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TITLE: The Clinical Development of Thalidomide as an Angiogenesis Inhibitor Therapy for Prostate Cancer

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<b>14. ABSTRACT</b>  Significant progress has been made in the understanding of key factors that regulate the cell-cell interaction in the context of the microenvironment of prostate cancer. This includes technical advances in getting information from small amounts of tissue to forward understanding of the molecular determinants of progression. We have developed tissue micro arrays (TMAs), and stained them for candidate factors implicated in stromal epithelial interaction and have demonstrated that they are expressed in the context of Thalidomide treated patients. This information will be used to compare these results to the expression patterns in similar prostate cancers not exposed to Thalidomide. We are requesting a no-cost extension of 6 months to allow completion of the planned studies. A formal letter will be sent separately.						
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## **INTRODUCTION**

Significant progress made toward the milestones outlined in the statement of work regarding DOD Grant DAMD17-01-1-0069. The clinical trial has been completed. No additional patients have been accrued. The primary goal on this trial was to demonstrate tolerance, efficacy and biologic effects either by imaging or serologic markers on the antitumor activity. Significant information has been gained toward achieving these goals. As in the past, no additional patients have been accrued and the trial has been completed. Data regarding the biologic effects of thalidomide have been generated and analysed.

## **BODY**

### **AIM 1. Assessment of safety and toxicity of preoperative thalidomide treatment**

#### **Methods**

Subjects in this prospective study of preoperative thalidomide were men with histological confirmed prostatic adenocarcinoma with no evidence of regional or distant metastases; disease could be clinical stage T1c-T2c with Gleason score of 7 or higher on initial biopsy or clinical stage T3. All gave informed consent to participate in this phase II study, which was approved by the institutional review board of The University of Texas M. D. Anderson Cancer Center.

Thalidomide was given once daily in the evening at a starting dose of 200 mg/day. This dose was escalated by 200 mg/day every week to a maximum of 600 mg/day if no toxicity greater than grade 2 ensued. Each treatment cycle lasted 42 days (6 weeks). At 6 weeks and 12 weeks, patients underwent digital rectal examination, transrectal sonography, and serum prostate-specific antigen (PSA) testing. If lesions showed no evidence of growth and serum PSA level had not increased at the 6-week interval, thalidomide treatment was continued for a maximum of 3 months (i.e., 2 cycles). PSA progression was defined as an increase in serum PSA of more than 25% over the baseline (pretreatment) value. Progression of measurable intraprostatic lesions was defined as an increase of more than 25% in two dimensions. Radical prostatectomy was performed after termination of the thalidomide treatment. For statistical analyses, the design of Thall, Simon, and Estey was used, and a success probability of 0.20 or larger was considered clinically promising. Clinical success was defined as stable disease (no increase in tumor mass) at 6 weeks followed by a decline in serum PSA of > 50% at 12 weeks. The maximum number of patients to be treated was set at n=40.

#### **Results**

The results of the trial are as follows and have not changed from past report. —

#### ***Clinical characteristics***

Total number of patients treated: 18

Total numbers of patients completed and going to prostatectomy are: 15

Total number of patients in whom tissue (prostate) has been collected from prostatectomy and serum are: 15 (15 primaries) (lymph nodes: 2).

Plasma samples have been collected from 16 patients.

Remaining goals: publication to be submitted within three months.

Results with regard to toxicity have been submitted in the previous annual report and are as follows:

**Drug related toxicity**

All patients were evaluable for toxicity. Drug dose was escalated to 600 mg thalidomide daily in all patients. Seventeen of the 18 patients (95%) completed treatment as scheduled. The incidence of adverse events ranged from 5% to 61 %. (Table 1)

Median time to surgery from thalidomide termination was 5 days (range 2 to 18 days). Prostatectomies were uneventful except for three cases involving difficulties in apical dissection, dissection from the rectum, or both.

**Table 1.** Toxicities

<b>Toxicities</b>	<b>G1</b>	<b>G2</b>	<b>G3</b>
Somnolence	10	2	
Constipation	6	5	
Fatigue	6	3	1
Pruritus	7	2	
Motor/ ataxia/ tremors	5	1	1
Xerostoma	7		
Dizziness	6	1	
Edema	6		
Pain	5	1	
Sensory	5		
Blurred vision	5		
Diarrhea	4		
Vasovagal episode/ bradycardia	1	1	1
Cardiac	4		
Memory loss	3		
Urinary frequency	3		
Dyspnoea/ diaphoresis	2	1	
Nausea/ vomiting	2		
Taste alteration	2		
Confusion		1	
Memory loss	2		
Insomnia	2		
Allergic rhinitis	2		
Hypomagnesemia	1	1	
Flatulence		1	
Confusion		1	
Depression	1		
Headache	1		
Hypotension	1		
Sexual function	1		
Dry eyes	1		
Hot flashes	1		
<b>Total</b>	<b>101</b>	<b>21</b>	<b>3</b>

**AIM 2. Assessment of efficacy and biologic effects with regard to serologic markers**

**Methods**

Pre Operative:

1. PSA time course.
2. Circulating Factors

Plasma Levels of circulating Vascular endothelial Growth Factor (VEGF) and Tumor Necrosis Factor (TNF)-α prior to and following thalidomide treatment have been assessed in 16 patients.

