cytometry analysis and cell sorting

The following directly conjugated mouse antihuman antibodies were used (all from Biolegend, San Diego, CA, USA): fluorescein isothiocyanate anti-CD31 (clone WM59), allophycocyanin anti-CD34 (clone 561), phycoerythrin anti-CD146 (clone SHM-57) and peridinin chlorophyll anti-CD45 (clone HI30). In short, cryopreserved cell suspension samples of adipose tissues and prostate were rapidly thawed and washed in PBS with 2% FBS (PBS-FBS). Approximately 2×10^6 adipose and cell suspensions were stained with a cocktail of previously optimized concentrations of antibodies for 30 min at 4°C. For each sample, isotype matched controls were analyzed to acquire the appropriate settings. Data were collected in a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) (one million gated events) and analyzed by FlowJo software (Ashland, OR, USA). Figure 1 depicts the identification criteria used for selecting the cellular populations of interest.

Live prostatic cells identified as $CD31^-CD34^+CD45^-CD146^-$ were purified by cell sorting in a BD FACSAria

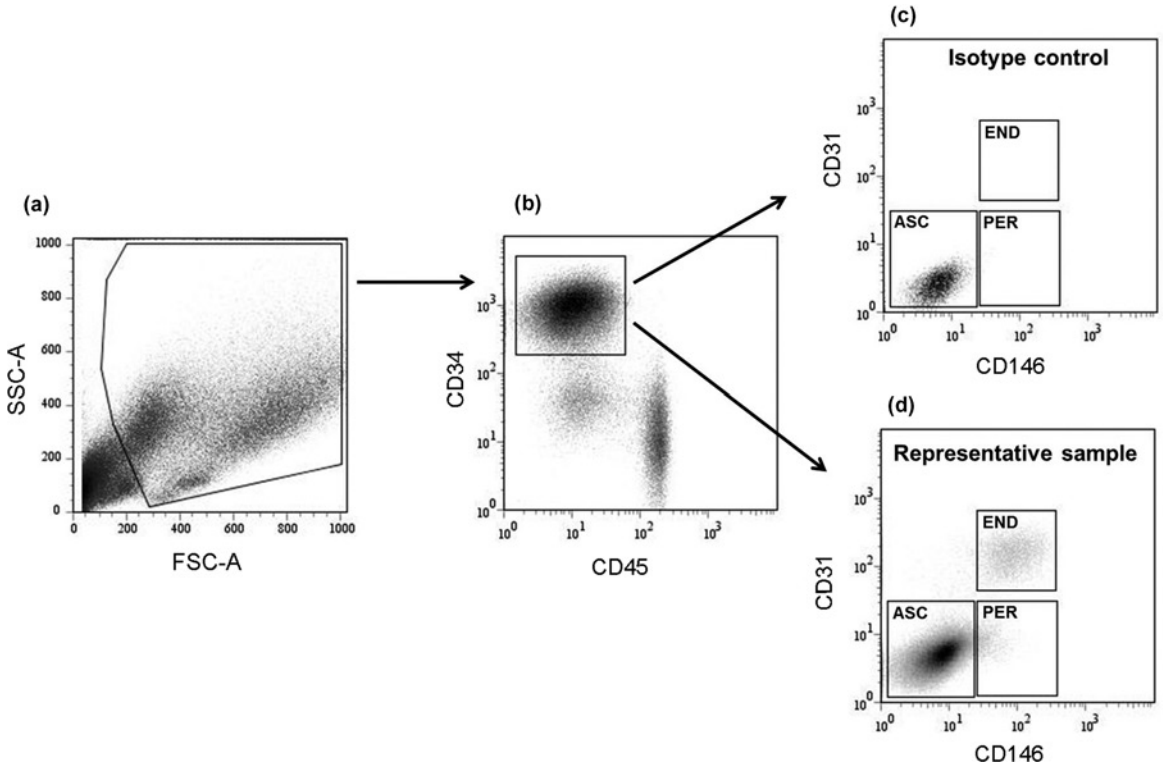

Figure 1 Flow cytometry quantification of adipose-derived stem cells. The gating strategy is represented: debris, dead cells, platelets and granulocytes were excluded in the first gating step (a). Subsequently, hematopoietic cells co-expressing CD45 antigens were excluded (b). Quantification of adipose tissue-derived mesenchymal stem cells (ASC; $CD31^-CD34^+CD45^-CD146^-$), while identifying endothelial precursor and mature cells (END; $CD45^-CD34^+CD31^+CD146^+$) and pericytes (PER; $CD45^-CD34^+CD31^-CD146^+$) was performed based on the surface expression of CD31 and CD146. An isotype control was used in all samples for gating (c). A representative example of visceral adipose tissue from a benign nodular hyperplasia patient is shown (d)

cytometer (BD Biosciences) ($n = 6$). Briefly, 2×10^6 cells were labeled with antibodies and 1 μg/mL of propidium iodide. Cell sorting was performed directly into RNeasy Micro Kit (Qiagen, Valencia, CA, USA) cell lysing solution and kept at -80°C.

RNA extraction and realtime polymerase chain reaction

