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1 **VASA NERVORUM ANGIOGENESIS IN PROSTATE CANCER WITH PERINEURAL**  
2 **INVASION**

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22  
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24 **VASA NERVORUM ANGIOGENESIS IN PROSTATE CANCER**

25 **Keywords**

26 Angiogenesis, CD34, D2-40, FGF-2, FSHR, locally advanced prostate cancer,  
27 neoangiogenic markers, perineural invasion, podoplanin, vasa nervorum, VEGF,  
28 VEGFR-2, vascular remodeling

29 **Abstract**

30 **Background.** Perineural invasion (PNI) is generally accepted as a major route of cancer  
31 dissemination in malignancies associated with highly innervated organs. However, the  
32 effect of cancer cells on vasa nervorum remains unknown. We studied this effect in  
33 locally advanced prostate cancer, a high-risk feature associated with approximately 20 %  
34 of prostate cancer specific mortality.

35 **Methods.** We used immunohistochemistry for CD34, fibroblast growth factor-2 (FGF-2),  
36 FSHR, podoplanin, vascular endothelial growth factor (VEGF), and VEGFR-2 as well as  
37 histochemical methods to examine the vasa nervorum of nerves invaded by cancer cells  
38 in tissue samples from 85 patients.

39 **Results.** The percentage of the nerve area occupied by CD34-positive vasa nervorum  
40 endothelial cells in nerves with PNI was much higher than in nerves without PNI ( $7.3 \pm$   
41  $1.2$  versus  $1.9 \pm 0.4$ ;  $p < 0.001$  and  $5.8 \pm 0.6$  versus  $1.23 \pm 0.8$ ;  $p < 0.001$  in pT3a and pT3b  
42 prostate cancer specimens, respectively). In 19/85 of the patients the CD34-positive vasa  
43 nervorum microvessels have a thick basement membrane, similar to the vessels in  
44 diabetic microangiopathy. This subendothelial layer contains collagen fibers. Vasa  
45 nervorum endothelia and Schwann cells express FGF-2 (nuclear localization) and FSHR  
46 (plasma membrane and cytoplasmic staining). Prostate cancer cells invading nerves  
47 express VEGF, a critical cytokine in tumor angiogenesis. The vasa nervorum of prostatic  
48 nerves with PNI did not express detectable levels of VEGFR-2. No podoplanin-positive  
49 lymphatic vessels were seen in nerves.

50 **Conclusion.** In locally advanced prostate cancer, perineural invasion of cancer cells is  
51 associated with formation of new endoneurial capillaries and changes of vasa nervorum  
52 morphology.

## INTRODUCTION

Tumor metastasis is a complex and highly selective process whereby cancer cells leave the primary tumor site and disseminate to other locations<sup>1</sup> via distinct routes involving blood vessels, lymphatic vessels, coelomatic cavities, and migration of cancer cells along nerves<sup>2</sup>. The latter route is encountered frequently in prostate and other cancers (pancreas, colon, rectum, biliary tract, stomach, skin, salivary gland, head and neck)<sup>3</sup>. Migration of cancer cells in close proximity of the nerve and/or within any of the three layers of the nerve (epineurium, perineurium, and endoneurium) constitutes the perineural invasion process<sup>4</sup>. Secreted neurotrophic factors that form a concentration gradient along nerves<sup>5,6</sup> may play a pivotal role in perineural invasion<sup>7</sup>. This is the case of the glial-derived growth factor that activates RET-receptor mediated cancer cell chemotaxis, which in turn guides the directional cancer cell migration towards and along the nerves<sup>8</sup>. Nerves not only harbor cancer cells, but they appear to actively promote cancer cell penetration of the nerves<sup>9</sup> and survival, and decreased apoptosis of the invading cells<sup>10</sup>.

Recent studies have demonstrated that perineural invasion results in i) cancer-related axogenesis/neurogenesis<sup>11</sup> and ii) cancer cell proliferation and migration<sup>11,12</sup>. These biological processes are highly dependent on a local supply of oxygen and nutrients provided by blood vessels that accompany the nerves and/or penetrate into the nerves (vasa nervorum). However, basic questions about blood supply for perineural invasion zones remain unanswered. For example, it is not known if the invading cancer cells in human tumors utilize the same strategies as those well documented in animal models of prostate cancer (ex., neovascularization by sprouting angiogenesis from pre-existing endothelial cells in established vessels)<sup>13</sup>. A potential alternative is that perineural invasion develops the ability to progress independently of neovascularization. The answers to these questions should critically impact the potential of new therapeutic

82 approaches to prevent cancer cells from leaving the primary tumor site and disseminate  
83 to distant organs.

84 Antibodies for endothelial cell markers (Table 2), were used to study the effect of  
85 perineural invasion on vasa nervorum in patients diagnosed with locally advanced  
86 prostate cancer. In this type of cancer the tumor cells penetrated through the  
87 fibromuscular pseudocapsule covering the prostate gland, but did not spread to lymph  
88 nodes or to distant areas (T3-N0-M0, in the TNM staging system<sup>14</sup>. We analyzed locally  
89 advanced prostate cancer specimens because this type of cancer comprises about 5-  
90 10 % of all newly diagnosed prostate cancers and it is associated with approximately  
91 20 % of prostate cancer specific mortality<sup>15</sup>. Modifications in the morphology of vasa  
92 nervorum have been also analyzed by histochemical methods.

## 94 **MATERIALS AND METHODS**

### 95 ***Tissue specimens***

96 Paraffin sections of locally advanced prostate cancer tissue fixed in 10% neutral  
97 formalin were obtained from the biorepositories of Paris Hospitals Lariboisière (57  
98 patients) and Tenon (28 patients) (Table1). The prostate cancer tissues were included in  
99 paraffin in the period 2009-2015. Two pathologists (C.C. and J-F.C.) reviewed all cases  
100 for the present study. The pathologic stage T3 (pT3) was assigned according to the  
101 World Health Organization guidelines<sup>16</sup>. Extraprostatic extension (pT3a) was diagnosed if  
102 tumor cells were present in the periprostatic soft tissue or penetrated through a  
103 fibromuscular pseudocapsule and came out on the other side. The seminal vesicle  
104 invasion (pT3b) was defined as tumor tissue present within the fibromuscular wall of the  
105 seminal vesicles. All pelvic lymph nodes were evaluated for the presence of metastatic  
106 disease. All cases were assigned a Gleason score.