Serum Levels of Interleukin (IL)-6 and basic Fibroblast Growth Factor (bFGF) prior to and following thalidomide treatment have been assessed in 15 patients.

## Results

1. The results of the trial with regards to this endpoint are as follows and have not changed from past report.

### PSA time course

At 6 weeks of treatment, PSA levels were a median 38% lower than at baseline (range, -12% to 49%), with eight patients showing a reduction of at least 40%. At 12 weeks, the median PSA reduction was 42% (range, -19% to 70.9%), and six patients (33%, 95% confidence interval, 16% to 56%) achieved a PSA reduction of at least 50%. Testosterone concentrations remained unaffected. Median testosterone levels were 308.85 ng/dL (range, 186.71–595) at baseline and 341.29 ng/dL (range, 208.88–923.97) at the end of the 12-week treatment period. (Figure 1).

## 2. Circulating Factors

Plasma levels of circulating TNF- $\alpha$  and VEGF measured in 16 thalidomide treated patients seemed to increase after treatment (Table 2). Serum levels of bFGF and IL-6 measured in 14 thalidomide treated patients remained unchanged. Interestingly, baseline VEGF levels were significantly lower in patients with a PSA drop >50% than in others and did not change after thalidomide treatment (Table 3) (Figure 2). Finally, bFGF levels dropped (though not significantly) in all the patients with a PSA response (Figure 3). Interestingly patients who had a PSA response had a higher bFGF at baseline (Table 3).

**Table 2.** Levels of circulating VEGF, bFGF, TNF- $\alpha$  and IL-6 before and after thalidomide treatment. Comparison of continuous variables by Mann-Whitney test ( $p < .05$  for significance).

	VEGF ng/ml (range)	bFGF pg/ml (range)	TNF- $\alpha$ pg/ml (range)	IL6 pg/ml (range)
PreTreatment	29.37 (11.37-95.47)	1.98 (0.4-5.00)	2.07 (1.15-5.07)	2.07 (0.67-14.70)
PostTreatment	41.37 (13.06-231.95)	1.83 (0.62-4.00)	3.15 (1.47-5.00)	2.67 (0.62-7.85)
p value	<b>0.015</b>	.9	<b>&lt;.0001</b>	.6

**Table 3.** Levels of circulating bFGF and VEGF in relation to response.

	VEGF		p	bFGF		P
	Responders	Non		Responders	Non	
PreTreatment	28.13	37.25	<b>0.02</b>	2.4	1.4	<b>0.01</b>
PostTreatment	28.1	58.6	<b>0.005</b>	1.7	2.1	<b>0.08</b>
p value	<b>0.1</b>	<b>0.03</b>		<b>0.44</b>	0.2	

## AIM 3. Assessment of thalidomide biologic effects in human tissue.

### Background

Even though the mechanism of action of thalidomide has been investigated extensively in the multiple myeloma context, it has not been fully clarified.<sup>1-8</sup> In particular, with regard to solid tumors a lot remains to be answered. Its mechanism of action is certainly more sophisticated than the originally thought anti-angiogenic effect. Thalidomide and its immunomodulatory analogs (IMiDs) have been suggested from in vitro data to induce apoptosis or growth arrest via more than one pathways, alter adhesion-at least of MM cells- to bone marrow stromal cells, inhibit the production of cytokines (interleukin-6 and vascular

endothelial growth factor). Even its qualities as a potent TNF down regulatory drug implicate numerous probable effects that are tissue specific.

The epithelial-stromal crosstalk is currently regarded as probably the determining factor of prostate tumor invasion and metastasis. We hypothesize that thalidomide initially targets the prostate tumor microenvironment and thus attains its anticancer effect. The focus of this research is to test this hypothesis for the first time in solid tumors and moreover in human tissue.

## Methods

### Tissue Microarray Technology

*Interrogating human tissue has improved dramatically since the submission of the grant in 2001.* These technological advances permit the expansion of the scope of interrogation and have been applied to the study of this tissue. *We have constructed* two tissue microarrays (TMAs). One Tissue microarray (TMA) was constructed from 15 radical prostatectomy specimens from the patients participating in the study. A second TMA, which served as a control, was constructed from prostatectomy specimens from patients matched for pathologic stage and Gleason score at surgery.