Fragments of adipose tissue were homogenized in QIAzol Reagent (Qiagen), and samples purified with the RNeasy Lipid Tissue Mini Kit (Qiagen). RNA was extracted from an aliquot of prostate cells in TriPure reagent (Roche Applied Science) and purified with the RNeasy Mini Kit (Qiagen). Sorted cells were purified with the RNeasy Micro Kit. All RNA samples were treated with DNase I (Qiagen).

The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality inspected using Experion RNA StdSens Chips in the ExperionTM automated microfluidic electrophoresis system (Bio-Rad, Hercules, CA, USA). First-strand cDNA was synthesized with 1 μg of total RNA from adipose tissues, 0.25 μg from prostate cell suspensions and 0.10 μg from sorted prostate cells using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). The transcript levels of adipsin (*CFD)* and *CD133* were quantified in duplicate by quantitative realtime polymerase chain reaction (PCR) using SYBR Green in an iCycler iQ5 Real-Time PCR (Bio-Rad). The expression of *PCA3* gene was evaluated using the Taqman assay (Applied Biosystems), in a StepOne Real-Time PCR (Applied Biosystems). Results were normalized, for adipose tissues and prostate, to the levels of the ribosomal *18S* rRNA and *28S* rRNA, respectively; whereas for *PCA3* we used *18S* and *ACTNB* as reference genes. Relative quantification was calculated using the REST 2009 software (Qiagen, Hilden, Germany), where relative mRNA expression was expressed as fold over the reference group.

Statistical analysis

Data are presented as mean $\pm$ standard error of mean, except for non-parametric variables where median (min–max) was used. Cell populations evaluated by flow cytometry are expressed as percent of CD45-/CD34+ cells adjusted by gram of tissue or only the percent of cells in blood samples analyses. Departure from normality was tested using Kolmogorov–Smirnov test. Values for percent of ASC per gram were $\log_{10}$ transformed to become normally distributed. Unpaired *t*-test was used for statistical comparison between means. The patients were re-assembled into one group bearing prostate cancer and

