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

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**14. ABSTRACT**

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.

**15. SUBJECT TERMS**
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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 antitumoral 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. Preliminary data regarding the biologic effects of thalidomide have been generated.

## 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.

**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-α 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-α and IL-6 before and after thalidomide treatment. Comparison of continuous variables by Mann-Whitney test ($p$ <·05 for significance).

| | **VEGF** | **bFGF** | **TNF-α** | **IL6** |
|---|---|---|---|---|
| | ng/ml (range) | pg/ml (range) | pg/ml (range) | 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 | **0·015** | ·9 | **<·0001** | ·6 |

## 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.[1-8] 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 attains 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)

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

**Laser Capture Microdissection Technology**

We employ Laser Capture Microdissection (LCM) on frozen specimens to isolate tumor and non-tumor epithelial or stromal cells adjacent to tumor or non tumor epithelium cells. We extract mRNA and perform real RT-PCR to assess expression of markers of interest especially for those that there is no commercially available antibody.

## Preliminary Results

With regard to thalidomide effect on the vasculature we have already 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 5b). 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. (Image 1)

**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.

| **a.** | CONTROL | TREATED | **SD** | **P** |
|---|---|---|---|---|
| | **Mean** | **Mean** | **Between Patients** | **VALUES** |
| VEGF | 2·24 | 1·63 | ·52 | ·0049 |
| VEGF stroma | 0·76 | ·34 | ·22 | <·0001 |
| IL6 | 1·68 | 1·23 | ·49 | ·04 |
| IL6 stroma | 1·53 | 1·41 | ·32 | ·279 |
| PDGF-a | 2·74 | 2·59 | ·29 | ·221 |
| IL8 | ·49 | 1·26 | ·5 | ·0008 |
| bFGF | 1·55 | 2·55 | ·43 | <·0001 |

| **b.** | **Control** | | **Treated** | | | |
|---|---|---|---|---|---|---|
| | Mean | Median (min,max) | Mean | Median (min,max) | | |
| MVD(CD31) | 32·6 | 30 (21,54) | 24·1 | 21 (11·5,46) | 8·8 | ·025 |

## 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 TNFa increase following thalidomide treatment in prostate cancer patients.
- Thalidomide has a profound antiangiogenic effect in prostate cancer that is most probably due to a reduction in both VEGF and IL-6 expression in both the tumor epithelium and the surrounding stroma.

## REPORTABLE OUTCOMES

This preliminary data has been submitted as an abstract and has been accepted as an oral presentation in the 2006 Prostate InterSpore Meeting.

## CONCLUSIONS

The clinical aims of this trial have been fulfilled. We are currently concluding interrogation of the biologic effects of thalidomide in the prostate tumor microenvironment. We have already observed a profound antiangiogenic effect. This is most probably driven by the reduction in VEGF and IL-6 expression bioth in the stromal and epithelial compartment.

The collection of translational data for a considerable number of factors implicated either directly in prostate cancer progression or associated with already known thalidomide mechanisms of action will be complete in 2 weeks. Analysis of this data, which will be complete in 1 month, is expected to forward the understanding of the thalidomide effect in the prostate tumor microenvironment including cell-to-cell interaction.

