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Angiotensin receptor autoantibodies as exposures that modify disease progression: Cross sectional, longitudinal and *in vitro* studies of prostate cancer

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ABSTRACT

Circulating angiotensin type I receptor (AT1R) agonistic autoantibodies (AT1RaAbs) that bind and chronically activate the receptor have been associated with a number of diseases suggesting that while the autoantibodies are not necessarily causative they may promote disease progression. The prostate has a local renin angiotensin system. The current study examines associations between AT1RaAbs and prostate cancer (PCA), disease-free survival (DFS), overall survival (OS) and AT1RaAb effects on PCA cell phenotype.

In a cross-sectional set of serum obtained from 151 men diagnosed with PCA, nonmalignant prostate disease or no disease, higher serum AT1RaAb levels were associated with PCA and non-organ confined PCA. The odds ratio for PCA was 6.3 (95% confidence interval 2.2 to 18) for a positive 1:1600 titer and 18 (95% confidence interval 6.9 to 45) at AT1RaAb levels > 1.04 μg/ml, ($p < 0.0001$). In a longitudinal set of pre-diagnosis samples from 109 men, DFS hazard ratios of 2.2 (95% confidence interval 1.4 to 3.5) and 1.6 (95% confidence interval 1.0 to 2.5) for most proximal to diagnosis and most distal to diagnosis samples, respectively, were found for high versus low AT1RaAb groups. Hazard ratios for OS in most proximal and distal samples were 2.4 (95% confidence interval 1.6 to 3.6) and 1.8 (95% confidence interval 1.1 to 2.8), respectively. Accelerated failure modeling of survival indicated that a 1 μg/ml increase in AT1RaAb levels was associated with a reduction of DFS and OS by 20% at the most proximal time point and by 15% at the most distal time points. Adjusting for age, did not affect the association with DFS in proximal samples but changed distal time point DFS and OS to a 10% decrease for every 1 μg/ml increase in AT1RaAb. Additional adjustments for body mass index, systolic blood pressure and prostate-specific antigen did not appreciably alter these associations. AT1RaAb treatment of PC3, DU145, and LNCaP cells significantly increased the maximal growth rate approximately 2-fold and invasiveness approximately 3-fold. *Conclusions:* These observations provide evidence supporting AT1RaAbs as exposures that may modify prostate cancer progression and indicate they may be predictive markers for risk stratification.

Abbreviations: Ang II, Angiotensin II; ANOVA, Analysis of variance; AT1R, Angiotensin type I receptor; AT1R aAbs, Angiotensin type I receptor autoantibodies; AUC, Area under the curve; BLSA, Baltimore Longitudinal Study of Aging; BMI, body mass index; BP, blood pressure; BPH, benign prostatic hyperplasia; CI, Confidence interval; CV, Coefficient of variance; DFS, disease-free survival; HG-PIN, high-grade prostatic intraepithelial neoplasia; NMD, nonmalignant prostate disease; NOC, Non-organ confined; OC, Organ confined; OS, overall survival; PCA, prostate cancer; PSA, prostate specific antigen; ROC, Receiver operating characteristic; RRP, radical retropubic prostatectomy; r_S, Spearman rank order correlation coefficient; TAAs, Tumor associated antigens.

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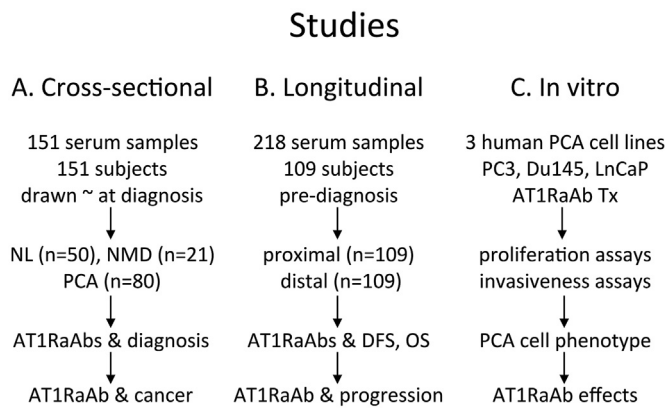

Fig. 1. Experimental approach. Three different studies are performed to test whether AT1RaAbs are exposures that can modify disease progression. (A) The association between AT1RaAb levels and prostate health is assessed using a cross-sectional study of serum from subjects diagnosed as being disease-free (NL), having nonmalignant prostate disease (NMD) or prostate cancer (PCA). (B) The association between AT1RaAb exposure and disease progression is explored in a longitudinal study using prediagnosis serum samples and determining disease-free survival (DFS) and overall survival (OS). (C) The biological effects of AT1RaAbs on prostate cancer cell phenotypes are examined *in vitro*.

1. Introduction

Circulating angiotensin type I receptor (AT1R) autoantibodies (AT1RaAbs) have been found in serum from patients with pre-eclampsia [1–3], transplant rejection [4–6], malignant hypertension [7–9], frailty [10], and Huntington's and Alzheimer's disease [11,12]. Additionally, serum AT1RaAb levels were significantly correlated with higher interleukin-6, blood pressure (BP) and body mass index (BMI), diminished physiologic capacity (weaker grip strength and slower walking speed), and adverse health outcomes (increased falls and mortality) in a study of community dwelling older adults [10]. Angiotensin II (Ang II) binding within the hydrophilic pocket formed by transmembrane domains of AT1R results in transient receptor activation [13]. In contrast, AT1RaAbs bind to the extracellular loop 2 of AT1R chronically activating the receptor [14]. The association of AT1RaAb levels with multiple diseases and negative health outcomes suggest that these exposures may not be causative to disease, but rather facilitate disease progression.

Based on 2013–2015 data, at some point during their lifetime approximately 11% of men will be diagnosed with prostate cancer (PCA) [15]. The prostate has a local renin angiotensin system [16]. The presence of Ang II in the basal layer of the epithelium and AT1R on stromal smooth muscle suggest that Ang II may mediate cellular growth and smooth muscle tone in normal human prostate [17]. A role for Ang II in promoting prostate cancer progression and aggressiveness was suggested by studies of Ang II induction of oxidative stress, PAX2 oncogene expression, cellular proliferation and protease secretion in PCA cell lines [18–20]. Renin angiotensin system components were found to be overexpressed in hormone refractory PCA tissue [21]. The above observations taken together led to an investigation of AT1RaAb association with (a) PCA in a cross sectional study, (b) disease-free and overall survival in a longitudinal study, and (c) altered cell phenotype using three distinct human PCA cell lines.

2. Methods

2.1. Reagents

Ang II was obtained from United States Biological (Salem, MA). Human serum antibodies against the loop 2 domain of AT1R (AT1RaAb) were initially enriched by Melon chromatography and then affinity purified using CAFHYESQN conjugated to SulfoLink coupling resin following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). A rabbit IgG antibody, termed AT1RpAb, was raised against the peptide CAFHYESQN and affinity purified (ABclonal, Woburn, MA). A rabbit gamma globulin isotype control (isoAb) was obtained from Thermo Fisher Scientific (Rockford, IL).

2.2. Human subjects

A cross-sectional set of serum samples (n = 151) was obtained under local approved protocols (Johns Hopkins Medicine Institutional Review Board) from the Department of Urology biospecimen bank. 101 specimens were collected from patients with scheduled appointments for either elevated prostate specific antigen (PSA) levels, a scheduled prostate biopsy or being seen as a newly diagnosed PCA patient in the Urology Outpatient Clinic. 80 of these patients were positive for PCA at biopsy, whereas 21 were biopsy benign and diagnosed as either atrophy, benign prostatic hyperplasia (BPH), high-grade prostatic intraepithelial neoplasia (HG-PIN) or inflammation. The remaining 50 specimens were collected from men visiting an offsite prostate cancer screening who were free of prostate disease. All subjects had PSA measured. Clinical measures at diagnosis included digital rectal exam, total Gleason score and clinical stage determined from the biopsy. The number of days between the blood draw generating the serum sample and the biopsy/diagnosis were also captured for each sample. Those with prostate cancer had pathological grading following radical retropubic prostatectomy (RRP) that included RRP Gleason score, surgical margin status, seminal vesicle or lymph node

Table 1
Characteristics, main predictors and outcomes of cross sectional and longitudinal sets.