Areas have been selected from all the available primary tumor foci and adjacent stroma as well as from non-malignant areas-both glandular and stromal - of the peripheral and/or transitional zone where applicable. Additionally, the TMA has been designed to include cores representing the whole spectrum of histologic patterns found on the different tumor foci. With this design we attempt to further the thorough study of the effect of thalidomide on the tumor microenvironment. We may also direct our analysis to look for differential effect according to histologic pattern, study the effect on the crosstalk between tumor and adjacent stroma including the vascular compartment, and possibly address the effect on the non-tumor epithelial and stromal compartment.

The control TMA we have constructed serves to not only compare differential expression or localization of expression of the various factors of interest, but most importantly it will guide the assessment of factors or populations whose expression may be lost after treatment and may not otherwise be identified, particularly since we aim to interrogate pathways and interactions that are currently still under investigation in the prostate cancer context).

To efficiently interrogate the thalidomide mechanism we created a panel of markers known to be implicated in prostate cancer biology. We actually subdivided them in 3 different groupings:

1. Those pertaining to the vasculature, i.e. markers of angiogenesis and the endothelial cell specific marker CD31. The panel of vascular markers include VEGF, IL-6, Platelet Derived Growth Factor-A (PDGF-A), IL-8, bFGF. Expression of the markers is assessed in both the epithelial and stromal compartment. (Analysis of these markers has been concluded)
2. Those implicated in broader stromal epithelial interaction. We include components of the hedgehog signaling pathway known to potentiate prostate cancer progression upon aberrant activation, matrix metalloproteinase (MMP)-2 and MMP-9, E-Cadherin and members of the Transforming Growth Factor (TGF)-beta superfamily. We assess expression of those markers both in the epithelial and stromal compartment.
3. Those related to the epithelial compartment. Here we include markers of proliferation (Ki67), apoptosis (active caspase 3) and survival (bcl2, p53 status, bclxl and others).

### Statistical Considerations

Descriptive statistical analysis will be calculated, including histograms or box-plots, proportions, means, standard deviations. Fisher's exact test and Wilcoxon test will be used in univariate analyses of categorical and continuous variables, respectively. For the data analysis on multiple observations from a patient, a mixed-effects model can be used to assess the biomarker expression with the correlated data. In addition, the multivariable analysis can be carried out for assessing the treatment effect on two

or three factors involved in one pathway, simultaneously. This also will apply when estimating treatment effect in proliferation and apoptosis.

*Power of the sample size to detect differences:* For the comparison of control vs. treatment groups, a sample size of 16 in each group will differentiate between proportions of expression from 0.01 (low expressive in treatment) to at least 0.38 (expressive in control) with 80% power at the significance level of 0.05, based on the two-group Fisher exact test in statistical software of nQuery Advisor 5.0 (1995 – 2002). **Table 4** depicts different scenarios and can be used as a guide for power assessments.

**Table 4.** Power calculation for binary outcomes at significance level of 0.05, based on two-group Fisher exact two-sided test of equal proportions (odds ratio = 1).

<b>Proportion 1</b>	0.01	0.01	0.01	0.01	0.01	0.05	0.05	0.05	0.05
<b>Proportion 2</b>	0.5	0.45	0.4	0.38	0.35	0.55	0.5	0.45	0.4
<b>Power (%)</b>	96	92	85	81	74	90	84	75	64
<b>n per group</b>	16	16	16	16	16	16	16	16	16

## Results

We have concluded our analysis on TMAs of markers related a. angiogenesis, b. stromal-epithelial interactions and c. the epithelial compartment. Exploratory hierarchical clustering analysis revealed two main clusters of markers, depending on whether they were upregulated or downregulated in the treated group with regard to the untreated control group. (**Figure 4**) Further analyses with standard *t* tests and a mixed model, allowing estimation of variability between and within individual samples, confirmed these results.

Results are briefly summarized below:

### a. Effects on angiogenesis and vascular markers

With regard to thalidomide effect on the vasculature we established that thalidomide treatment has a profound antiangiogenic effect in prostate tissue. We have compared Microvessel density (MVD) between the thalidomide treated group of specimens and the control and have found it significantly lower following thalidomide treatment (**Table 5a**). Table 5a summarizes the involvement (extent of staining) of vascular markers in samples from the control group and the treated group. Expression of VEGF and IL-6, markers strongly implicated in prostate cancer angiogenesis, was lower in both the tumor epithelium and the stroma in samples from the thalidomide-treated group than in samples from the control group. IL-6 was consistently expressed in the treated samples but to a lesser extent in comparison to the control. Expression of IL-8 and bFGF was higher in the treated group than in the untreated group. PDGF-A expression was high and not different in samples from both the control and treated groups. (**Figure 5**)