107 The protocol was approved by the institutional ethics committee at each study site.  
108 Written informed consent was obtained at the time of surgery from all living donors from  
109 whom samples were obtained.  
110

### 111 ***Immunohistochemistry***

112 Paraffin sections (5  $\mu$ m) of both pT3a and pT3b tumors were immunolabeled with  
113 antibodies directed against several endothelial markers (Table 2). Immunohistochemistry  
114 was carried out using an automated immunohistochemical stainer according to the  
115 manufacturer's guidelines (Leica Bond RX, Leica Biosystems). Antigen retrieval was  
116 conducted by treatment with high temperature at pH 6 or pH 9 (Table 2).

### 117 118 ***Image and statistical analysis***

119  
120  
121 In our study we have analyzed all the nerves (invaded or not invaded by cancer  
122 cells) present on the tissue paraffin sections (the range was 3 – 20 nerve profiles per  
123 section). The normal nerve profiles were located at least 500  $\mu$ m from the tumor border,  
124 outside the prostatic stroma. The nerves with PNI were analyzed inside and outside the  
125 prostatic stroma. We have quantitatively determined the density of FSHR-, CD34-, D2-  
126 40-positive blood vessels. This was done by counting the number of marker-positive  
127 vessels on digital images from whole images of serial sections obtained by using the  
128 Philips Digital Ultra-Fast Scanner 1.6 RA and Philips Image Management System 2.2RA,  
129 available in Curie Institute. The area occupied by CD34-positive endoneurial endothelial  
130 cells was calculated using the formula: % of endoneurial endothelial cells area =  $\Sigma$  pixels  
131 of endoneurial endothelial cells area  $\times$  100 /  $\Sigma$  pixels of the nerve area, as previously  
132 described<sup>17</sup>. The pixel density of the image was measured with the "color deconvolution  
133 function" of FIJI software in the range of 0 – 255, where 0 means presence of endothelial  
134 cells and 255 means absence. The computations were performed separately for nerve  
135 areas in which tumoral invasion was present or absent. The values presented are means

136 ± standard deviations (SD). Relationships between the vascular expression of CD34 and  
137 FSHR, and clinicopathological data were examined by Pearson's correlation coefficient.  
138 The statistical significance was evaluated using the 2-tailed t-test.

## 140 RESULTS

### 141 *Effect of cancer invasion of nerves on vasa nervorum*

142 In order to obtain reliable results, we had to analyze the effect of PNI on vasa  
143 nervorum in nerve-reach prostate areas. Published data indicate that the innervation of  
144 prostate is richest in the “capsular” regions, with density of innervation increasing towards  
145 the seminal vesicles<sup>18</sup>. These areas correspond to the pathological T3a and T3b prostate  
146 tumors<sup>16</sup>, which we have analyzed in the present study. We noticed the presence of  
147 prostate cancer cells along these nerves (Fig. 1A). In the majority of the cases PNI was  
148 detected before metastasis, which is the result of dissemination through lymphatic or  
149 blood vessels.

150 The presence of cancer cells within any of the three layers of the nerve (i.e.,  
151 epineurium, perineurium, and endoneurium) may cause compression and destabilization  
152 of vasa nervorum, which should lead to reduced perfusion and hypoxia. Hypoxia, in turn,  
153 is known to induce secretion of angiogenic factors involved in prostate cancer  
154 progression and metastasis<sup>19</sup>. Because the formation of new endoneurial capillaries *in*  
155 *vitro* depends on the presence of angiogenic factors<sup>20</sup>, we have analyzed by  
156 immunohistochemistry the expression of two potent angiogenic factors: VEGF and FGF-  
157 2. VEGF was present in the cytoplasm of most cancer cells, including those surrounding  
158 the nerves (Fig. 1B). FGF-2 was observed in the nuclei of vasa nervorum and Schwann  
159 cells, both in nerves that have or do not have perineural invasion (Fig. 1C). A strong  
160 FGF-2 staining was also noticed in both tumor and peritumoral endothelial cells (not  
161 illustrated). These results suggest that VEGF and FGF-2, via their endothelial receptors,

162 may activate pathways leading to endothelial cell proliferation and new vasa nervorum  
163 formation.

164         Angiogenesis of vasa nervorum induced by PNI is expected to lead to increased  
165 number of endothelial cells per mm<sup>2</sup> of nerves. To find out whether this is the case, we  
166 determined the percentage of nerve area occupied by endothelial cells, as detected by  
167 the endothelial marker CD34<sup>21</sup>. The pictures in Figs. 2A and 2B are representative for the  
168 distribution of CD34-positive vasa nervorum of nerves without or without PNI,  
169 respectively. A similar pattern was noticed for two other neurotropic cancers, pancreatic  
170 cancer and colon cancer (not illustrated).

171         In the pT3a prostate cancer specimens, the percentage of the area occupied by  
172 CD34-positive endothelial cells associated to the nerves with PNI was much higher than  
173 that in nerves without PNI ( $7.3 \pm 1.2\%$  *versus*  $1.9 \pm 0.4\%$ ;  $p < 0.001$ ) (Fig. 2C). In the  
174 pT3b prostate cancer specimens, the percentage of the area occupied by CD34-positive  
175 endothelial cells associated to the nerves with PNI was also much higher than that in  
176 nerves without PNI ( $5.8 \pm 0.6$  *versus*  $1.23 \pm 0.8$ ;  $p < 0.001$ ). No significant difference was  
177 found between pT3a and pT3b cases ( $p = 0.59$ ). The percentage of the nerve area  
178 occupied by CD34-positive endothelial cells in the nerves with PNI correlated with the  
179 clinical stage ( $r = 0.62$ ;  $p = 0.008$ ) and with the tumor size ( $r = 0.62$ ;  $p = 0.01$ ).

180         Although it is generally thought that cancer is associated with increased  
181 microvessel density in a variety of human cancers, this view is controversial for prostate  
182 cancer<sup>22</sup>. We have determined the CD34-positive vessel density in the prostate in regions  
183 of cancer and in the benign peritumoral area. Our results showed that tissue sections  
184 without cancer were significantly more vascularized than were tumors ( $3.28 \pm 0.5 \%$   
185 *versus*  $2.01 \pm 0.32\%$ ;  $p = 0.03$ ).