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**Table 1**. Toxicities

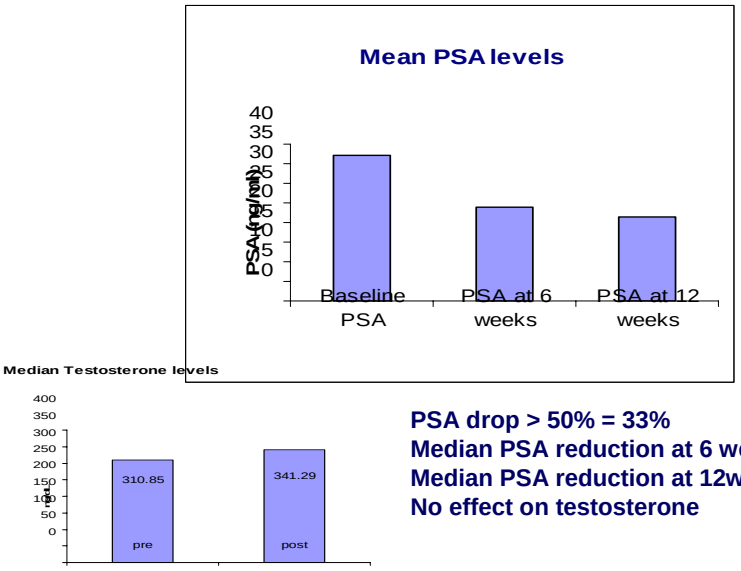
| **Toxicities** | ***G1*** | ***G2*** | ***G3*** |
|---|---|---|---|
| Somnolence | 10 | 2 | |
| Constipation | 6 | 5 | |
| Fatigue | 6 | 3 | 1 |
| Pruritus | 7 | 2 | |
| Motor/ ataxia/ tremors | 5 | 1 | 1 |
| Xerostoma | 7 | | |
| Dizziness | 6 | 1 | |
| Edema | 6 | | |
| Pain | 5 | 1 | |
| Sensory | 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 | | |
| ***Total*** | ***101*** | ***21*** | ***3*** |

Table 3:
Levels of Circulating bFGF and VEGF in relation to response

| | | ***VEGF*** | | | ***bFGF*** | |
|---|---|---|---|---|---|---|
| | **Responders** | **Non** | **p** | **Responders** | **Non** | **p** |
| **PreTreatment** | **28.13** | **37.25** | **0.02** | **2.4** | **1.4** | **0.01** |
| **PostTreatment** | **28.1** | **58.6** | **0.005** | **1.7** | **2.1** | **0.08** |
| **p value** | **0.1** | **0.03** | | **0.44** | **0.2** | |
| | | | | | | |

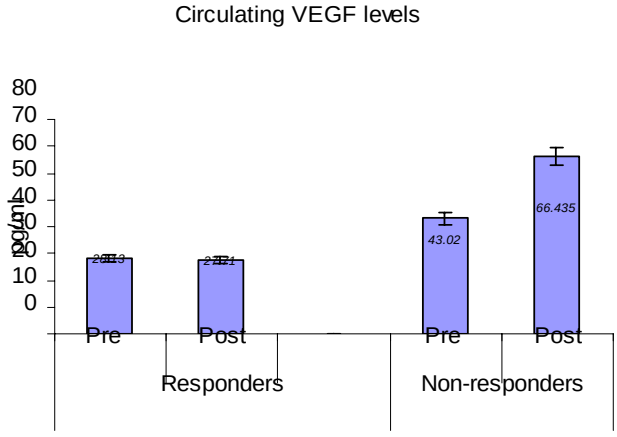
**Figure 1**

# Change in PSA Levels



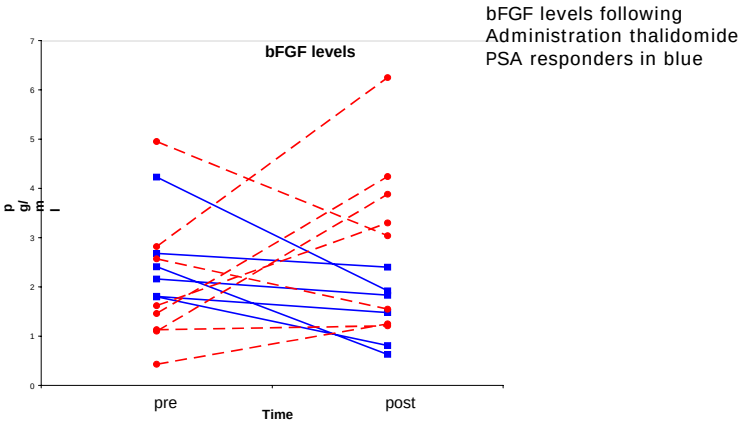

**PSA drop > 50% = 33%**
**Median PSA reduction at 6 weeks 38.28%**
**Median PSA reduction at 12weeks 41.82%**
**No effect on testosterone**