### b. Effects on broader stromal-epithelial interaction

Comparison of the expression of markers of stromal-epithelial interaction between thalidomide treated and control cases suggested a modulation of hedgehog signaling and the matrix metalloproteinase (MMP) to E cadherin ratio by thalidomide. Results are summarized briefly below. (**Table 5b**)

**Hedgehog Signaling:** We assessed the expression of three main components of the sonic hedgehog (Shh) pathway—gli2, Smoothed (Smo), and the Shh ligand (**Figure 6**). Thalidomide treatment attenuated hedgehog signaling. The transcription factor gli2, the main downstream effector of the Shh pathway, was consistently expressed in the control in both the nucleus and cytoplasm of tumor cells; in contrast, gli2 expression was significantly lower in thalidomide-treated and localization was predominantly cytoplasmic.

**Table 5.** Involvement (extent of staining) of: a) angiogenic markers assessed by a 4-point system b) Microvessel density (MVD) assessed by CD31 staining expressed in absolute numbers per core.

<b>a. Angiogenesis</b>	<b>CONTROL</b>		<b>TREATED</b>		Standard Deviation (SD) Between Patients	<b>P VALUES</b>
	(Mean)		(Mean)			
VEGF	2.24		1.63		0.52	0.0049
VEGF stroma	0.76		0.34		0.22	< 0.0001
IL-6	1.68		1.23		0.49	0.04
IL-6 stroma	1.53		1.41		0.32	0.279
PDGF-A	2.74		2.59		0.29	0.221
IL-8	0.49		1.26		0.5	0.0008
bFGF	1.55		2.55		0.43	< 0.0001

<b>Microvessel Density (CD31)</b>	Control		Treated		SD Between Patients	<b>p Values</b>
	Mean	Median (min,max)	Mean	Median (min,max)		
	32.6	30 (21,54)	24.1	21 (11.5,46)	8.8	0.025

<b>b. Stromal–Epithelial Interactions</b>	<b>CONTROL</b>		<b>TREATED</b>		SD Between Patients	<b>p Values</b>
	(Mean)		(Mean)			
Gli2	2.11		1.2		0.36	< 0.0001
Smo	2.84		2.33		0.32	0.0005
Shh	2.11		2.32		0.49	0.7782
E-cadherin	2.59		2.84		0.15	0.0041
MMP-9	1.86		0.21		0.51	< 0.0001
MMP-2	2.94		2.1		0.49	0.0002
MMP-2 stroma	1.06		1.02		0.58	0.871
TGF-β	2.61		2.34		0.42	0.08
β-catenin	2.08		2.32		0.31	0.15
TNF-α	1.12		2.08		0.73	0.0018
TNF-α stroma	0.12		0.62		0.22	< 0.0001

<b>c. Epithelial Compartment</b>	Control		Treated		SD Between Patients	p values
	Mean	Median (min, max)	Mean	Median (min, max)		
Ki-67 (%)	8.9	5.0 (0,23)	6.0	3.0 (0,33.5)	7.8	0.333
Active caspase 3 (%)	0.64	0 (0,3)	1.9	0 (0,3)	3.8	0.064

The Shh ligand and the transmembrane protein Smo are upstream components of the pathway responsible for the level of Shh activation. Expression of the ligand was the same in both groups, but expression of Smo, considered the determining factor of aberrant activation of hedgehog signaling in prostate cancer, was much higher in the control. **(Figure 6) (Table 5b)**

MMP: E-cadherin Ratio. Expression of MMP-2 and MMP-9 were significantly lower in the thalidomide-treated group than in the control group. E-cadherin, a marker of cellular adhesion, was consistently higher in the treated (Table 5b). A three-way scatter plot (**Figure 7**) by discriminant analysis of the relative expression of MMP-9, MMP-2 and E-cadherin, that may predict prostate cancer phenotype more accurately than conventional variables of disease stage and tumor grade (10,11,22), distinguished between thalidomide-treated and control samples with 93% accuracy.

Other Markers of Stromal–Epithelial Interactions. TNF- $\alpha$  expression was higher in the thalidomide-treated group. No significant differences were detected in  $\beta$ -catenin or transforming growth factor (TGF)- $\beta$  expression (**Table 5b**).

### c. Effects on the epithelial compartment

The apoptotic index of tumors, assessed with an antibody to active caspase 3, was low in both groups, although slightly higher in the treated (mean  $\pm$  s.d., 1.9%  $\pm$  2.6%; range, 0% to 3%) than in the control (mean  $\pm$  s.d., 0.64%  $\pm$  0.75%; range, 0% to 3%) ( $p=0.064$ ) (**Table 5c**). No statistically meaningful difference was found between treated and control tumors with regard to proliferative index; mean Ki-67 expression in the treated was 6.0% ( $\pm$  8.8; range, < 1% to 33.5%; median, 3.0%) as compared with 8.9% ( $\pm$  7.1; range, < 1% to 23.5%; median, 5.0%) in the control ( $p = 0.333$ ) (**Table 5c**). No difference was detected in bcl2 expression and androgen receptor expression and localization.