Model Covariates	Cross Sectional Set, Pre-treatment			Longitudinal Set, Pre-diagnosis	
	Normal N = 50	NMD[a] N = 21	PCA N = 80	Proximal N = 109	Distal N = 109
Age at visit, mean (SD),y	55 (8)	64 (7)	58 (7)	81 (8)	69 (10)
BMI, mean (SD), kg/m^2				24.9 (3.0)	24.9 (2.5)
Systolic BP, mean (SD), mm Hg	–	–	–	145 (24)	138 (25)
Diastolic BP, mean (SD), mm Hg	–	–	–	78 (12)	82 (12)
PSA, mean (SD), ng/ml median (range)	1.0 (0.6)	7.7 (4.7)	7.4 (5.4)	11.5 (39)	2.4 (2.0)
	0.7 (0.2–2.4)	6.7 (2.0–19.4)	5.9 (0.6–32)	3.5 (0.4–352)	2.0 (0.2–10.5)
Main predictor					
AT1RaAb, mean (SD) median (range) μg/ml	0.81 (0.37)	0.87 (0.33)	1.76 (0.93)	1.96 (1.56)	1.31 (1.25)
	0.75 (0.25–2.17)	0.78 (0.41–1.60)	1.48 (0.58–4.50)	1.46 (0.10–8.98)	0.80 (0.20–6.69)
Outcomes					
PCA Diagnosis (%)	0	0	80 (100)	81 (73%)	81 (73%)
Deaths (%)	0	0	0	109 (100%)	109 (100%)
Time to diagnosis, y	–	–	–	4 ± 4	15 ± 6
Time to death, y	–	–	–	7 ± 5	18 ± 8

[a] Abbreviations: NMD, non-malignant disease; PCA, prostate cancer; SD, standard deviation; BP, blood pressure; BMI, body mass index; PSA, prostate specific antigen.

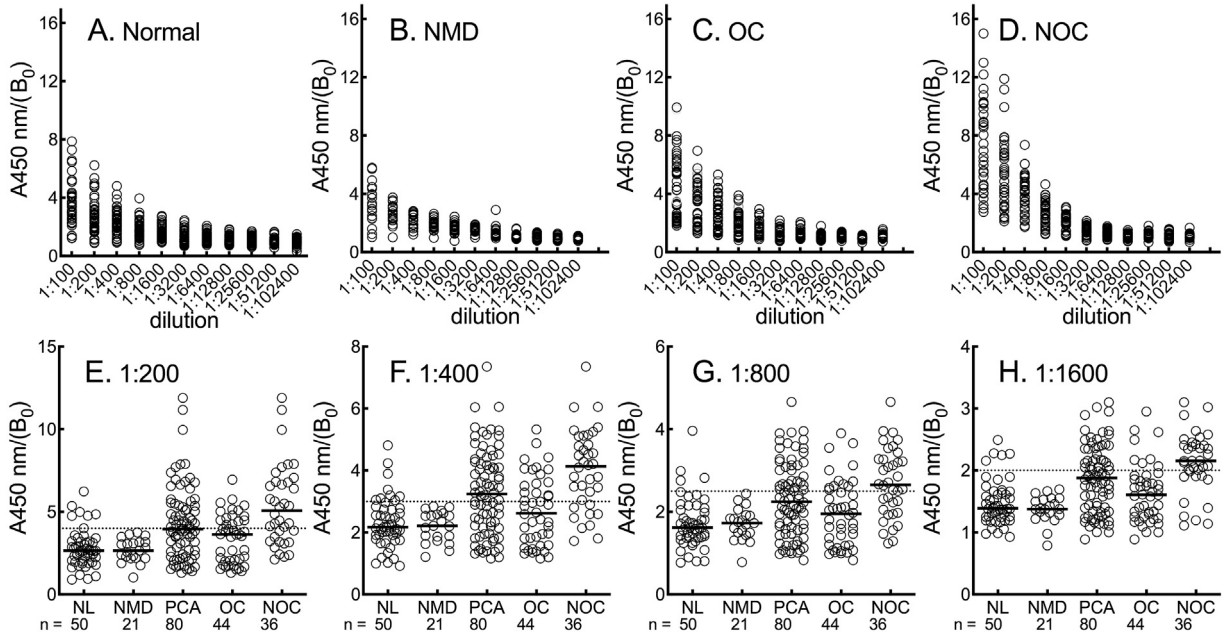

Fig. 2. AT1R autoantibody titer assays. Serum titer assays on subjects who at biopsy were determined to be (A) normal (NL), have (B) nonmalignant disease (NMD), (C) organ confined cancer (OC), or (D) non-organ confined cancer (NOC) were performed. The distribution of the serum AT1RaAb levels by titer assay and by prostate status were profiled at (E) 1:200 and (F) 1:400. (G) 1:800 and (H) 1:1600 dilutions. In panels E to H, the heavy horizontal lines mark median levels while the dotted line depict the optimum cut off for that dilution.

Table 2
Sensitivity and Specificity of titer assay and ELISA.

A. Titer	Cut off (A450/B_0)	Sensitivity	Specificity	Odds Ratio	P value
PCA vs NL					
1:200	4.0	40 (29–52)	86 (73–94)	4.1 (1.6–10)	<0.005
1:400	3.0	40 (29–52)	82 (69–91)	3.0 (1.3–7.2)	<0.05
1:800	2.5	39 (28–51)	92 (81–98)	7.2 (2.4–22)	<0.0001
1:1600	2.0	41 (30–53)	90 (78–97)	6.3 (2.2–18)	<0.0001
1:3200	1.3	52 (41–64)	88 (76–95)	8.1 (3.1–21)	<0.0005
NOC vs OC					
1:200	4.0	67 (49–81)	85 (69–94)	11 (3.6–34)	<0.0001
1:400	3.0	75 (58–88)	92 (79–98)	3.6 (0.9–15)	<0.0001
1:800	2.5	61 (43–77)	82 (66–92)	7.2 (2.5–21)	<0.0005
1:1600	2.0	69 (52–84)	82 (66–92)	12 (4.1–38)	<0.0001
1:3200	1.3	56 (44–67)	72 (58–84)	3.2 (1.5–7.1)	<0.005
B. ELISA	Cut off (μg/ml)	Sensitivity (95% CI)	Specificity (95% CI)	OR (95% CI)	
PCA vs NL	1.04	80 (69–88)	82 (67–92)	18 (6.9–45)	<0.0001
NOC vs OC	1.04	86 (70–95)	26 (14–41)	2.1 (0.7–6.8)	<0.0001
	1.7	64 (46–79)	81 (67–92)	7.7 (2.8–22)	<0.0001

Abbreviations: NL, normal; NMD, nonmalignant prostatic disease; PCA, prostate cancer; NOC, non-organ confined prostate cancer; OC, organ confined prostate cancer.

involvement, established capsular penetration, focal capsular penetration, and organ confined status of the tumor.

A longitudinal set of pre-diagnosis serum samples from 109 men was obtained from the Baltimore Longitudinal Study of Aging (BLSA). Participants under age 60 were assessed every 4 years; those aged 60–79 years every 2 years, and those aged 80 and older were assessed annually. Assessments include comprehensive health, cognitive, and functional evaluations that take nearly 3 days to complete. Measures include physical examination, medical interview medications, electrocardiogram, vitals and anthropometry, clinical labs, core cognitive battery, physical performance and strength testing. For visits performed before 1991, PSA levels were measured using frozen sera samples stored at −80 °C [22]. Since 1991, PSA measurements and digital rectal examination (DRE) were performed at each evaluation. Participants with a PSA level above 4.0 ng/ml and/or abnormality on DRE also underwent a transrectal ultrasound-directed prostate biopsy for diagnostic purposes. This prospective cohort study, run by the National Institute on Aging, has been previously used to assess markers of risk status for PCA [22,23]. Clinical measures associated with each BLSA sample include age, BMI, blood pressure, and PSA levels while for subjects who later developed PCA, additional clinical measures included diagnosis date and disease stage. Data on clinical staging was missing for 25 subjects. All subjects in this set are deceased with known date of death and post-mortem pathological status of the prostate (disease-free or PCA present).