186         All these results indicate that an angiogenic process occurred for vasa nervorum  
187 of cancer invaded nerves.

188 In 19/85 (22%) of patients with pT3 tumors, the vasa nervorum in nerves with  
189 perineural invasion had a thick subendothelial layer containing collagen fibers (Fig. 3A),  
190 in comparison with non-invaded vasa nervorum) (Fig. 3B). Standard histochemical  
191 staining (hematoxylin-eosin-saffron staining, periodic acid-Schiff reaction and Masson's  
192 trichrome technique) indicated that in 19/85 (22%) of patients with pT3 tumors, the vasa  
193 nervorum in nerves with perineural invasion had a thick subendothelial layer containing  
194 collagen fibers, characterized by the absence of pericytes (Fig. 3).

195 The vasa nervorum of prostatic nerves with PNI did not express detectable levels  
196 of VEGFR-2. No podoplanin-positive lymphatic vessels were seen in nerves.

197 Work from our laboratory showed that FSHR is expressed by the tumor  
198 endothelial cells, most frequently at the periphery of prostate cancer tissue<sup>23</sup>. However,  
199 no information was available for the vascular FSHR expression in zones of perineural  
200 invasion in locally advanced prostate cancer. The density of FSHR-positive vasa  
201 nervorum (number of vessels/mm<sup>2</sup> of nerve tissue) of nerves with perineural invasion was  
202 not significantly different from that of nerves without perineural invasion ( $47.8 \pm 12.5$   
203 *versus*  $46.8 \pm 10.4$ ;  $p = 0.9$ ). The density of FSHR-positive vasa nervorum correlated with  
204 both the final Gleason score ( $r = 0.48$ ;  $p = 0.02$ ) and tumor size ( $r = 0.69$ ;  $p = 0.003$ ). We  
205 noticed also intense FSHR staining of Schwann cells in all nerve ganglia and nerve fibers  
206 in peripheral areas affected by perineural invasion (Fig. 3D). No immunohistochemical  
207 signal was obtained by using an irrelevant mouse antibody of the same IgG2a subtype as  
208 FSHRA02 (not illustrated). In benign peritumoral tissues of cancer patients the nerves  
209 show also intense FSHR staining of Schwann cells and vasa nervorum at all analyzed  
210 Gleason scores (5-9). On a radical prostatectomy specimen obtained for a non tumor  
211 related disease, the majority of nerves and their associated vessels in the peripheral  
212 areas of the prostate do not express FSHR (not illustrated). The absence of staining in  
213 nerves and blood vessels was also noticed in a nerve ganglion in the peripheral zone.

214 The above results suggest that perineural invasion by prostate cancer cells could  
215 have important effects on the nerve microvessels by inducing angiogenesis and vascular  
216 remodeling.

## 218 **DISCUSSION**

219 Our study indicates that an angiogenic process occurs at the level of vasa  
220 nervorum of cancer invaded nerves, in comparison with the nerves that are not invaded.  
221 The increased blood supply is expected to facilitate further growth and invasion of tumor  
222 cells along the nerves, and progression of the disease.

223 The density of blood vessels in the cancer invaded nerves is higher than in the  
224 non-invaded nerves. This indicates that the angiogenic process in the cancer invaded  
225 nerves is caused by the proximity of both nerve and tumor cells. Angiogenesis could be  
226 induced by angiogenic factors released by the tumor cells, and the release can be  
227 induced by the adjacent nerves, or vice-versa. Angiogenesis was shown to occur in  
228 nerve cell grafts and at spinal cord lesions during peripheral nerve regeneration, and can  
229 be triggered by physiological tasks that increase neural function and synaptic activity<sup>24,25</sup>.  
230 It is possible that such increased neural function is induced by the tumor cells that invade  
231 the nerves.

232 In mouse models of prostate cancer, noradrenaline generated by nerves induces  
233 angiogenesis by activating adrenergic receptors on the endothelial cells, which in turn  
234 alter the endothelial cell metabolism<sup>26</sup>. Therapies attempting to starve the tumor by  
235 inhibiting angiogenesis had limited long term therapeutic benefit in various cancers,  
236 including that of the prostate<sup>27</sup>, most likely due to resistance mechanisms<sup>28</sup>. Co-targeting  
237 angiogenesis, neural signals and/or endothelial cell metabolism may provide a  
238 multipronged therapeutic approach to inhibit cancer invasion via the nerves<sup>26</sup>.

239 Analysis of the morphology of blood vessels in nerves with perineural invasion  
240 indicated that in 22% of patients with locally advanced prostate cancer the vasa

241 nervorum had a thick collagenous subendothelial layer in comparison with normal vasa  
242 nervorum. Another characteristic of these microvessels is the absence of pericytes  
243 associated with the capillaries and postcapillary venules. Pericytes are distinctive  
244 regulators of angiogenesis and are thought to provide vessel stability and control of  
245 endothelial proliferation<sup>29</sup>. The modified walls of these vessels could constitute an  
246 adaptation to ensure constant blood flow. The walls of normal vessels constantly adjust  
247 the vessel diameter to adapt to the metabolic needs of the tissue. The thick walls and the  
248 absence of contractile cells indicate that this process does not occur in the modified  
249 vessels, ensuring a constant, possibly maximal blood flow.

250 Thickening of the capillary basement membrane is a hallmark of diabetic  
251 microangiopathy, and may lead to occlusive angiopathy and to tissue hypoxia and  
252 damage<sup>30</sup>.

253 The vasa nervorum of prostatic nerves with perineural invasion do not express  
254 detectable levels of VEGFR-2. This observation indicates that the angiogenic process  
255 induced by perineural invasion could not involve VEGF/VEGFR-2 /signaling.

256 The data were obtained from locally advanced prostate cancer, which is  
257 associated with approximately 20% of prostate cancer specific mortality. Most likely the  
258 data have a more general significance, based on the fact that migration of cancer cells  
259 along the nerves is encountered in other cancers in highly enervated organs (pancreas,  
260 colon, rectum, biliary tract, stomach, skin, salivary gland, head and neck). This study  
261 should stimulate investigations in these other cancer types.