### Work in Progress

We still have not concluded our work on frozen tissue specimens that was described in previous reports and includes Laser capture Microdissection technology, Real time PCR and high throughput proteomics assays. This is underway concurrently with our work on tissue from other preoperative studies for the interest of maintaining the same experimental conditions.

### Expense Report

1. Consumables used for the TMAs manufacturing: **6,500 USD**
2. Expenses made for detection Kits: **2,000 USD**
3. Expenses made for the purchase of antibodies, reagents, controls and other consumables (pipets, pipet aid, tips, containers etc) used for Immunohistochemistry: **18,000 USD**
4. Expenses for consumables reagents used in LCM (Laser Capture Microdissection) template preparation of frozen tissue, RNA extraction amplification, primers and controls: **22,500 USD**

### Personnel receiving pay for the research effort

Sherrie Hodges, a Senior Research Assistant in the department of Genitourinary Medical Oncology is receiving salary support from this grant.

### KEY RESEARCH ACCOMPLISHMENTS

- We have a confirmed effect of preoperative thalidomide on levels of PSA Patients participating in the trial had a median reduction of 42% in PSA concentrations after 12 weeks of thalidomide treatment, while testosterone levels remained unchanged.
- Low circulating levels of VEGF predict for PSA response (decline in PSA  $\geq$  50%) and patients with such a response don't experience an increase in VEGF levels following treatment.
- Levels of Circulating TNF $\alpha$  increase following thalidomide treatment in prostate cancer patients.
- Thalidomide has a profound effect on the tumor microenvironment that precedes the effect on the epithelial compartment. This consists of both antiangiogenesis and of inhibition of stromal epithelial interactions favorable to prostate cancer progression. We are providing the first evidence in human solid tumors to support this concept.

## REPORTABLE OUTCOMES

- Preliminary data have presented as an oral presentation in the 2006 Prostate InterSpore Meeting.
- The data have been presented as a poster in the in the Prostate cancer Foundation Scientific Retreat held in Scottsdale Arizona, October 19-21, 2006.
- A manuscript generated from this work has been accepted for publication in Clinical Cancer Research (**Initial Modulation of the Tumor Microenvironment Accounts for Thalidomide Activity in Prostate Cancer**. Eleni Efstathiou, Patricia Troncoso, Sijin Wen, Kim-Anh Do, Curtis A. Pettaway, Louis L. Pisters, Timothy J McDonnell, and Christopher J Logothetis CCR06-1938).

## CONCLUSIONS

The clinical aims of this trial have been fulfilled. Thalidomide was well tolerated and didn't delay surgery or cause excessive perioperative toxicity. More importantly our observations on a molecular level are the first evidence in human solid tumors that thalidomide affects the tumor microenvironment and this may be a therapeutically valid approach in adult solid tumors as thalidomide has exhibited clinical efficacy in the metastatic setting.

Our observations on molecular effects suggest that thalidomide affects the tumor microenvironment in a manner that may transform the tumor phenotype to less invasive. The components of the microenvironment that appear to be modulated by thalidomide at the time of analysis include both angiogenesis and the broader stromal–epithelial cell interaction, as reflected by changes in Shh signaling and MMP: E-cadherin ratio. The observations we report suggest that targeting the microenvironment as a component of a rational co-targeting strategy may enhance the efficacy of more traditional epithelial targeting strategies in prostate cancer.