2.3. Immunoassays

Analytical assays were performed without knowledge of the patient's clinical details. An AT1RaAb titer assay was performed by coating 96 well serocluster plates with 100 ng/well purified capture antigen, CAFHYESQN overnight at 4 C followed by blocking with Neptune blocking reagent (ImmunoChemistry Technologies, Bloomington, MN, USA). Plates were incubated with serum that was serially diluted starting at 1:100 with ten subsequent 1:2 dilutions. A final set of wells received no serum (B_0). A 1:2000 dilution of a secondary goat anti-human IgG detection antibody conjugated to horseradish peroxidase was used in conjunction with 3,3′,5,5′-tetramethylbezidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) to detect immunoreactive material. The absorbance at 450 nm for each subject and dilution were normalized to the corresponding B_0 and A450/B_0 was plotted versus dilution to analyze the titer profile.

AT1RaAb levels were quantified using a modification to a recently described ELISA [10]. Briefly, the assay employed CAFHYESQN as the capture antigen coated at 100 ng/well. To facilitate the solubility of the capture antigen, the peptide solution was sonicated for 2 min in an ice water bath prior to its addition to the wells. Affinity purified rabbit IgG raised against CAFHYESQN (AT1RpAb) was used as a calibrator and was reconstituted in SeraSub (CST Technologies, Great Neck NY). Standards (1:2 serial dilutions starting from 100 ng/ml of AT1RpAb) and 2 serum

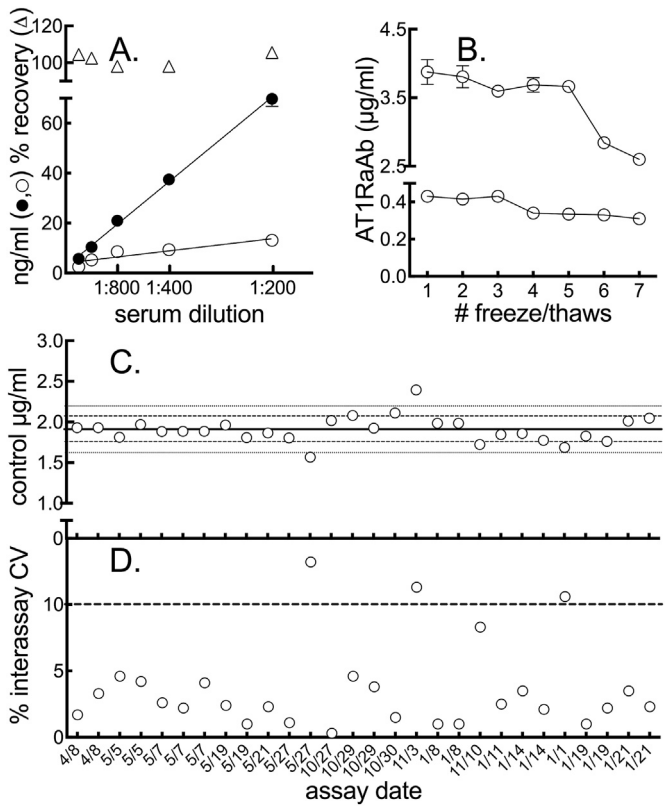

Fig. 3. AT1R autoantibody ELISA performance. (A) Serum samples (open circle) and aliquots of the same sample spiked with 50 ng of purified AT1RaAb (closed circle) were serially diluted, analyzed by the ELISA and % recovery (triangle) was determined. (B) Two serum samples (low and medium range) were subjected to multiple freeze thaw cycles and then assayed to determine the effect on recovery. (C) The variation of an internal mid-range control serum sample that had a known concentration of 1.9 µg/ml AT1RaAb and was included with each assay was followed over ten months. The solid line represents the mean, the dashed line is one standard deviation and the dotted line is two standard deviations from the mean. (D) The inter-assay coefficient of variance (CV) for a mid-range control sample was plotted over the same time frame. The dashed line marks the acceptable cut off point for % CV.

controls were included in each plate. Following a 2 h incubation of 1:200 diluted samples, immunoreactive material was detected after washing and by sequential incubation with goat anti-human IgG antibody conjugated to horseradish peroxidase and 3,3′,5,5′-tetramethylbezidine substrate. The absorbance values of standards were fit to a four parameter logistic function and sample AT1RaAb levels were interpolated.

2.4. Cell culture and in vitro assays

PC3, DU145 and LNCaP cells were obtained from John T. Isaacs, Johns Hopkins School of Medicine. Cells were grown in complete RPMI-1640 medium containing 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.0) and 10% heat inactivated fetal bovine serum. For all *in vitro* studies, cells were seeded in a complete RPMI medium. PC3, DU145 and LNCaP cells have all been previously shown to produce AT1R [10].

2.4.1. Cell proliferation assays

Cells were treated 24 h after seeding with affinity purified AT1RaAb, AT1RpAb, IgG isotype control antibody (isoAb) or Ang II in serum-free RPMI-1640 medium for 1 h at 37 C. An equal volume of complete RPMI medium was then added and cell proliferation was measured using a crystal violet assay, 48 well plates and triplicate wells per treatment [24]. At each time point a set of wells were fixed with 1% gluteraldehyde. At the end of the time course, cells were stained with 0.02% crystal violet, the absorbance at 560 nm of dye extracted from wells was read, normalized to the absorbance of the first time point, log transformed and fitted to modified logistic to determine maximal growth rate [24].

A commonly used surrogate for following cell proliferation - reduction of WST-8 by cellular dehydrogenases - was used to profile the response of prostate cancer cells to different doses of AT1RpAb. Briefly, cells were incubated with seven serial dilutions of 50 nM AT1RpAb in serum-free basal RPMI medium for 1 h. WST-8 and the electron mediator 1-methoxy-5-methylphenazinium methylsulfate were then added in complete RPMI medium following the manufacturer's protocol (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Absorbance at 450 nm was measured over 56 h.

2.4.2. Cell invasiveness assays

The effect of AT1RaAbs on invasive phenotype was assessed by measuring cell invasiveness by transwell matrigel invasion assay as described [25], except that 10^6 cells/ml were pre-labeled with 10 µM DiIC12(3) at 37 C for 1 h. Cells were treated with vehicle (control), 50 nM Ang II, 50 nM AT1RpAb, or 50 nM isoAb in serum-free RPMI medium for 1 h at 37 C. Each upper chamber of the fluoroBlok inserts (8 µ, uncoated and coated with matrigel) received 1.7×10^5 cells/cm^2. RPMI-1640 medium containing 5% FBS was added to the lower chamber. After incubation, the migrating cells (uncoated inserts) and invading cells (matrigel coated inserts) were measured by fluorescence using a Victor II Multichannel plate reader with excitation set at 530 nm and emission at 560 nm. The percent cell invasion for a given treatment was calculated as the mean relative fluorescence of cells that invaded through a matrigel coated membrane divided by the mean relative fluorescence of cells that migrated through an uncoated membrane, multiplied by 100.

2.5. Statistical analysis

For the cross-sectional sample set, the sensitivity, specificity, and odds ratio of immunoassays at specific cut off values were determined by contingency table analysis using Fisher's exact test with a two-tailed P value. Comparisons of covariate values between groups were by Kruskal-Wallis ANOVA with Dunn's multiple comparisons test. Receiver operating characteristic (ROC) curve analysis was used for identifying the optimal cut off value of AT1RaAb based on the Youden's J statistic. Pairwise correlations between AT1RaAb and clinical correlates or pathological measurements were assessed using Spearman rank correlations. For the longitudinal sample set, comparison of variables between subjects' most proximal and distal samples was performed by Wilcoxon matched pairs signed rank test. Time to events (diagnosis or death) were modeled nonparametrically by Kaplan-Meier survival curves and dichotomizing samples into either low or high AT1RaAb levels and analyzing serum samples grouped as most proximal or distal to timed event. Survival estimates between groups were compared by Gehan-Breslow-Wilcoxin test, Mantel-Cox (logrank) and Tarone-Ware tests which give varying weights to early versus late events. Hazard ratios for disease-free survival (DFS) and overall survival (OS) were compared by Log-rank and Mantel-Haenszel tests. Accelerated failure estimates were used to assess the effects of other predictors on AT1RaAb association with DFS and OS at most proximal or most distal time points. For the *in vitro* studies of maximal growth rates and percent cell invasion, means of preselected treatments (control and Ang II, isoAb and AT1RaAb) were compared by ANOVA with Sidak's multiple comparisons test and multiplicity adjusted P values reported.