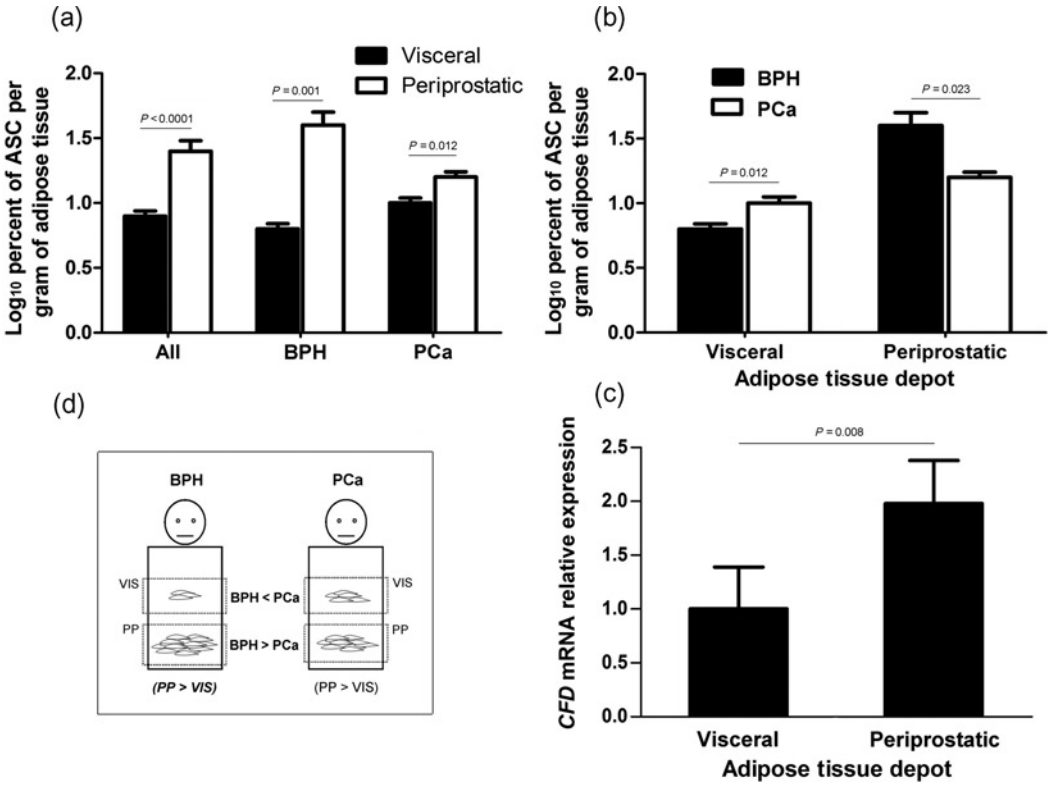

Figure 2 Adipose-derived stem cell (ASC) quantity and *CFD* gene expression in periprostatic and visceral adipose tissue. (a) Comparison of ASC quantity per gram of tissue between visceral and periprostatic fat depots, overall and for BPH and prostate cancer groups, using the Student *t*-test. (b) Differences in the $\log_{10}$ percent of ASC per gram of adipose tissue between BPH and prostate cancer patients within each fat depot, by the Student *t*-test. (c) Comparison of adipsin (*CFD*) gene expression between periprostatic and visceral depots (expression ratio results were tested for significance by a randomization test, with 10,000 iterations). (d) Scheme depicting the relative quantities of ASC among adipose depots and according to prostatic disease. Bars represent mean ± standard error of mean from 12 men (BPH, $n = 6$ and prostate cancer, $n = 6$). PCa, prostate cancer; BPH, benign nodular hyperplasia; VIS, visceral; PP, periprostatic

simultaneously body mass index (BMI) $\geq 25\ kg/m^2$ (obese/overweight, OB/OW), and another including all other subjects (without cancer and BMI $< 25\ kg/m^2$ [NP], with cancer and NP, and without cancer and OB/OW). Spearman's correlation coefficients were determined to assess the statistical dependence between gene expression data (Cq of target gene normalized to reference gene) and the logarithmically transformed percent of ASC per gram of tissue.

The REST 2009 software (Qiagen) was used to determine gene expression ratios. We used 10,000 iterations to evaluate if significant differences existed between comparison groups. Analyzes were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered significant.