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## **FIGURES**

Figure 1

## Change in PSA Levels

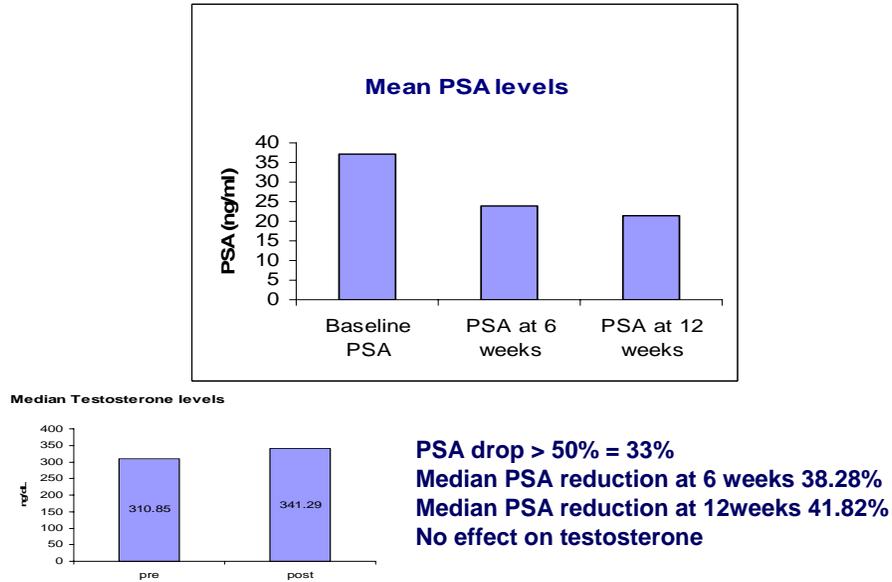
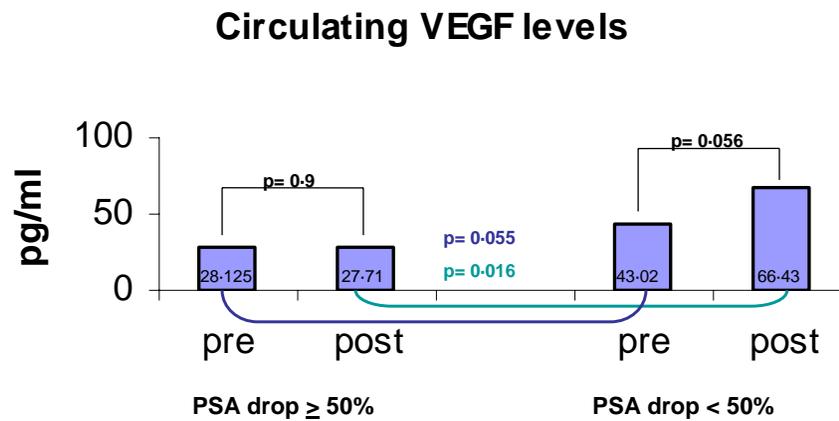
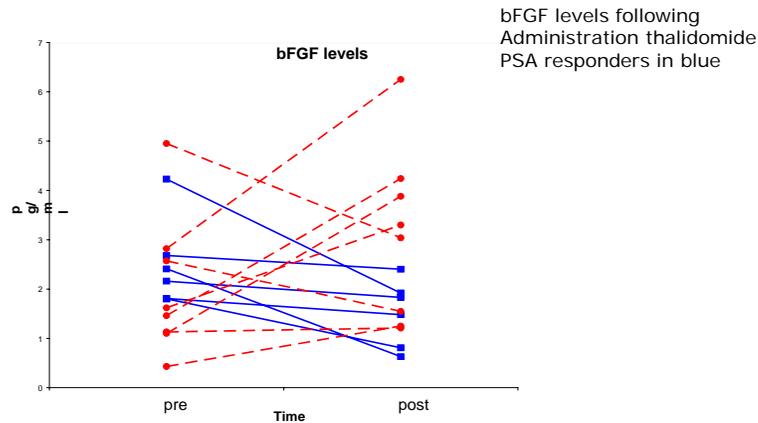