## 262

## 263 **CONCLUSION**

264 Perineural invasion of cancer cells is associated with angiogenesis and vascular  
265 remodeling of vasa nervorum in locally advanced prostate cancer.

## 266

## 267 **ACKNOWLEDGMENT**

268 Preliminary portions of this work have been presented elsewhere<sup>31</sup>.

269  
270 **CONFLICT OF INTEREST**

271 None

272  
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275  
276 ***Author Contributions:***

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279 *Analysis and interpretation of data:* Robin, Leclere, Nicolas, Pichon, Chnecker, Côté,  
280 Guillonneau, Radu, Ghinea

281 *Drafting of the manuscript:* Ghinea, Radu

282 *Critical revision of the manuscript for important intellectual content:* Robin, Leclere,  
283 Nicolas, Pichon, Chnecker, Côté, Guillonneau, Radu, Ghinea

284 *Statistical analysis:* Robin, Ghinea

285 *Supervision:* Ghinea, Guillonneau

286 *Other:* None

287  
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## 367 **FIGURE LEGENDS**

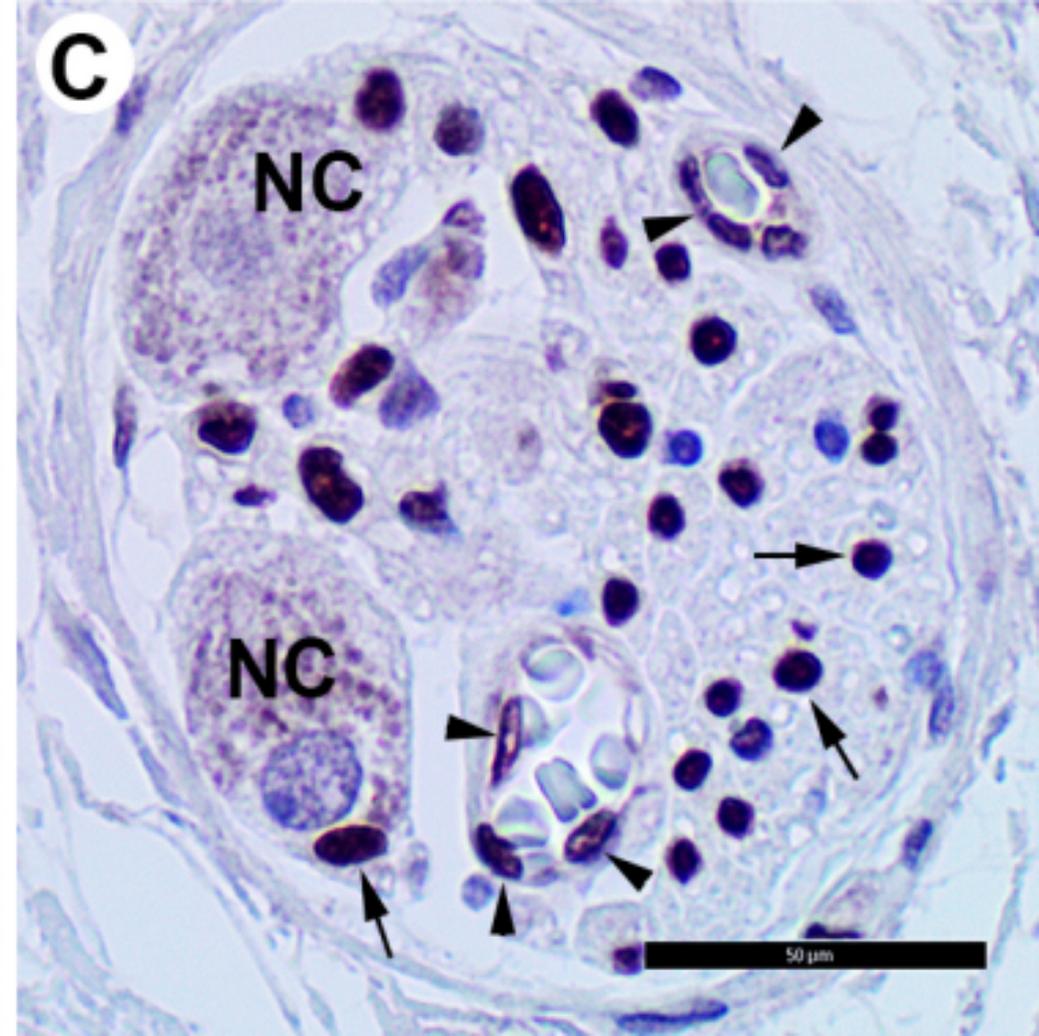
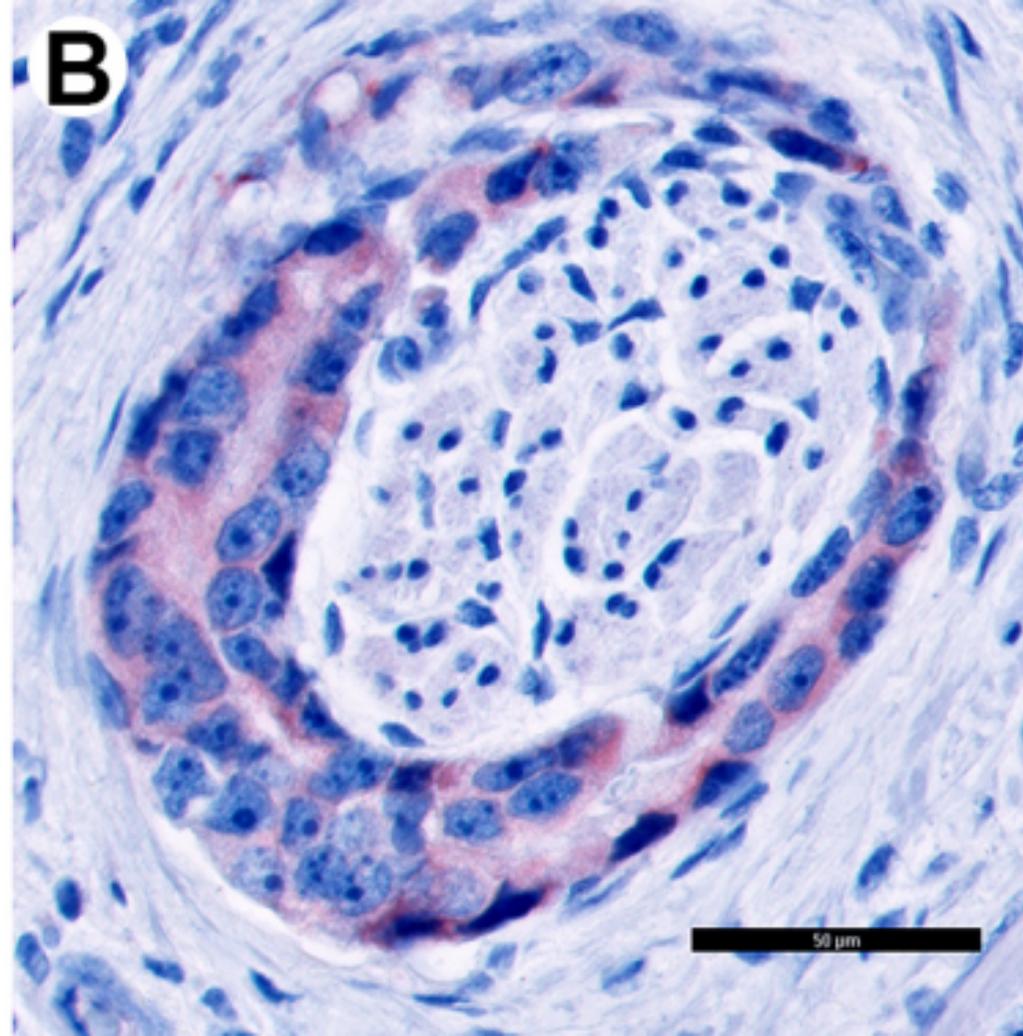
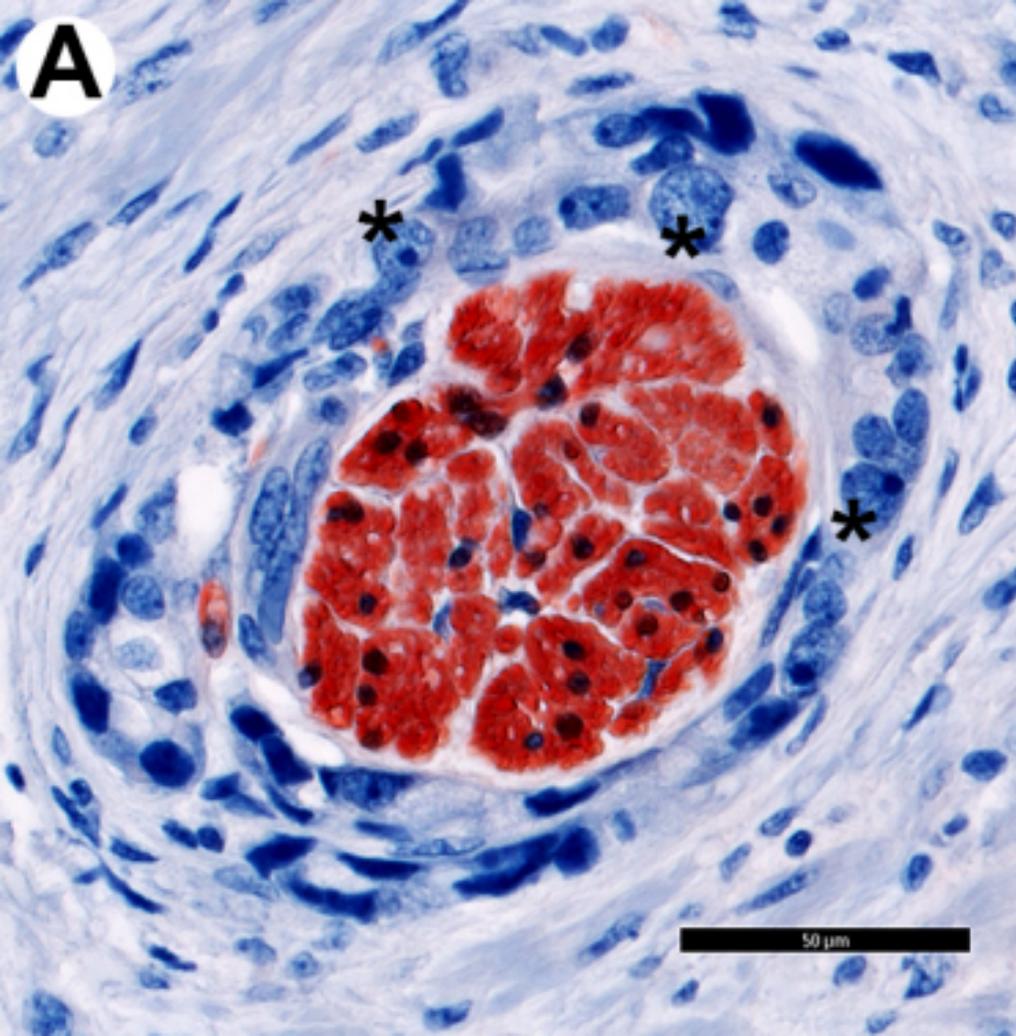
368 **FIGURE 1. Prostate cancer cells that left the primary tumor site are visible along**  
369 **nerves.** Immunohistochemical analysis was performed on paraffin-embedded sections of