Results

When comparing the amount of ASC between distinct anatomic origins, we found it was significantly increased in PP versus visceral adipose depot ($P < 0.0001$), even after stratified analyses by prostatic disease (BPH, $P = 0.001$; prostate cancer, $P = 0.012$) (Figure 2a). Interestingly, in PP adipose tissue, the quantity of ASC was significantly lower for prostate cancer compared with BPH patients ($P = 0.023$), whereas the inverse was observed in visceral adipose tissue ($P = 0.012$) (Figure 2b). To strengthen these findings, we evaluated adipsin (*CFD*) mRNA transcript abundance in adipose tissue, whose expression was shown to be highly up-regulated in ASC. In agreement with Figure 2a, *CFD* was overexpressed by two-fold in PP compared with visceral adipose tissue ($P = 0.008$) (Figure 2c). Figure 2d schematically represents the relative ASC frequencies found in adipose depots, according to prostatic disease.

In the prostate, the amount of ASC was increased in cancers compared with BPH ($P = 0.043$) (Figure 3a). Furthermore, *CFD* expression was 10-fold increased in prostate tumors from cancer patients ($P = 0.186$), whereas in isolated ASC (sorted cells) from the prostate, it was also increased in tumors compared with prostatic tissue of BPH ($P = 0.192$) (Figure 3b). Moreover, the normalized *CFD* expression was well correlated with the amount of ASC cells in the prostatic tissue (Spearman coefficient, $r = -0.71$, $P = 0.047$). CD133 is a specific stem and progenitor cell marker for a variety of cells, albeit it is not expressed in ASC. Interestingly, *CD133* expression was up-regulated in prostatic tumors of cancer patients by 3.6-fold ($P = 0.025$) (Figure 3c), but not in isolated ASC (sorted cells) (Figure 3d).

In the peripheral blood, although the ASC cell population was barely detectable, we found a trend for increased ASC-like cells in cancer compared with BPH patients (0.05 ± 0.01 and 0.03 ± 0.005, respectively, $P = 0.068$).