Figure 2

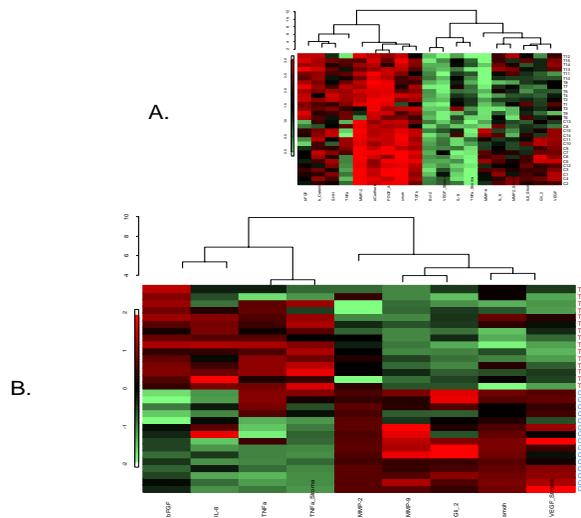


**Figure 3**

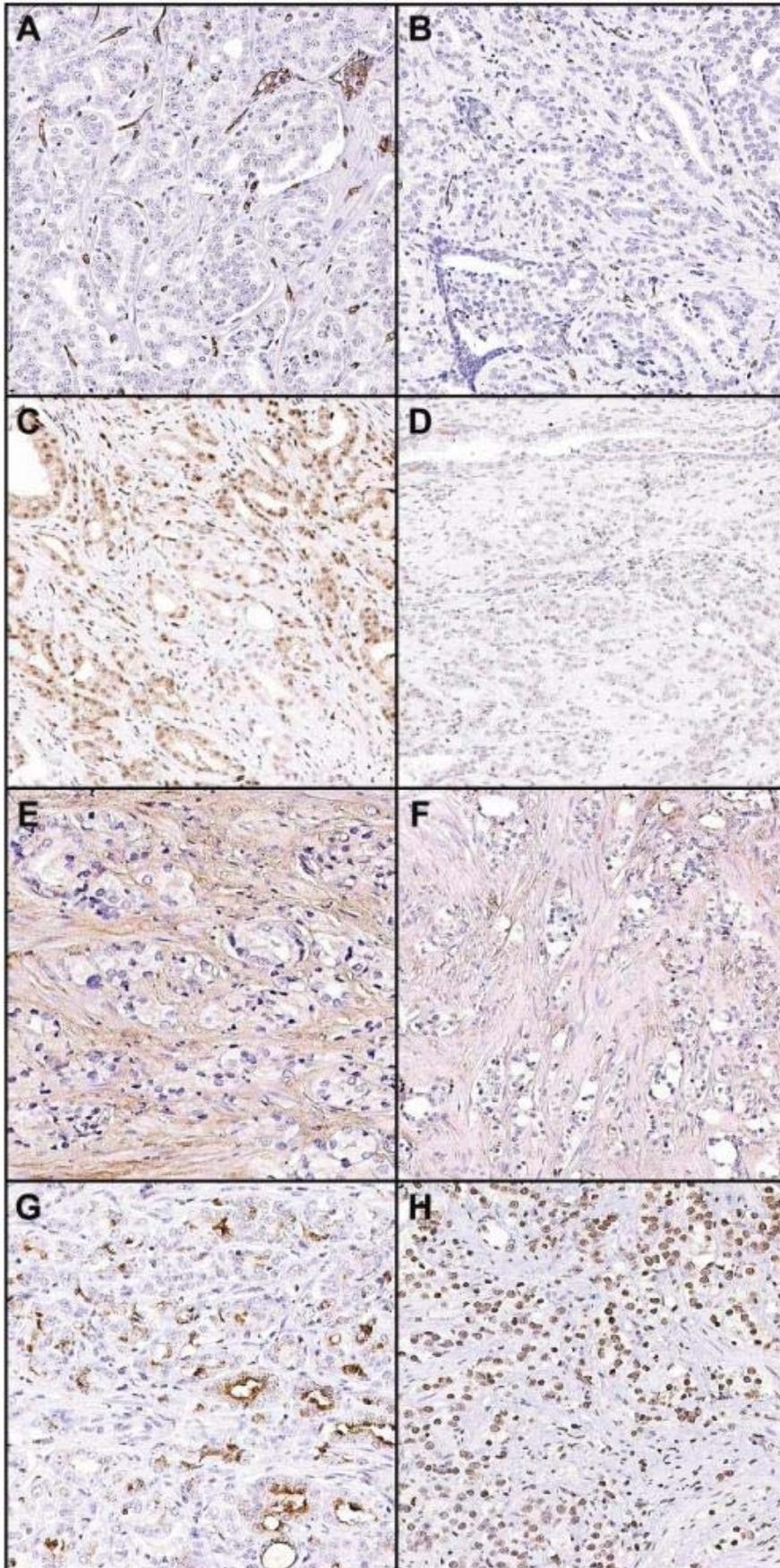
**Levels of bFGF prior and following thalidomide treatment**