3. Theory

Chronic inflammation is believed to contribute to the development of a number of diseases [26]. AT1RaAbs have been associated with an increased inflammatory burden [10,27,28] involving increased reactive oxygen species, NADPH oxidase expression, and nuclear factor-kappa B

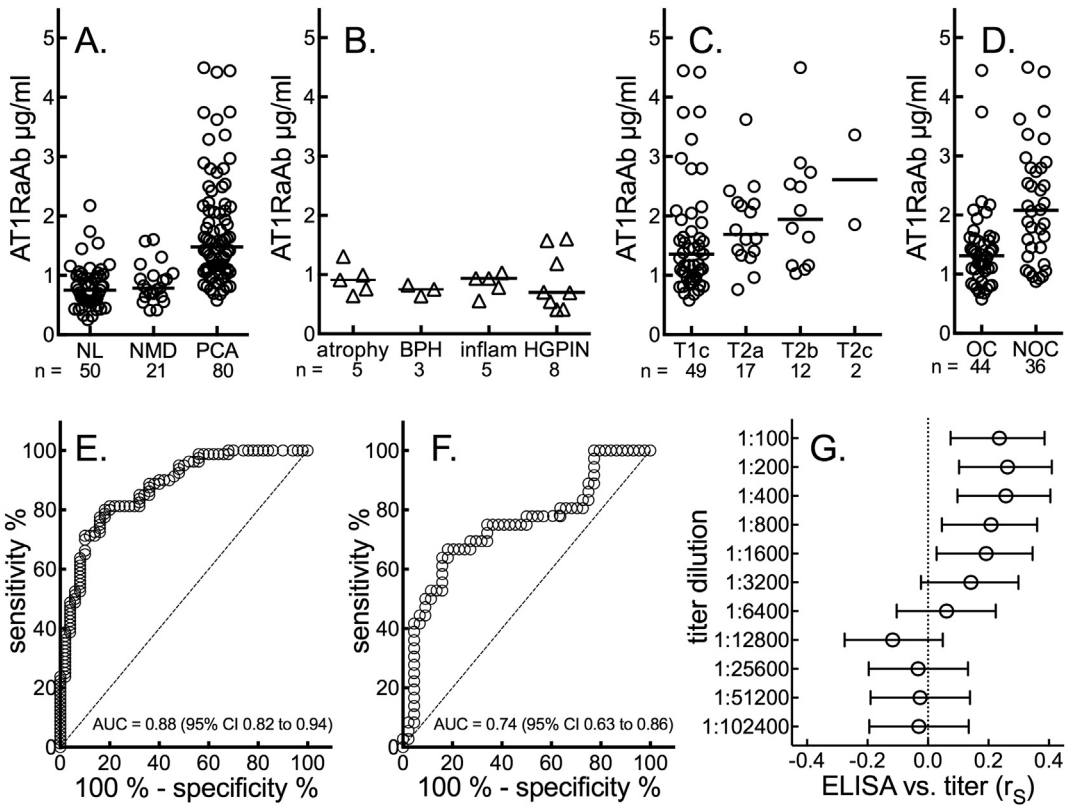

Fig. 4. AT1R autoantibody ELISA assay. AT1RaAb levels in serum from subjects who at biopsy were determined to be disease-free (NL, n = 50), have nonmalignant prostate disease (NMD, n = 21) or who were diagnosed with prostate cancer (PCA, n = 80) were quantified using an antigen capture ELISA. ELISA results were segregated into groups to compare the distribution of AT1RaAb values in (A) NL and PCA, (B) among NMD types, (C) different clinical stages of PCA, and (D) organ confined cancer (OC) and non-organ confined (NOC) cancer. Receiver operating characteristic curves were used to profile sensitivity and specificity for discriminating (E) PCA from NL and (F) NOC from OC PCA. The association between ELISA AT1RaAb levels and titer assay values ($A450/B_0$) were assessed by determining Spearman rank order correlations (r_S) values (G). Abbreviations: atrophy, prostatic atrophy; BPH, benign prostatic hyperplasia; inflam, prostate inflammation; HG-PIN, high-grade intraepithelial neoplasia; AUC, area under the curve.

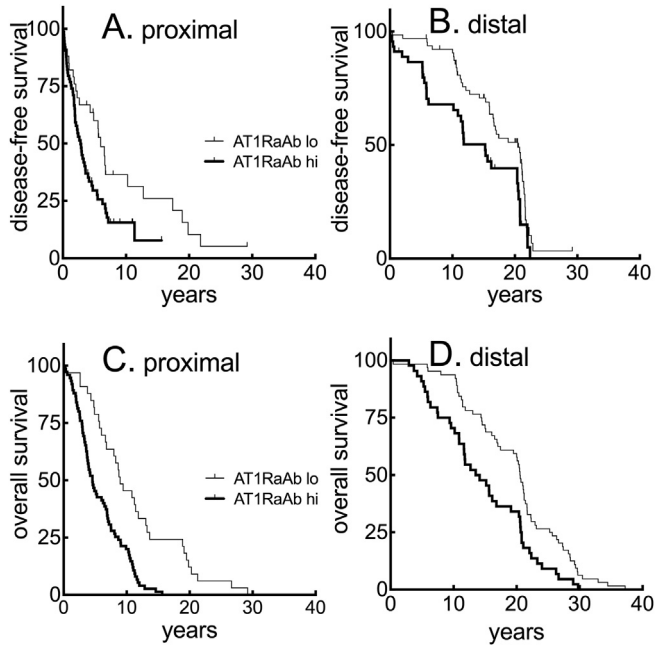

Fig. 5. Kaplan Meier modeling of disease-free survival and overall survival. Pre-diagnostic longitudinal samples were stratified to most proximal (A, C) and distal (B, D) to timed events (diagnosis, death). The exposure group was defined as "lo" AT1RaAb levels (≤1.04 µg/ml) or "hi" AT1RaAb levels (>1.04 µg/ml).

Table 3
Kaplan-Meier disease-free and overall survival and hazards ratios.

AT1RaAb[a]	Most proximal samples Median Survival		Most distal samples Median Survival	
	DFS (y)	OS (y)	DFS (y)	OS (y)
Low	4.9	9.0	16.2	20.6
High	2.0	4.6	11.8	15.6
# Events[b]	81	108	72	99
P value[c]				
Mantel-Cox	P < 0.005	P < 0.0001	P = 0.1	P < 0.05
Gehan-Breslow-Wilcoxin	P < 0.05	P < 0.0001	P = 0.06	P < 0.05
Tarone-Ware	P < 0.01	P < 0.0001	P = 0.08	P < 0.05
HR[d]				
Mantel-Haenszel, 95% CI	2.2 (1.4–3.5)	2.4 (1.6–3.6)	1.6 (1.0–2.5)	1.8 (1.1–2.8)
Log-rank, 95% CI	2.0 (1.4–3.4)	2.2 (1.7–3.5)	1.52 (1.0–2.5)	1.7 (1.2–2.8)

[a] Low AT1RaAb levels were ≤1.04 µg/ml and high values were >1.04 µg/ml.
[b] One sample in the proximal set and 10 samples in the distal set were omitted to match the data analyzed in the parametric survival estimates where missing clinical data precluded inclusion.
[c] Survival estimates between low and high groups were compared by Gehan-Breslow-Wilcoxin test, Mantel-Cox (logrank) and Tarone-Ware tests which give varying weights to early versus late events. The Gehan-Breslow-Wilcoxin test is less sensitive to late events when few subjects remain.
[d] HR, hazard ratio (95% confidence interval) by Mantel-Haenszel and log-rank method.

Table 4
Modeling AT1RaAb and survival by Ln (time to event).