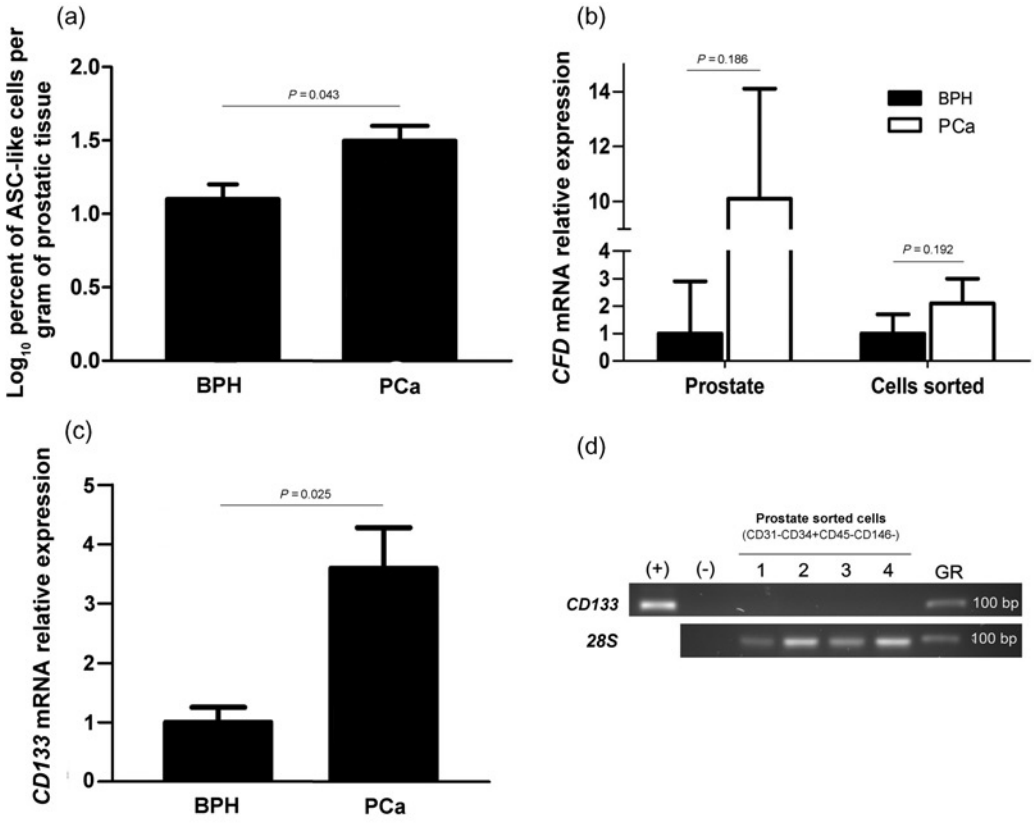

Figure 3 Quantification of adipose-derived stem cells (ASC) in prostatic tissue and gene expression in prostate tissue and $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ sorted cells. (a) Log_{10}-transformed amount of ASC per gram of prostatic tissue in BPH and prostate cancer patients (comparisons between groups with Student's *t*-test). (b) Adipsin gene (*CFD*) expression in prostatic tissue and $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ cells sorted from prostate samples, according to prostatic disease (BPH, $P = 0.186$ and prostate cancer, $P = 0.192$). (c) Comparison of *CD133* gene expression in prostatic tissue between BPH and prostate cancers. Gene expression ratios were tested for significance by a randomization test with 10,000 iterations. Bars represent mean ± standard error of mean from 12 men (BPH, $n = 6$ and prostate cancer, $n = 6$), except for sorted samples (3 BPH and 3 PCa). (d) Representative gel electrophoresis of target *CD133* and reference *28S* quantitative realtime polymerase chain reaction products from $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ cells sorted from prostate cell suspensions (HBP, 1–2 and prostate tumor, 3–4). (+), positive control, using RNA from prostate tumor sample; (−), negative control; GR, gene ruler; PCa, prostate cancer; BPH, benign nodular hyperplasia

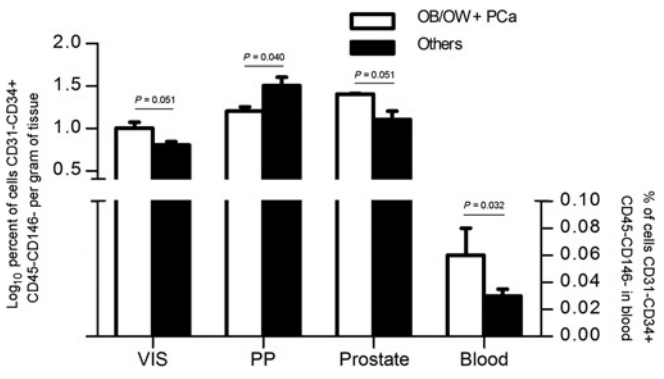

Figure 4 $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ progenitor cells in adipose tissue, prostate and peripheral blood and prostate cancer-obesity phenotype. The *t*-test was used in all comparisons. OB/OW+PCa includes obese/overweight (OB/OW) patients with prostate cancer (PCa) ($n = 4$); Others includes OB/OW ($n = 5$) and lean controls ($n = 1$), and lean patients with cancer ($n = 2$). PP, periprostatic; VIS, visceral

We evaluated *PCA3* mRNA transcript levels, since it provides information about the presence of prostate tumor cells, and found that tumor samples used for ASC quantification from prostate cancer patients overexpressed *PCA3* (659-fold, $P = 0.011$), thus confirming that tumor samples are representative of malignancy areas.