**Figure 2**



# Figure 3.

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



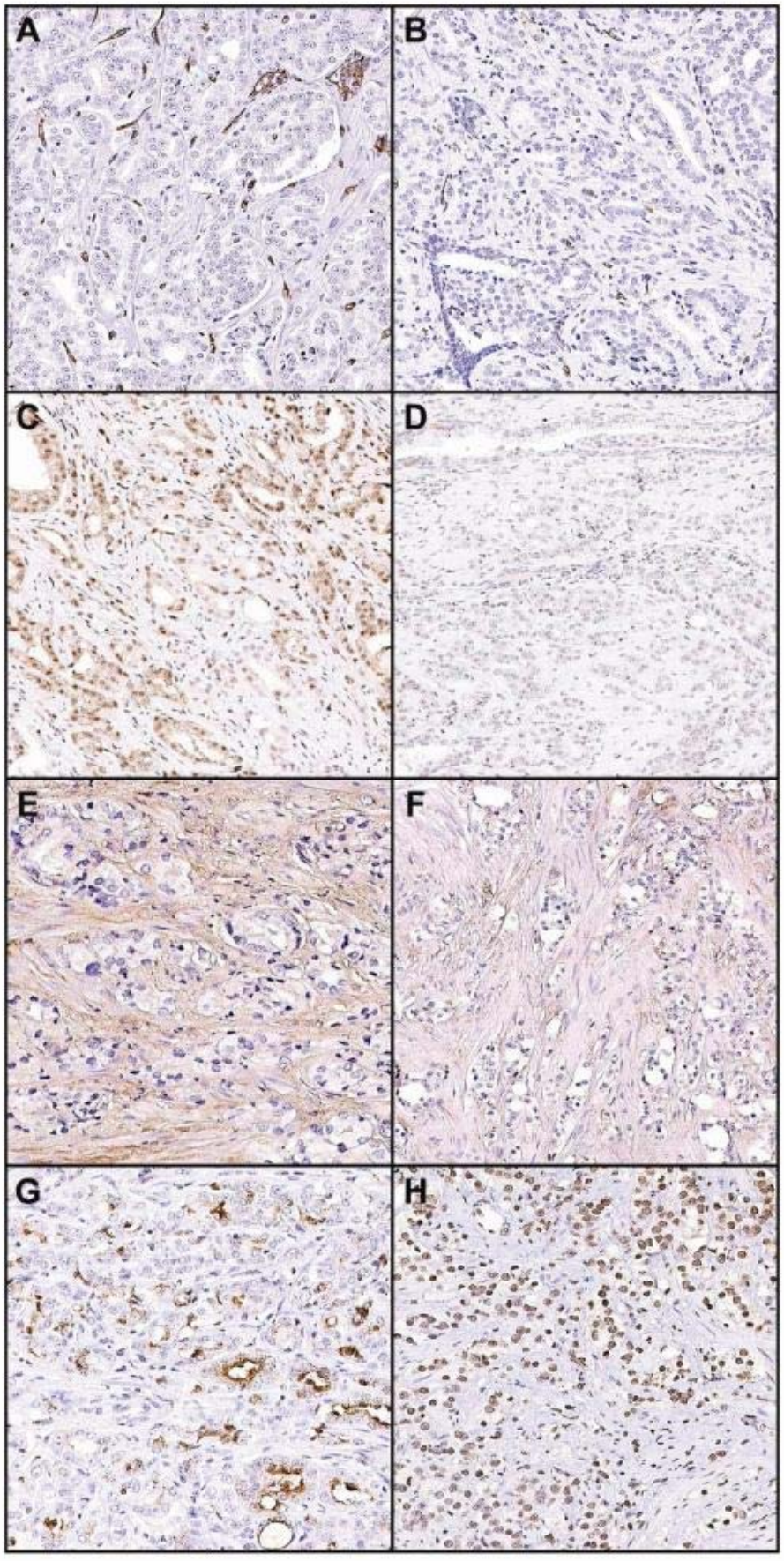



**Image 1**. Representative images of Microvessel density (MVD) and vascular marker expression in control and thalidomide treated samples. Images on the left (A, C, E, G) are representative of control samples while those on the right (B, D, F, H) of thalidomide treated. A, B are representative of CD31 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 localisation was nuclear, contrary to the cytoplasmic already reported in prostate cancer and observed in our control samples.