		Time to diagnosis			% Decrease in DFS[a]	
	Model[b]	$\beta_{AT1RaAb}$	95% CI	P value	%	95% CI
proximal	1	−0.23	(-0.40 to −0.07)	<0.005	−21%	(-33 to −7)
	2	−0.22	(-0.38 to −0.04)	<0.01	−20%	(-32 to −4)
	3	−0.22	(-0.39 to −0.05)	<0.01	−20%	(-32 to −4)
	4	−0.22	(-0.39 to −0.05)	<0.01	−20%	(-32 to −5)
	5	−0.23	(-0.40 to −0.05)	<0.01	−20%	(-33 to −5)
distal	1	−0.16	(-0.24 to −0.09)	<0.0001	−15%	(-21 to −8)
	2	−0.10	(-0.17 to −0.04)	<0.005	−10%	(-16 to −4)
	3	−0.11	(-0.17 to −0.04)	<0.005	−10%	(-16 to −4)
	4	−0.11	(-0.17 to −0.04)	<0.005	−10%	(-16 to −4)
	5	−0.10	(-0.17 to −0.02)	<0.05	−9%	(-15 to −2)
		Time to death			**% Decrease in OS[c]**	
	Model	**$\beta_{AT1RaAb}$**	**95% CI**	**P value**	**%**	**95% CI**
proximal	1	−0.25	(-0.35 to −0.15)	<0.0001	−21%	(-29 to −13)
	2	−0.18	(-0.27 to −0.10)	<0.0005	−15%	(-23 to −8)
	3	−0.18	(-0.27 to −0.10)	<0.0005	−15%	(-23 to −8)
	4	−0.18	(-0.27 to −0.09)	<0.0005	−15%	(-23 to −8)
	5	−0.18	(-0.28 to −0.09)	<0.0005	−15%	(-24 to −8)
distal	1	−0.18	(-0.25 to −0.11)	<0.0001	−16%	(-22 to −10)
	2	−0.11	(-0.18 to −0.06)	<0.0001	−10%	(-17 to −6)
	3	−0.11	(-0.16 to −0.06)	<0.0001	−11%	(-15 to −6)
	4	−0.11	(-0.16 to −0.06)	<0.0001	−11%	(-15 to −5)
	5	−0.11	(-0.16 to −0.06)	<0.0005	−10%	(-15 to −5)

[a] DFS, disease-free survival.
[b] Model covariates: 1 = AT1RaAb alone, 2 = AT1RaAb + age at visit, 3 = AT1RaAb + age at visit + BMI, 4 = AT1RaAb + age at visit + BMI + BP, 5 = AT1RaAb + age at visit + BMI + BP + PSA.
[c] OS, overall survival.

activation [2]. A multifaceted approach was used to test the hypothesis that the presence of circulating AT1RaAbs facilitate disease progression by determining the (a) association between AT1RaAb levels and disease in a cross-sectional study, (b) temporal association between AT1RaAb exposure and disease progression in a longitudinal study and (c) biological effects of AT1RaAbs on human prostate cancer cell lines *in vitro* (Fig. 1).

4. Results

4.1. Cross-sectional sample set characteristics

Serum was obtained from a set of 151 subjects who were diagnosed with PCA, nonmalignant prostate disease (NMD) or no prostate disease (disease-free). Most of these subjects were Caucasian (92%) 1% were African American, 3% were Asian and 4% were unknown. Other characteristics of this cross-sectional group are given in Table 1. The NMD group was significantly older than either the normal ($P < 0.0001$) or PCA group ($P < 0.005$). Median PSA values differed between the disease-free and the NMD groups ($P < 0.0001$) and the disease-free and PCA groups ($P < 0.0001$). The NMD and PCA groups did not differ in median PSA values. Post RRP pathology determined that of the 80 subjects diagnosed with PCA, 6% had focal capsular penetration, 42% had established capsular penetration, 28% had positive surgical margins, 9% had seminal vesicle involvement, 8% had lymph node metastasis and 47% had non-organ confined disease.

4.2. AT1RaAb titer analysis

The serum samples were analyzed using a titer assay (Fig. 2). There was no difference between serum titer values ($A450/B_0$) from normal subjects and subjects diagnosed with NMD. Subjects found to have PCA exhibited a degree of overlap with disease-free and NMD titers. Separating PCA samples into organ confined (OC) versus non-organ confined (NOC) groups revealed that high titers were associated with NOC. The relationship between serum dilution, titer and discriminatory capacity was profiled by determining the sensitivity, specificity and odds ratio at various cut offs (Table 2). Overall, the titer assay exhibited a specificity ranging between 72 and 92% while the range in sensitivity was 40–75% at different dilutions and cutoffs. The risk of a subject with a positive 1:1600 titer ($A450/B_0 > 2.0$) having PCA was 6.3 (95% CI 2.2 to 18) times the risk of a subject with a negative titer having PCA. At a 1:1600 dilution, the risk of a subject with a titer >2.0 having NOC-PCA was 12 (95% CI 4.1 to 38) times the risk of a subject with a negative titer. An antigen capture ELISA was employed to examine the association of AT1RaAb with clinical covariates.

4.3. AT1RaAb ELISA analysis

4.3.1. AT1RaAb ELISA performance characteristics

A limit of blank of 0.8 ng/ml, lower limit of detection of 1.2 ng/ml and lower limit of quantification of 7.2 ng/ml were determined for the AT1RaAb ELISA following standardized procedures [29,30]. To assess both linearity and recovery, serum samples were separated into multiple aliquots, half of which were spiked with AT1RaAb standards. The spiked and unspiked samples were serially diluted and the levels of AT1RaAb measured. The measured ng/ml values were tightly linear (Fig. 3A). The average recovery across the five dilutions was 101 ± 4%. To determine the effect of repetitive freezing and thawing of serum on immunoassay results, two samples were subjected to 7 freeze/thaw cycles (Fig. 3B). From the first through the fifth freeze/thaw cycle, the average value was 3.7 ± 0.1 μg/ml (<10% variance). After 5 freeze thaw cycles, the value dropped. Thus, the number of freeze/thaw cycles that serum samples could have gone through was restricted to ≤5.

Included in each assay was control serum with an AT1RaAb level of 1.9 μg/ml which was used to verify that the calculated values were within an acceptable range, defined as between 1.6 and 2.2 μg/ml. Over a ten month period, the value of this control fell within the acceptable limit with the exception of two assays (Fig. 3C). Additionally, the control samples enabled calculation of an inter-assay coefficient of variance (% CV). Over the same ten month time period, three assay dates yielded an inter-assay %CV that was unacceptable, i.e. >10% (Fig. 3D). A failure of either criteria (control μg/ml value in a given assay or inter-assay %CV) necessitated a rerunning of the samples and assay.

4.3.2. AT1RaAb ELISA results

The AT1RaAb ELISA was applied to the 151 cross sectional serum samples. AT1RaAb serum values in NMD were not significantly different from normal serum values (Fig. 4A and B, Table 2). AT1RaAb serum values in PCA with a median of 1.51 and range of 0.58–4.50 μg/ml, were significantly different from those in normal or NMD ($P < 0.0001$). AT1RaAb levels in PCA samples were not significantly correlated with disease stage (Fig. 4C). AT1RaAb levels in OC-PCA and NOC-PCA were significantly different, $P < 0.0001$ (Fig. 4D).

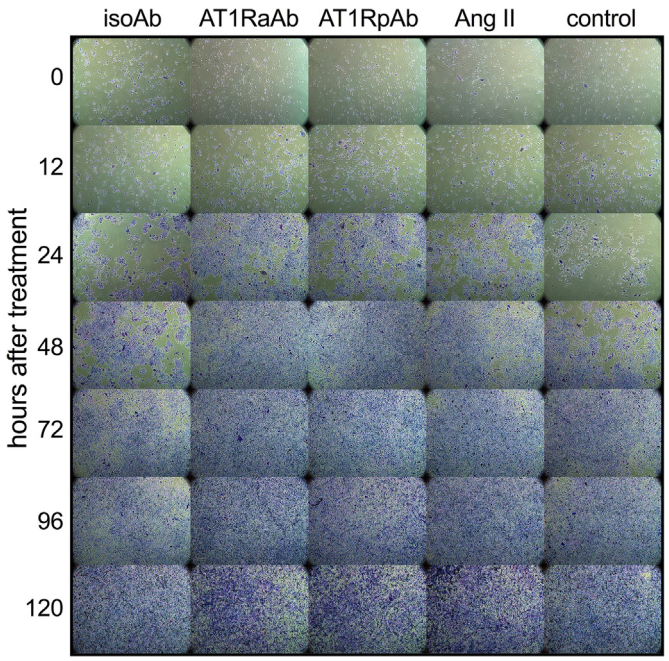

Fig. 6. AT1R antibodies and PC3 cell proliferation. The proliferation of PC3 cells treated with 50 nM human IgG isotype antibody control (isoAb), 50 nM human-derived AT1R autoantibody (AT1RaAb), 50 nM rabbit-derived AT1R antibody (AT1RpAb), 50 nM angiotensin II (Ang II) or control was followed by crystal violet staining of fixed cells in triplicate wells at different time points.