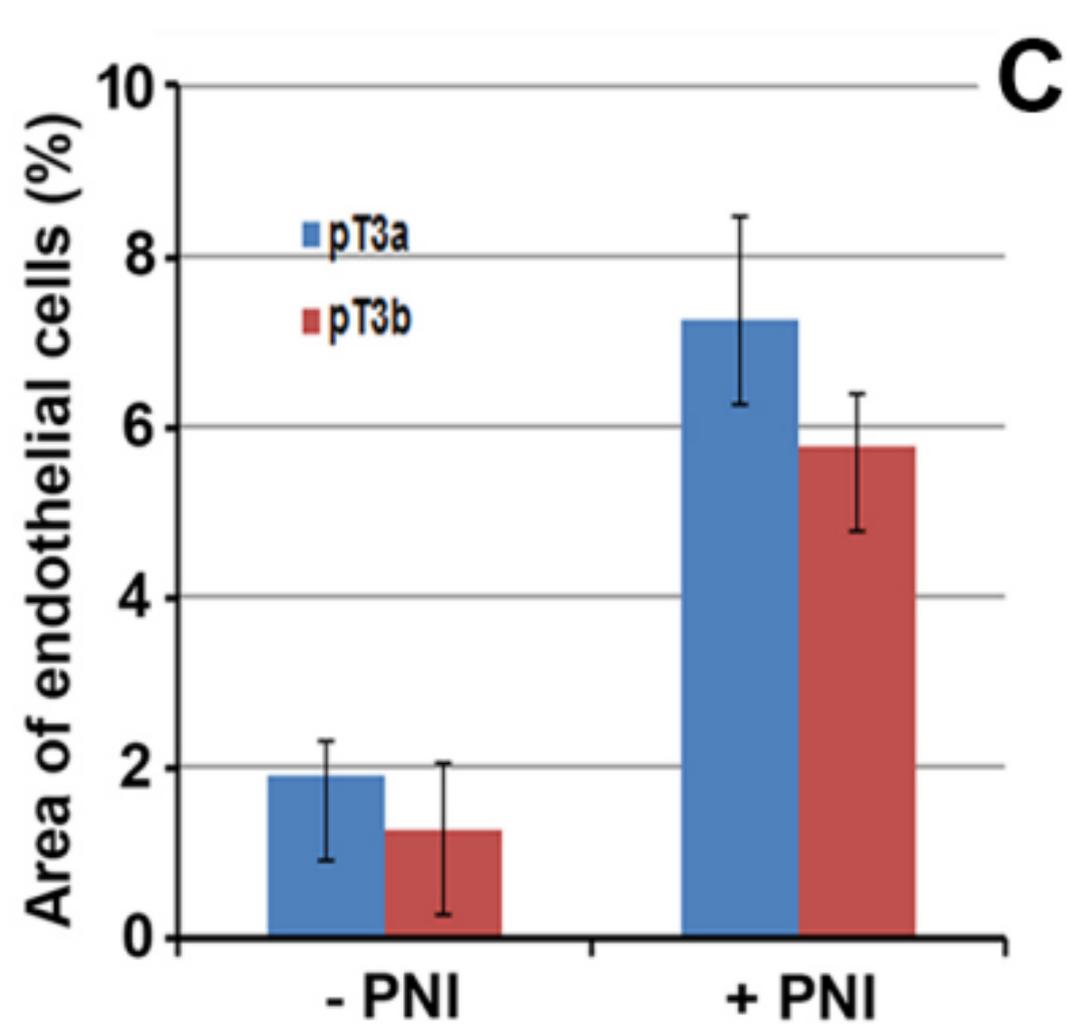
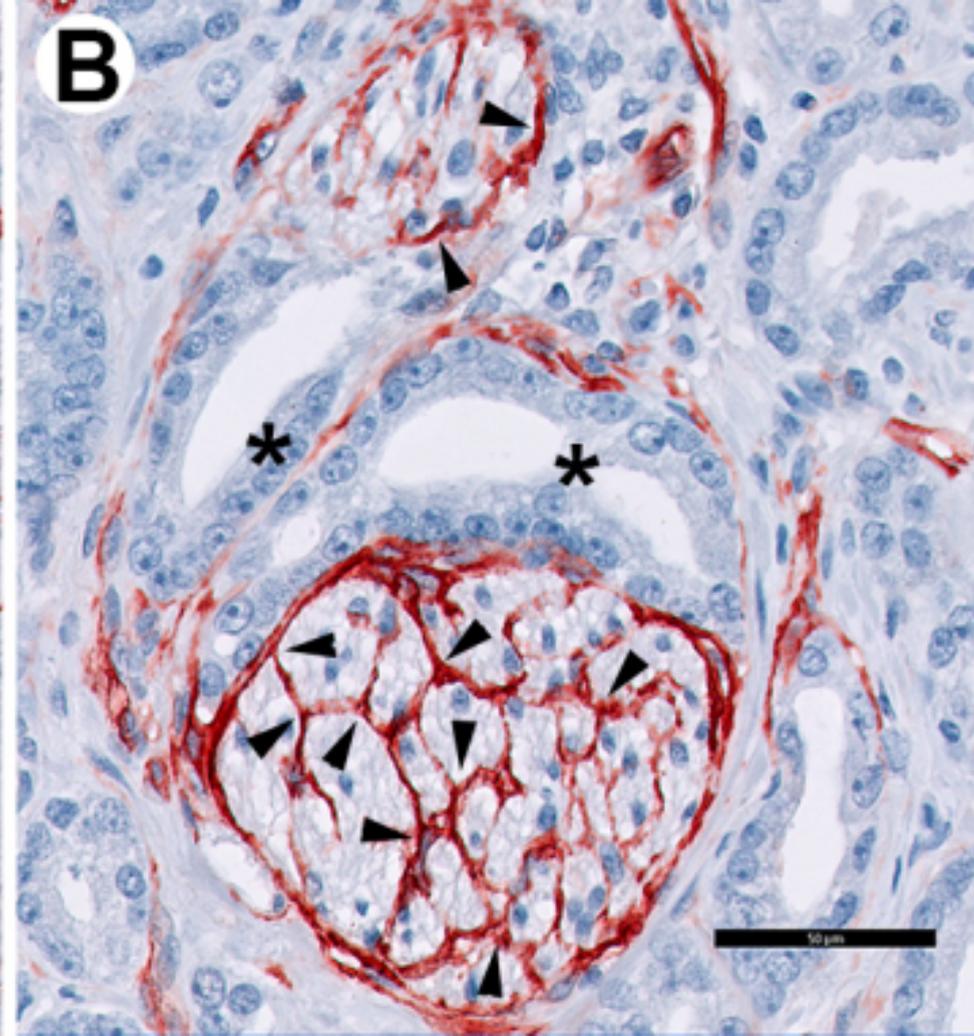
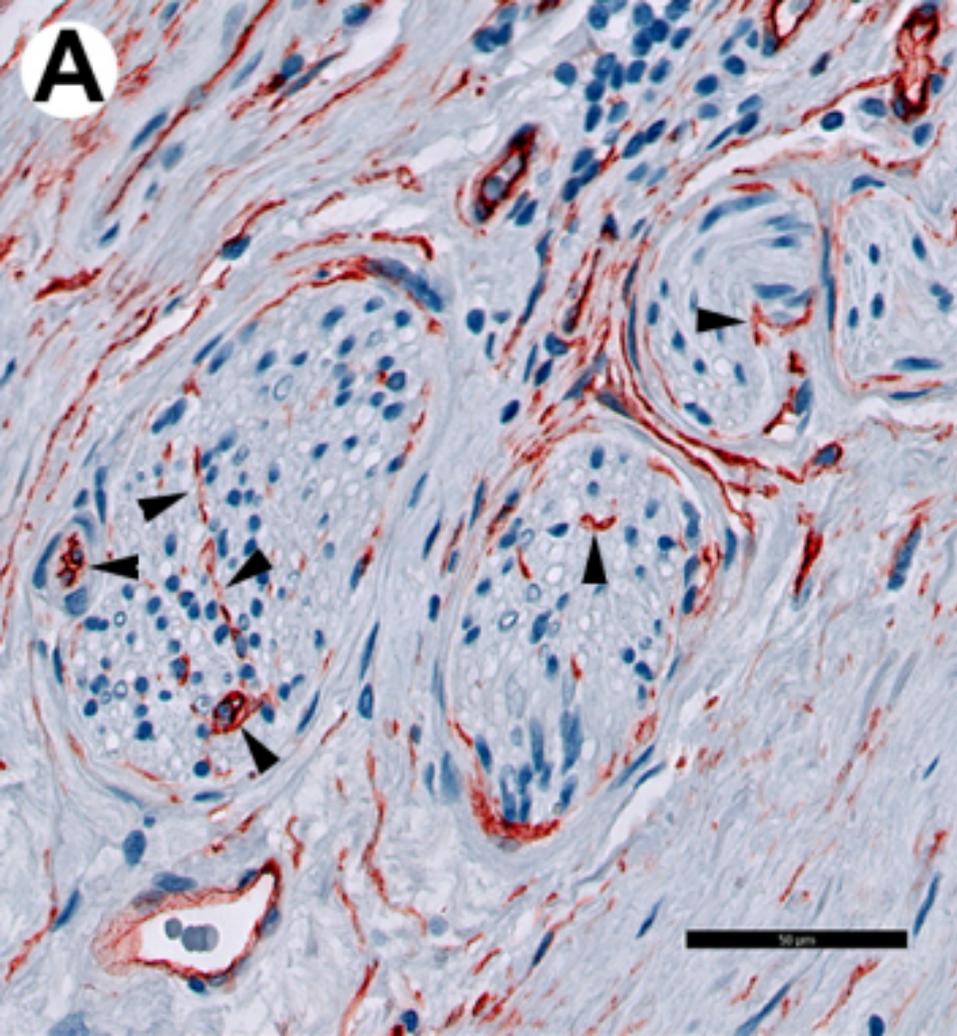
370 human prostate cancer tissues using as primary antibodies a rabbit polyclonal antibody  
371 against S100 (a nerve marker), a rabbit polyclonal antibody against VEGF, and a mouse  
372 monoclonal antibody against FGF-2. Panel A: Nerve fibers (red color) surrounded by  
373 cancerous prostatic glands (asterisks). Panel B: Epithelial cancer cells (red-brown color)  
374 expressing VEGF. Panel C: Vasa nervorum endothelial cells (arrowheads) and Schwann  
375 cells (arrows) expressing FGF-2 (brown color). NC, nerve cells. Bars: 50  $\mu$ m.  
376