In order to analyze the simultaneous influence of being overweight/obesity and bearing cancer in ASC frequencies, we compared subjects with these characteristics (OB/OW+ PCa) to the other patients combined into one group (Others) (Figure 4). Subjects that were OB/OW and had prostate cancer presented increased quantities of ASC in peripheral blood ($P = 0.032$) and a statistical trend in visceral adipose tissue ($P = 0.051$) and prostatic tissue ($P = 0.051$). Noteworthy, the amount of ASC in PP adipose tissue was decreased in OB/OW with cancer ($P = 0.040$).

Discussion

White adipose tissue is starting to receive further attention, mainly because of accumulating evidence over the years that it is a highly active endocrine organ that secretes numerous factors, results in inflammation and modulates insulin signaling and lipid deregulation.[21,22] Recently, adipose tissue was found to also contain progenitor $CD31^{-}CD34^{+}CD45^{-}CD146^{-}$ cells, which are highly

represented in adipose tissue stromal vascular fraction (SVF), and display multipotency and proliferative capacity.[10,23,24] One recently published study demonstrated that the neighboring adipose tissue considerably contributes with ASC to tumor vascular and fibrovascular stroma,[8] while another study found that different fat depots possess unique capacities to promote tumor growth.[25] Hence, we sought to investigate the accumulation of ASC in human PP adipose tissue, and to evaluate if these cells were found in the prostate of cancer or BPH patients.

In prostate cancer, the extension beyond the organ into PP fat has been associated with worst prognosis.[26] This anatomic depot of adipose tissue, which lies in close proximity to the prostate, induces tumor cell aggressive behavior and is modulated by prostate tumor cell-derived factors,[1–3] therefore resulting in interaction towards cancer progression. Furthermore, clinical studies revealed increased PP thickness in subjects with prostate cancer and advanced disease.[4,5] In the present study, we found increased amounts of ASC in PP adipose tissue compared with another fat depot (visceral), regardless of prostatic disease. This finding suggests unique progenitor cell population amounts residing in PP adipose tissue and differences between anatomic adipose depots, which has been previously appreciated for other anatomic origins.[25,27] We hypothesize that the hypoxia, inflammation and oxidative stress conditions arising over the course of prostatic gland cancerous or hyperplasic development,[28,29] may induce recruitment of ASC to the involving PP adipose tissue from blood and eventually other fat depots or stimulate their local proliferation.

In the prostatic tissue, we found higher amounts of $CD31^-CD34^+CD45^-CD146^-$ progenitor cells in tumors, whereas a trend for increased numbers in circulation was observed for cancer patients compared with BPH subjects. Our findings in the human prostate are concordant with previous results in murine models, which demonstrated that ASC from adipose tissue home to tumors, through the modulation of the stromal-derived factor-1/C-X-C chemokine receptor type 4 axis.[15,16,30] This axis was shown to be highly activated both in prostate cancer and benign hyperplasia,[31,32] driving the recruitment of ASC.

The most accessible option for tumors that necessitate overcoming growth demands is to exploit resources in close proximity to the site of tumor development.[8] In the case of prostate cancer, the enveloping PP adipose tissue may be such a reservoir of ASC. However, local recruitment of these cells towards the tumor may result, as we observed, in a reduced amount of ASC in the PP fat depot compared with BPH. Whether ASC migrate from PP adipose tissue into the prostate through the visceral pelvis fascia, which is conceivable in light of their previously described capacity to move and ability to produce matrix proteinases[33] or through the blood, is an interesting issue that remains to be established. Further studies detailing the characterization of the progenitor cell population from each fat depot (using additional markers, e.g. alpha smooth muscle actin, CD90),[34] may reveal the potential of differentiation (adipogenic or vascular) of these cells and correlation with migration capacity. Our findings seem to reflect an interactive migratory pattern between PP adipose tissue and prostate tumor cells. In our study, all except one of the prostate cancer men disclosed clinical and pathologically prostate-confined tumors, agreeing with the increased amount of ASC found in prostate tumors while decreased in PP adipose tissue of prostate cancer patients. Larger confirmatory studies are warranted.