The discriminatory capacity of the ELISA was investigated by ROC curve analysis. The area under the curve (AUC) was 0.88 (95% CI 0.82 to 0.94), $P < 0.0001$ for distinguishing between normal and PCA (Fig. 4E). For discriminating between OC-PCA and NOC-PCA the AUC was 0.74 (95% CI 0.63 to 0.86), $P < 0.0005$ (Fig. 4F). Serum level cut offs, sensitivity, specificity and odds ratios are given in Table 2. The risk of an individual with AT1RaAb levels >1.04 μg/ml being diagnosed with PCA was over 18 times the risk for individuals with lower AT1RaAb levels. When AT1RaAb levels were >1.04 μg/ml the odds ratio for a subject diagnosed with NOC-PCA was 2.1 (95% CI 0.7 to 6.8). The odds ratio for NOC-PCA was 7.7 (95% CI 2.8 to 22) when AT1RaAb levels were >1.7 μg/ml.

The relationship between the titer assay results ($A450/B_0$) and the ELISA results (μg/ml) were investigated by Spearman rank order correlation. AT1RaAb levels determined by the ELISA were significantly correlated with titer values up to the 1:3200 dilution where the Spearman rank order correlation (r_s values) was 0.15 (95% CI 0.30 to −0.02) with a P value of 0.08 (Fig. 4G). Serum levels of AT1RaAb by ELISA were also significantly correlated with several clinical and pathological variables based on r_s values. AT1RaAb levels were correlated with PSA ($r_s = 0.32$, 95% CI 0.16 to 0.46, $P < 0.0001$) and with post treatment pathological findings of RRP Gleason score ($r_s = 0.31$, 95% CI = 0.09 to 0.50, $P < 0.05$), established capsular penetration ($r_s = 0.38$, 95% CI = 0.17 to 0.56, $P < 0.0001$), surgical margin status ($r_s = 0.28$, 95% CI 0.06 to 0.48, $P < 0.05$), and OC status ($r_s = -0.38$, 95% CI = −0.56 to −0.17, $P < 0.001$). AT1RaAb serum levels were not correlated with age, focal capsular penetration, seminal vesicle or lymph node involvement. Given the lack of racial diversity in the sample donors, the association of AT1RaAb levels with race could not be assessed. There was no significant association between AT1RaAb levels and the time interval between blood draw and biopsy. Furthermore, there were no significant differences between the levels of AT1RaAb in samples drawn before, at or after biopsy indicating that the procedure did not influence AT1RaAb levels (data not shown). A summary table reporting the cross-sectional study design and results following the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [54] is given in Appendix A. Table 5 A dataset containing all the cross-sectional data is available at [55].

4.4. Longitudinal sample set analysis

4.4.1. Longitudinal sample set characteristics

The association of elevated AT1RaAbs with PCA and especially NOC status is consistent with research indicating that Ang II activation of AT1R promoted prostate cancer cell growth and spread [18–20]. The association could also occur because the cancer is out and about and provoking the immune system and thus the autoantibodies could be reflecting the immune response to the cancer. Since most of the samples in the cross sectional set were obtained around the time of diagnosis, this sample set does not directly address associations with disease progression. The temporal nature of associations were investigated by using pre-diagnosis serum samples from 109 men from the BLSA who were disease-free on enrollment and longitudinally followed until death. Most were Caucasian (97%) while the remaining were African American. These samples were, on average, from older subjects at time of visit with a broader age range (33–95 years) than in the cross sectional sample set. Median values of PSA and AT1RaAb also exhibited a much broader range (0.2–352 ng/ml and 0.10–8.99 μg/ml, respectively) than the cross sectional sample set. The median (and range) for time to diagnosis was 6.6 (0.1–25.6) years and for time to death was 11.0 (0.2–40.0) years.

Longitudinal samples from each subject were stratified into most proximal or distal to time of diagnosis and time to death. Group characteristics by relative time point are given in Table 1. AT1RaAb and PSA levels were significantly higher at the most proximal time point, $P < 0.001$ by Wilcoxon matched pairs signed rank test. BMI was not different between the two groups, while systolic and diastolic blood pressure were significantly different between proximal and distal time points, $P < 0.05$. AT1RaAb levels were significantly correlated with a number of measures. For the proximal group, AT1RaAb and age at visit had a r_s value of 0.24 (95% CI 0.05 to 0.42) with $P < 0.01$ while AT1RaAb and PSA had a r_s value of 0.24 (95% CI 0.04 to 0.42) with $P < 0.05$. AT1RaAb and time to diagnosis had a r_s of −0.34 (95% CI -0.53 to −0.13), $P < 0.005$ while AT1RaAb and time to death had a r_s of −0.40 (95% CI -0.55 to −0.22), $P < 0.0001$. In the distal group, AT1RaAb and age at visit exhibited a significant association with a r_s of 0.25 (95% CI 0.05 to 0.42), $P < 0.01$. AT1RaAb and PSA were not significantly correlated in the distal group. AT1RaAb and time to diagnosis had a r_s value of −0.37 (95% CI -055 to −0.16) with $P < 0.001$ and a r_s value of −0.35 (95% CI -0.51 to −0.17) with $P < 0.0005$ for AT1RaAb and time to death in the distal group.

4.4.2. Longitudinal sample set Kaplan-Meier estimates

AT1RaAb levels were segregated into high (>1.04 μg/ml, using the cut off identified in the cross sectional sample set) versus low (≤1.04 μg/ml) and DFS and OS were profiled by Kaplan-Meier survival curves (Fig. 5). High AT1RaAb levels were significantly associated with reduced DFS in the most proximal samples and with reduced OS in both proximal and distal samples (Table 3). Comparisons between low and high AT1RaAb groups for DFS in the distal samples were not significant. Median DFS and OS were reduced by approximately half in proximal samples with high AT1RaAb levels, while median OS was reduced by one-quarter in distal samples. The Log-rank and Mantel-Haenszel tests yielded similar hazard ratios within each group. For proximal DFS, a subject with high AT1RaAb who had not been diagnosed with PCA at a given time point had twice the probability of being diagnosed by the next time point compared to a subject in the low AT1RaAb group. OS hazard ratios were over 2.0 for the proximal group. For the distal group, a subject with high AT1RaAb had a 1.7 to 1.8 probability of dying by the next time point compared to subjects with low AT1RaAb.