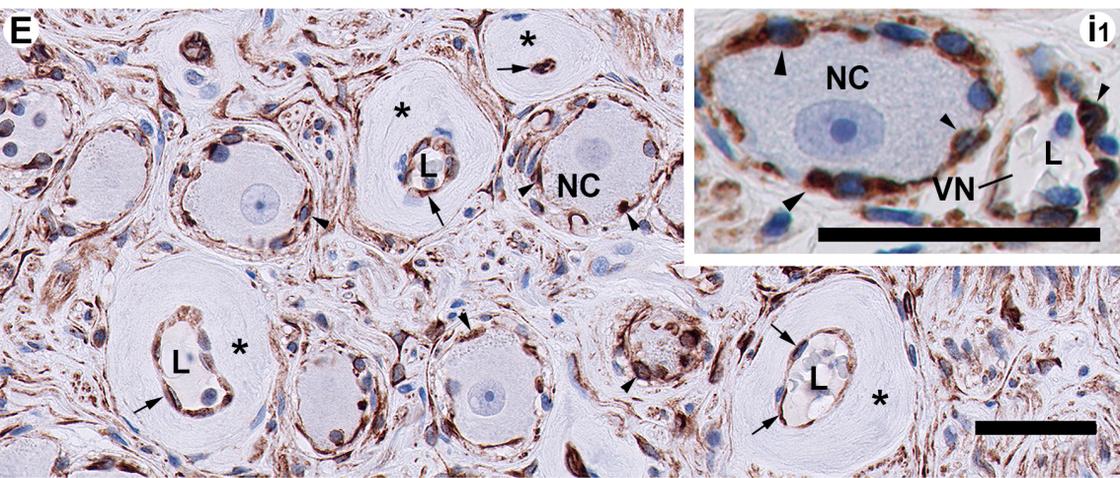
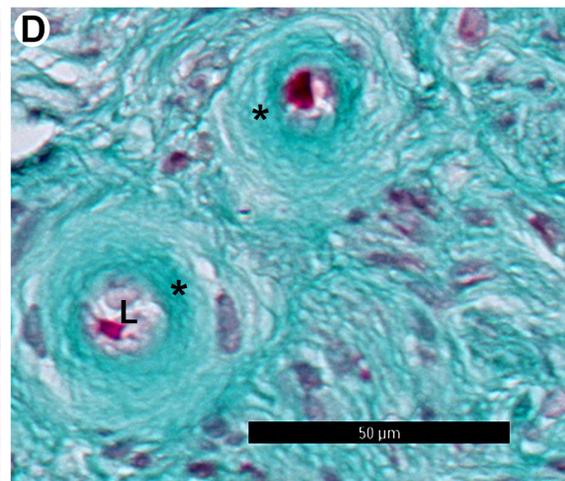
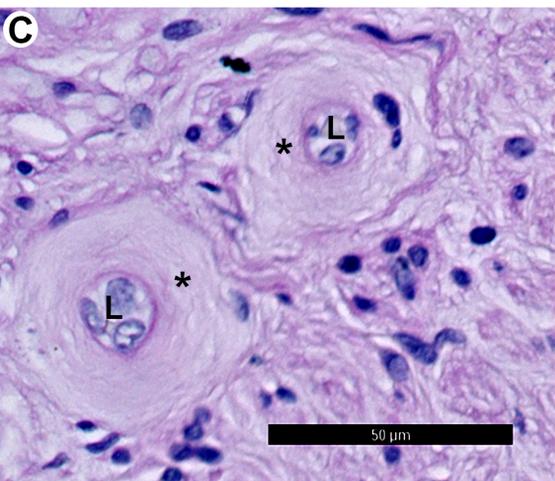
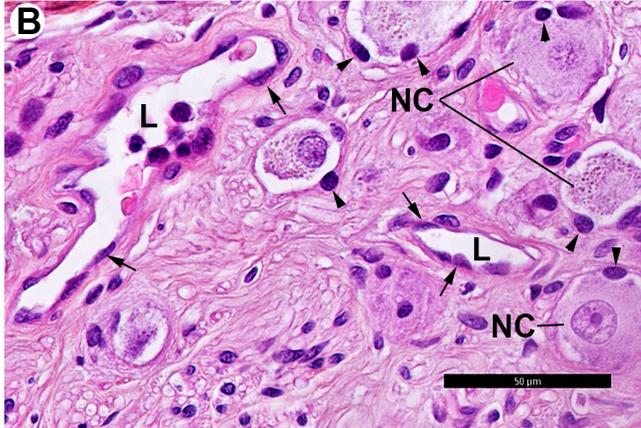
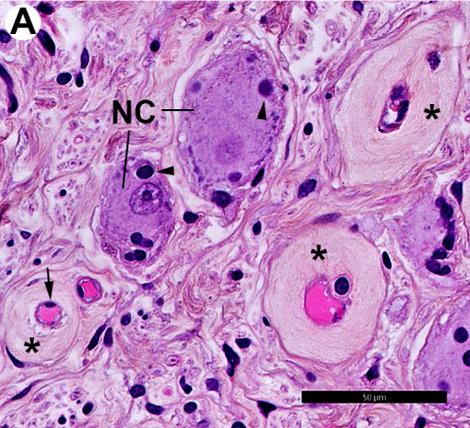
377 **FIGURE 2. Expression of CD34 by vasa nervorum endothelial cells in locally**  
378 **advanced prostate cancer.** Immunohistochemical analysis was performed on paraffin-  
379 embedded sections of prostate tumors using the monoclonal antibody CD34 followed by  
380 a secondary peroxidase-coupled goat anti-mouse immunoglobulin visualized by the red-  
381 brown peroxidase-reaction product of aminoethyl-carbazole. Sections were also stained  
382 with hematoxylin. Vasa nervorum (arrowheads) of prostatic nerves without perineural  
383 invasion (panel A) showed faint CD34 staining, whereas a strong presence of CD34 was  
384 noticed on vasa nervorum of prostatic nerves with perineural invasion (panel B).  
385 Asterisks: cancerous glands. Bars: 50  $\mu$ m. Panel C: quantitation of CD34-positive vasa  
386 nervorum endothelial cells in nerves with perineural invasion (+PNI) or without perineural  
387 invasion (-PNI). No differences are noticed between the pT3a (blue bars) and pT3b  
388 tumors (red bars).  
389