# Appendix

**Thalidomide as a Modulator of the Stromal Epithelial Interaction of High-Grade Prostate Cancer**

Eleni Efstathiou[1], Patricia Troncoso[2], Timothy J. McDonnell[3], and Christopher J. Logothetis[1].
[1]Department of Genitourinary Medical Oncology, [2]Department of Pathology, and [3]Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center.

We used the preoperative model to correlate the modulation of the growth promoting stromal-epithelial (S-E) interaction with efficacy of thalidomide in prostate cancer (PCa).

**Design:** We constructed two Tisssue MicroArrays (TMA): one from 15 prostatectomy specimens of thalidomide treated patients and one that served as a control from prostatectomy specimens of non treated prostate cancer patients. The cases were matched for pathologic stage and Gleason score. All cases were locally advanced with a Gleason score $\geq$ 7.

The components of the S-E interaction we tested included:

Tumor associated angiogenesis. We interrogated the effect on angiogenesis modulation by MVD (microvessel density) assessment and also evaluated for the thalidomide effect on VEGF, IL6, IL8, bFGF, both in the epithelial and stromal compartments. Fibrous stroma: In a hypothesis generating search we evaluated candidate pathways implicated in the S-E interaction; a) The Sonic hedgehog (Shh,smoothened,gli) pathway known to be activated in epithelial mesenchymal boundaries during vertebrate organ development and recently implicated in tumor related S-E interaction. Smoothened has been suggested as the rate limiting step of Shh activation in PCa and thus as a critical point of tumor growth regulation, b) Tgfb (smad, PDGF), c) mmps (mmp2, mmp9) MMP-2 in particular has been strongly implicated in S-E crosstalk and has been identified as an independent predictor of decreased progression free survival in patients with high-grade PCa, d) TNFa, e) IGF, f) beta-catenin/e-cadherin. In addition we assessed the effect of S-E modulation on the epithelial proliferation (Ki67), apoptosis (activated caspase3) and related pathways (p53, bcl2, bclxl).

| | Marker Modulation | | |
|---|---|---|---|
| **# Modulated Markers/ Total Markers** | **Increase 1/20** | **Decrease 8/20** | **No Change 11/20** |
| **Angiogenic** | 0/5 | 2/5 | 3/5 |
| CD31 (MVD) | | √ | |
| VEGF | | √ | |
| IL6 | | | √ |
| bFGF | | | √ |
| IL8 | | | √ |
| **Stromal-Epithelial Interaction** | 1/9 | 3/9 | 5/9 |
| Smoothened | | √ | |
| Shh | | | √ |
| MMP-2 | | √ | |
| MMP-9 | | √ | |
| TNF-a | √ | | |
| TGFbeta | | | √ |
| IGFR | | | √ |
| beta-catenin | | | √ |
| e-cadherin | | | √ |
| **Epithelial** | 0/6 | 2/6 | 4/6 |
| Ki67 | | √ | |
| Caspase 3 | | | √ |
| p53 | | | √ |
| Bcl2 | | √ | |
| BclXL | | | √ |
| Bax | | | √ |

**Preliminary Results:** The table depicts the differential expression of the markers evaluated in the treated TMA when compared to that observed in the control.

**Conclusion**: We provide evidence to support the hypothesis that thalidomide modulates S-E interaction in human prostate cancer. The findings suggest that the efficacy of thalidomide is mediated by targeting the stromal- epithelial interaction.