4.4.3. Longitudinal sample set accelerated failure model estimates

An accelerated failure model was used to assess the effect of other

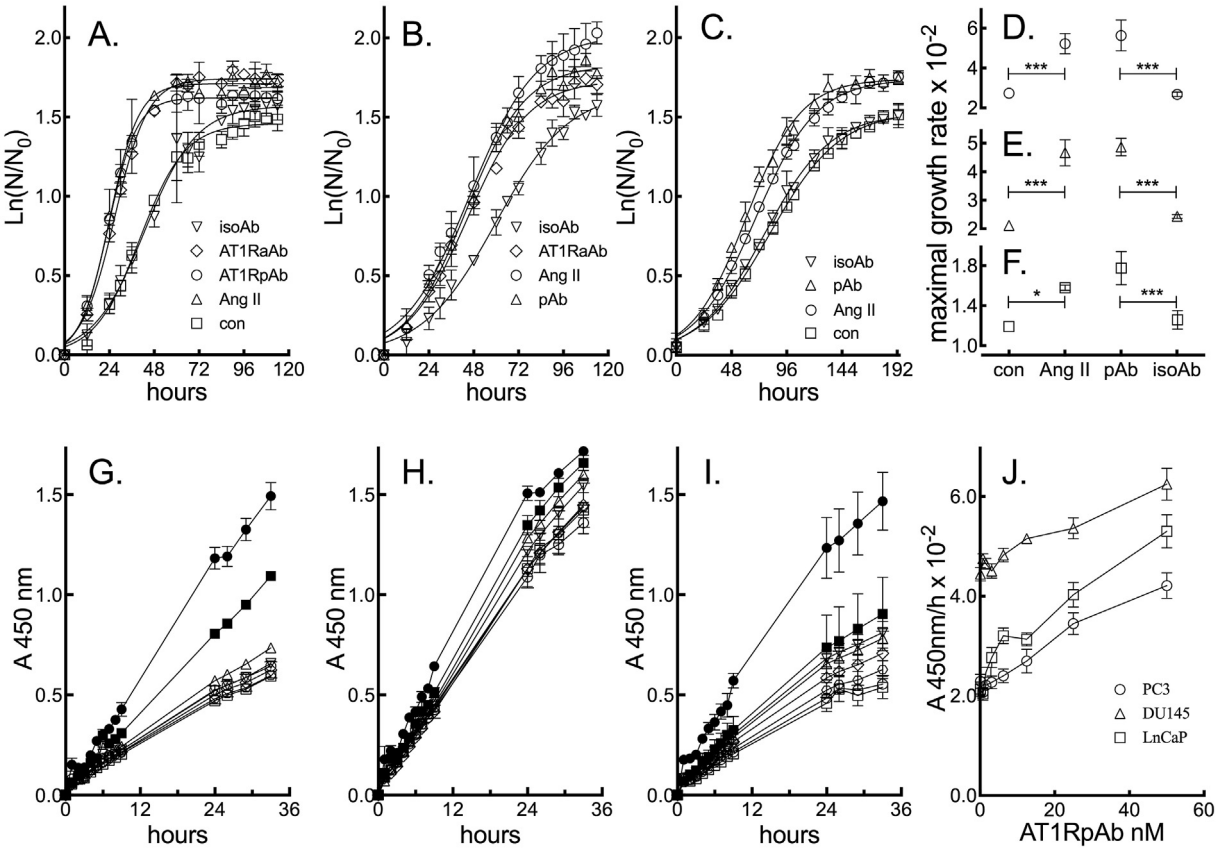

Fig. 7. AT1R antibodies and proliferation. The growth curves of (A) PC3, (B) DU145 and (C) LNCaP cells treated with 50 nM human-derived AT1RaAb, rabbit-derived AT1RpAb (pAb), human IgG isotype control antibody (isoAb), angiotensin II (Ang II) or control. Maximal growth rates were determined for (D) PC3, (E) DU145 and (F) LNCaP. The means of preselected treatments (control and AngII, isoAb and AT1RaAb) were compared by ANOVA with Sidak's multiple comparisons test. WST-8 reduction by cellular dehydrogenases in (G) PC3, (H) DU145 and (I) LNCaP cells treated with 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.12 nM, 1.56 nM, 0.78 nM or 0 nM AT1RpAb was also followed as a continuous time course. Error bars represent standard deviation. (J) The linear rates of dehydrogenase activity (ΔA450 nm/hour) over the first 9 h were plotted as a function of AT1RpAb dose for PC3 (circle), DU145 (triangle) and LNCaP (square) cells. Error bars represent standard error of the slope (J).

covariates on the association of AT1RaAb levels with survival. All subjects in the longitudinal sample set are deceased and the date of death was known for everyone in the study. Additionally, post-mortem pathological analysis of subjects was performed to identify occult PCA in subjects who at the time of death did not have a diagnosis of PCA. Time to death was known for all subjects/samples as was the disease status of the prostate at time of death. Thus, censoring of data was not necessary. A special case of the accelerated failure model, the natural log of event time without censoring was used to model survival and the parameters of the model were estimated using a conventional linear regression approach at either proximal or distal time points. Solving for $\beta_{AT1RaAb}$ enabled the % change in time to event to be determined as e^{β}AT1RaAb - 1 while other predictors (age at visit, BMI, systolic blood pressure and PSA) were added to the model (Table 4). Data for disease stage were missing in 25 subjects, so staging was not studied in the model. Clinical data was missing for one proximal sample and ten distal samples so those samples were omitted from analysis. The minimal model for DFS at the most proximal time point had a $\beta_{AT1RaAb}$ of −0.23 (95% CI -0.40 to −0.07, $P < 0.01$) which corresponds to a 21% reduction in DFS for every 1 μg/ml increase in AT1RaAb. Adding age of visit, BMI and BP had little effect as $\beta_{AT1RaAb}$ became −0.22 (95% CI -0.39 to −0.05, $P < 0.05$) and reduction in DFS per μg/ml increase in AT1RaAb was 20% at the proximal time points. At the distal time points using the minimal model, the reduction in DFS was 15% for a 1 μg/ml increase in AT1RaAbs and adding age of visit to the model altered the reduction in DFS to 10%. Further adjustments for BMI, systolic BP and PSA had minimal effect as reduction in DFS per μg/ml increase in AT1RaAb was 10% for the distal time points. The base model for the association of AT1RaAbs with OS yielded a 22% reduction for proximal time to death and 16% for distal time point time to death. Adjusting for age at visit, yielded a 17% and 10% reduction in time to diagnosis at proximal and distal time points, respectively. Further adjustments for BMI, BP and PSA had minimal effect on $\beta_{AT1RaAb}$ values and percent change in overall survival (Table 4). A summary table reporting the longitudinal study design and results following the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines is given in Appendix B. Table 6 A dataset containing all the longitudinal data is available at [55].

4.5. AT1R antibody effects on prostate cancer cell proliferation

To investigate potential biological actions of AT1RaAbs, 50 nM affinity purified AT1R antibodies (AT1RaAb - from human serum, or AT1RpAb - from rabbits immunized with CAFHYESQN), an isotype control antibody, Ang II or vehicle alone (control) were added to PC3 cells and growth curves profiled by crystal violet staining (Fig. 6). The growth curves for the AT1RaAb and AT1RpAb treatment were not significantly different, but they were shifted to the left of isotype antibody and control treated cultures (Fig. 7A). Since both AT1R antibodies caused a similar magnitude of effect, all subsequent assays were performed using AT1RpAb. Two other PCA cell lines, DU145 and LNCaP, were treated with AT1RpAb, isotype antibody, Ang II and control and the growth curves analyzed. Both cell lines exhibited a shift in growth curves towards faster proliferation with AT1RpAb or Ang II treatment (Fig. 7B and C). The increase in maximal growth rates for cells treated with