390 **FIGURE 3. Vasa nervorum remodeling in patients diagnosed with locally advanced**  
391 **prostate cancer.** Standard histochemical methods with haematoxylin-eosin-saffron  
392 staining of cell inclusions and nuclei (panels A and B), periodic acid-Schiff reaction for  
393 glycogen, glycoproteins, glycolipids, and proteoglycans (panel C), and Masson's  
394 trichrome technique for collagen (panel D). In 22% of patients the subendothelial layer  
395 (asterisks) of vasa nervorum contains collagen fibers and is characterized by the  
396 absence of pericytes. For most vasa nervorum (panel B) the intima consists of

397 endothelial cells and their basal membrane. In context of cancer, the vasa nervorum  
398 endothelial cells (arrows) and Schwann cells (arrowheads) of prostatic nerve ganglions  
399 showed strong presence of FSHR (panel E and inset i1). L, capillary lumen; NC, nerve  
400 cells; Bars: 50  $\mu\text{m}$ .







**TABLE 1.** Clinical characteristics of the study patients

<b>Clinical characteristics of patients</b>	<b>All cases (n = 85)</b>	<b>pT3a (n = 70)</b>	<b>pT3b (n = 15)</b>
Age at prostatectomy [median (range)]	67.3 (58 - 78)	68 (58 - 78)	67 (62 -70)
PSA at diagnosis [median (range)]	7.3 (3.6 - 26)	7 (3.6 - 26)	7.4 (6 - 18.6)
Gleason score			
< 7	22	17	5
≥ 7	63	53	10
Seminal vesicle extension			
No	70	70	0
Yes	15	0	15

**TABLE 2.** Primary antibodies used in present the immunohistochemical present study

Antibody	Specificity	Description	Isotype	Antigen retrieval	Incubation time	Dilution	Supplier
Qbend-10	CD34	mouse monoclonal	IgG1	Tris-EDTA pH9	30 min	1/200	DAKO
RB-9031	VEGF	rabbit polyclonal		Citrate pH6	60 min	1/100	Thermo / LabVision
11B55	VEGFR-2	rabbit polyclonal		Tris EDTA pH9	60 min	1/100	Cell Signaling
bFM-2	FGF-2	mouse monoclonal	IgG1	Citrate pH6	30 min	1/100	MERCK
D2-40	Podoplanin	mouse monoclonal	IgG1	Citrate pH6	30 min	1/500	DAKO
S-2644	S100	rabbit polyclonal		Citrate pH6	30 min	1/3000	Sigma
FSHRA02	FSHR	mouse monoclonal	IgG2a	Citrate pH6	30 min	50 ng/ml	Institut Curie