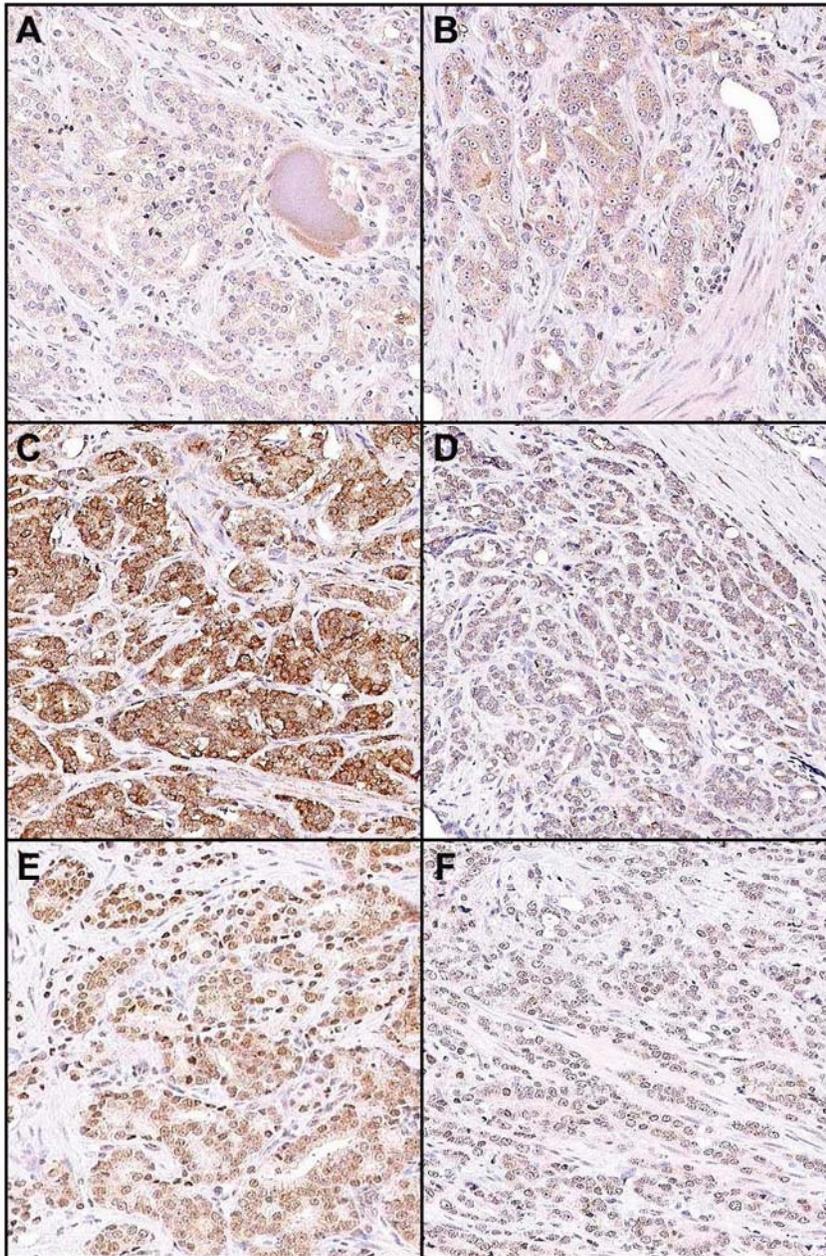
**Figure 4**



**Figure 4. Hierarchical clustering.** Exploratory hierarchical clustering analysis revealed two main clusters of markers, depending on whether they were upregulated or downregulated in the treated group with regard to the untreated control group. Further analyses with standard *t* tests and a mixed model, allowing estimation of variability between and within individual samples, confirmed these results. (A) Image plot of the initial exploratory hierarchical clustering of the raw data. Involvement (extent of staining) for each marker (columns) and per sample (rows) is represented by color. The range of green to red represents the range of involvement from 0 to 3. (B) Image plot of relative involvement of only the differentially expressed markers between the treated group (upper half) and the control untreated group (lower half). Red signifies higher expression than the mean; green, lower; and black, no difference from the mean.



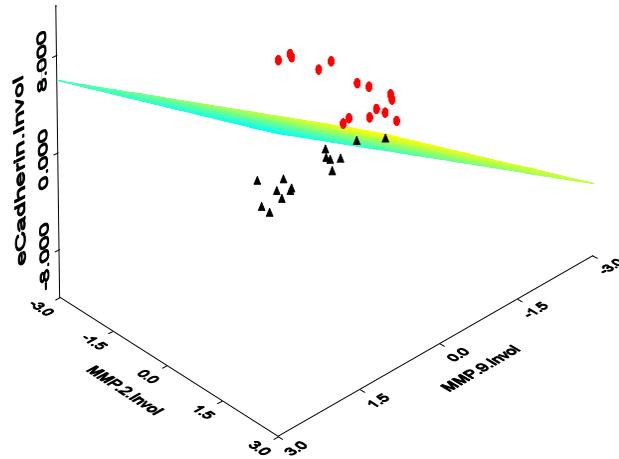
**Figure 5.** Representative images of Microvessel density (MVD) and vascular marker expression in control and thalidomide treated samples. Images on the left (A, C, E, G) are representative of control samples while those on the right (B, D, F, H) of thalidomide treated. A, B are representative of CD31 staining, C, D of VEGF, E, F of IL-6 and G, H of IL-8. As described MVD, VEGF and IL-6 expression were significantly higher in the control samples. Interleukin 8 expression was higher in the thalidomide treated samples and predominant localization was nuclear, contrary to the cytoplasmic already reported in prostate cancer and observed in our control samples.



**Figure 6. Shh signaling.** After thalidomide treatment (B), Sonic hedgehog ligand expression in tumor specimens was no different from that in the control (A). Expression of both the transmembrane protein smoothened and the transcription factor gli2 in the treated (D and F respectively) was lower than in the control (C and E) suggesting an attenuation of hedgehog pathway signaling after thalidomide treatment.

**Figure 6**

Figure 7



**Figure 7. MMP-to-E-cadherin ratio.** Three-way scatter plot of relative expression of MMP-9, MMP-2, and E-cadherin. The surface is determined by discriminant analysis ( $R=3MMP9+MMP2/E-Cadherin$ ). Red dots represent samples from the treated group, and black triangles indicate samples from the control group.