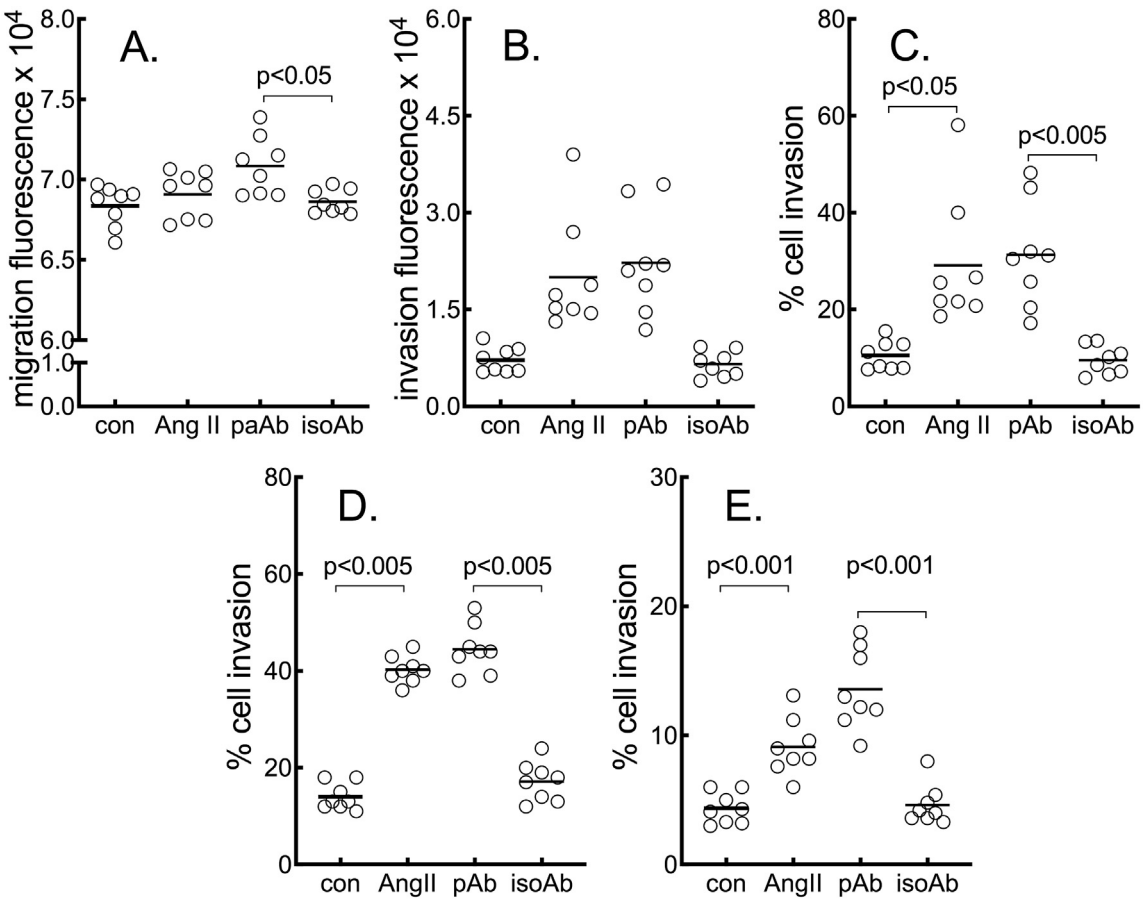

Fig. 8. AT1R antibodies and invasive phenotype. Fluorescently labeled prostate cancer cell lines were treated with 50 nM isotype antibody (isoAb), AT1RpAb (pAb), angiotensin II (AngII), or control and the fluorescent signal from PC3 cells that traversed both (A) uncoated and (B) matrigel coated membranes was measured. The % invasion for (C) PC3, (D) DU145 and (E) LNCaP were determined for each condition. The invasion results are from two separate experiments with quadruplicate wells for each condition. The heavy horizontal line represents the mean. The means of preselected treatments (control and Ang II, isoAb and AT1RaAb) were compared by ANOVA with Sidak's multiple comparisons test.

AT1RaAb (4.9 ± 0.3) and AT1RpAb (5.6 ± 0.8) were significant ($P < 0.0001$) relative to isoAb treatment (2.7 ± 0.1) and the increase in maximal growth rates for Ang II treated cells was significant ($P < 0.0001$) compared to control (Fig. 7D). Treatment with AT1RpAb and Ang II significantly increased ($P < 0.005$) maximal growth rates for DU145 cells (Fig. 6E). LNCaP cells also exhibited increased maximal growth rates with AT1RpAb ($P < 0.0005$) and Ang II ($P < 0.05$) treatments (Fig. 7F).

An assay measuring the activity of cellular dehydrogenases that is directly proportional to the number of living cells was employed to measure the dose response of PC3, DU145 and LNCaP cells to AT1RpAb treatment. PC3 and LNCaP cultures exhibited a broad linear response while DU145 cells exhibited a higher basal rate of dehydrogenase activity (Fig. 7G, H and I). The rate of WST-8 reduction over 9 h was used to profile the relative rates of dehydrogenase activity as the change in WST-8 absorbance at 450 nm per hour (Fig. 7J) in response to AT1RpAb dose. The correlation between dose and dehydrogenase activity was significant ($P < 0.0001$) for all three cell types with a r^2 value of 0.98 for PC3, 0.96 for DU145 and 0.94 for LNCaP.

4.6. AT1RpAb effects on cancer cell invasive phenotype

The prostate cancer cell lines were used to study the effect of AT1RpAb on an invasive phenotype (Fig. 8). When PC3 cells were treated with control, Ang II, AT1RpAb, or isotype antibody, the relative fluorescence values for PC3 invasion through matrigel were significantly different between Ang II and control as well as between AT1RpAb and isoAb. Relative fluorescence for migration was only significantly different between AT1RaAb and isoAb. Determining the % cell invasion for all three cell types revealed similar results where Ang II and AT1RpAb treatment increased invasion between two- and three-fold relative to control and isotype antibody, respectively.

5. Discussion

The role of tumor extrinsic factors such as host immune function has become a productive venue for both diagnostics (eg. autoantibodies against tumor-associated antigens, TAAs) and therapeutics (eg. engineered drug conjugated TAAs, chimeric antigen receptor T cell therapy) [31–33]. The paradigm for TAAs are intracellular or cell-surface cancer cell components which are either overexpressed, expressed in a novel location, or have an altered structure or sequence which yields an immunogenic response. In the current study, autoantibodies that can predate clinical disease, correlate with time to diagnosis and time to death and have receptor agonistic activity were observed. This suggests that AT1RaAbs are distinct from TAAs and may be informative biomarkers.

As noted earlier in section 1, serum levels of AT1RaAb have been associated with a number of different diseases. Biomarkers associated with multiple pathologies will have reduced specificity. A potential common thread underlying these diseases is chronic immune activation/inflammation [34–38]. It must be noted that chronic inflammation has been associated with other disease states where correlations with AT1RaAb have not been reported (e.g. - stroke, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease). A common

observation from studies measuring AT1RaAbs using different assays has been significant overlap in levels between those with and without a particular disease. For example, see Figs. 2 and 4 in the current results and data in other publications [10–12,27,39,40]. Biomarkers with a wide distribution and an appreciable overlap between groups will have diminished diagnostic capacity. Thus, AT1RaAbs are not ideal diagnostic markers. They are, however potential candidates for use in risk stratification for disease progression. The most common clinical use of measured AT1RaAbs is as an adjunct to HLA typing in screening to assess risk of allograft dysfunction [41].

The observed associations and actions of AT1RaAbs in this current study are consistent with reports that AT1R, angiotensin II converting enzyme, and angiotensinogen are overexpressed in hormone refractory prostate cancer tissue [21] and that in other cancer types, AT1R has been implicated in cancer cell proliferation, survival, migration and invasiveness [42–45]. Additional *in vitro* works has shown that Ang II, the normal ligand for AT1R, stimulates LNCaP and PC3 prostate cancer cell line proliferation and invasiveness [20] and that reducing AT1R expression inhibits cell growth in LNCaP cells [46]. The presence of activating antibodies targeting AT1R may be expected to enhance AT1R effects on cancer. An elevated AT1RaAb titer in serum was associated with ovarian cancer stage and grade and an enriched pool of patient IgGs stimulated cancer cell migration and angiogenesis [47].

In our cross sectional study of samples obtained around the time of diagnosis, higher serum AT1RaAb levels were associated with PSA levels and pathological markers of disease. In our longitudinal study of pre-diagnosis samples, those most proximal to diagnosis samples exhibited significant association between AT1RaAb and PSA. However, adjusting for PSA in the accelerated failure model did not attenuate the association of AT1RaAbs with DFS or OS. The lack of an effect when adjusting for PSA in the accelerated failure estimates could reflect that the information content of AT1RaAbs is distinct from that of PSA. An invasive phenotype, defined by increased cell invasion was observed after AT1RpAb treatment of three PCA cell lines. This is consistent with published stimulatory effects of Ang II on cancer cell invasion [48–50].

The limitations to the cross sectional sample set study include an imbalance in sample size of different types of NMD as well as different cancer stages. Although all samples were obtained prior to treatment, the time between serum draw and biopsy was variable. Prostate biopsies have been associated with increased local inflammation and infection [51–53] and this could significantly influence an immune-related biomarker. In the current study there was no significant difference in AT1RaAb levels obtained before or after biopsy. Limitations to the longitudinal sample set study include a relatively modest sample size ($n = 109$ subjects, 218 samples) where 11 samples were omitted from accelerated failure modeling due to missing PSA values. Ten of those samples were in the most distal to diagnosis group which may have reduced the estimates power to detect associations. 31% of samples from subjects eventually diagnosed with PCA lacked staging data, precluding the inclusion of staging in the accelerated failure modeling. The analysis of a larger longitudinal sample set would enable a number of these concerns to be addressed. Limitations to the *in vitro* work include the nature of the surrogate markers used. It is possible that AT1RpAb treatment effects the energy metabolism and reductive potential of the cell, complicating the interpretation of the dehydrogenase activity assay as a surogate for proliferation.

6. Conclusion

Serum AT1RaAb levels correlated with prostate cancer status and clinical and pathological measures in a cross sectional study and with DFS and OS in a longitudinal study at proximal and distal timed events. The *in vitro* presence of AT1RaAbs modified cancer cell behavior. These results are consistent with host agonistic autoantibodies acting as disease modifying agents (exposures) that bind to and chronically activate a specific G protein coupled receptor resulting in conditions that favor cancer expansion, survival and malignancy. Varying exposure to AT1RaAbs may be one of the factors that contribute to disease heterogeneity in progression rates.

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Appendix A and B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2019.100008.

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