As initially suggested,[23] a recent study confirmed an increased frequency of progenitor $CD34^+$ cells in the circulation of obese (BMI $\geq 30\ kg/m^2$) patients with colorectal cancer.[35] A similar analysis was conducted in our group of patients, although the low number of obese patients prompted us to use a distinct cut-off (overweight and obese subjects with BMI $\geq 25\ kg/m^2$), which has been reported before.[36,37] We found a significantly higher frequency of $CD31^-CD34^+CD45^-CD146^-$ progenitors in the circulation of OB/OW patients with prostate cancer, than in lean patients with prostate cancer and OB/OW controls. Since we also observed that ASC frequency is decreased in PP adipose tissue and increased in the prostate of OB/OW men with prostate cancer, their increase in blood may account for one possible route for ASC migration. Some reports indicate that ASC play a role in obesity and are regulated by diet and obesity.[27,38] If confirmed by further studies, the source of tumor stroma from adipose tissue depots that lie in close proximity to cancer foci, besides revealing a mechanistic link between obesity and cancer progression, may identify surgical and medical interventions to disrupt tumor-stroma interactions.

To our knowledge, no prior reports have evaluated the ASC pool in human prostate and PP adipose tissue. Nevertheless, our initial, hypothesis-generating study should be interpreted in the context of some potential limitations including lack of *in vitro* evaluation of ASC functional characteristics. Concurrently, the small size of the studied cohort of patients limited the possibility of statistical adjustments for eventual confounding factors (e.g. age, clinical stage, Gleason score) and advises cautious interpretation of findings also after disease and obesity-stratified analysis. Further work should be performed in a larger series of prostate cancer patients. In addition, the apparent heterogeneity of mesenchymal cells reflected by CD34 expression on stromal and perivascular cells depending on the organ and vasculature type also alerts for cautious interpretations. Nevertheless, CD34 is a frequently used marker to distinguish ASC from other cells in the SVF, and by adding CD31 it is possible to discriminate vascular endothelial cells.[39] In blood and prostate, this panel allowed discrimination of ASC ($CD31^-CD34^+CD45^-CD146^-$), while excluding hematopoietic mesenchymal cells ($CD45^+$). Moreover, the lack of a specific ASC marker prompted us to investigate adipsin gene (*CFD*) expression, since it is overexpressed in low-passage and fresh ASC, and in comparison with extra-fat mesenchymal stem cells.[18,40] Additionally, *CFD* gene expression was correlated with the amount of ASC in the prostate. Conversely, it is known that the *CD133* gene is expressed by progenitor cells in the prostate,[41] but not by ASC.[42] Noteworthy, we found that *CD133* expression was up-regulated in tumors compared with prostatic tissue of

BPH patients, albeit its expression was abolished in the sorted prostatic $CD31^-CD34^+CD45^-CD146^-$ progenitor cells. By demonstrating absence of *CD133* expression in the $CD31^-CD34^+CD45^-CD146^-$ cells sorted from the prostate, we showed that these cells may be ASC. Moreover, the increased expression of *CD133* in the prostate tumors is a molecular feature characteristic of tumor environment, which confirms the distinctive characteristics of tumor and non-cancer samples in our study. These findings support that the progenitor cells identified in the prostate by flow cytometry with the panel $CD31^-CD34^+CD45^-CD146^-$ may indeed correspond to the ASC population. Further studies are warranted to confirm the ideal markers to identify the ASC population in prostate tissue.

Our findings suggest that the PP fat depot may represent a source of progenitor cells to prostate tumor stroma. The present study further supports ASC migration through the blood in obese men with prostate cancer, although contiguous paths should be considered. If confirmed and extended by following studies, these findings may impact prostate cancer therapeutic interventions aimed at disrupting tumor-stroma crosstalk.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. RR, CM, RS and AC conducted the experiments; and RR wrote the manuscript. RR and CM contributed equally to this study.

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