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Original Article

E-cadherin expression is inversely correlated with aging and inflammation in the prostate

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Abstract: Introduction and Objective: Benign prostatic hyperplasia (BPH) is a prostatic disease that is significantly associated with aging. However, it is not well understood how aging contributes to BPH pathogenesis. Several factors associated with an increased risk of BPH are also associated with increasing age, including chronic inflammation and declining epithelial barrier function. Thus, this study explored the potential associations between aging, loss of adherens junction protein E-cadherin and the presence of inflammatory mediators in prostate tissue specimens from healthy young donor and BPH patients. Methods: Serial prostate sections from a cohort of five donors aged 15-26 years and 13 BPH patients aged 50-77 years were immunostained with E-cadherin, COX-2, CD4, CD8, CD20 and CD68. E-cadherin and COX-2 H-Scores and the number of inflammatory cells were calculated for the same area in donor, normal adjacent prostate to BPH (NAP) and BPH specimens. Quantification and statistical correlation analyses were performed for comparisons between groups. Results: E-cadherin was decreased in aged NAP tissues and in BPH compared to young donor tissue. E-cadherin was inversely correlated with age and infiltration of inflammatory cells in NAP compared to young healthy donor prostate. Stromal COX-2 was positively correlated with age and inflammation. E-cadherin was further down-regulated in BPH, while COX-2 H-Scores were not significantly altered in BPH compared to NAP. Conclusions: These findings suggest that aging is associated with down-regulation of E-cadherin and up-regulation of stromal COX-2 immunostaining in the prostate. E-cadherin immunostaining was inversely associated with age and inflammation, while stromal COX-2 immunostaining was positively associated with age and inflammation in the prostate. These findings suggest that the prostate epithelial barrier is altered and inflammation is increased with age in the prostate. These changes are further exacerbated in BPH, and may be involved in BPH pathogenesis.

Keywords: E-cadherin, COX-2, prostatic inflammation, BPH, aging

Introduction

Aging is associated with an increase in inflammation and a decline in epithelial barrier function in various organs, potentially contributing to the development of age-related diseases. Age-dependent changes in cell adhesion complexes have been associated with aging and an increase in barrier permeability (Reviewed in [1]). Decreased function and repair of the aging epithelial barrier is likely due to multiple mechanisms and could contribute to the development and/or progression of age-related diseases. Studies of epithelial barrier function in the prostate and the impact of aging are limited. However, in age-related prostatic diseases such as benign prostatic hyperplasia (BPH), adherens junction protein E-cadherin down-regulation is frequently observed [2-6] and has been associated with inflammation [7, 8]. E-cadherin is an adherens junction protein expressed by both basal and luminal prostate epithelial cells [9] that is important for maintaining cell-cell adhesion and is critical for development and maintenance of the prostate epithelium [10-12]. We previously reported the

presence of prostate secretory proteins in the stromal compartment of BPH, but not in prostate cancer or normal adjacent prostate (NAP), suggesting that the epithelial barrier in BPH glands may be altered [6]. In benign prostatic epithelial cell lines BPHrE1 and BPH-1, knockdown of E-cadherin induced an increase in epithelial barrier permeability and a decrease in the formation of tight junction 'kiss points' [4], suggesting that E-cadherin down-regulation in BPH might result in a leaky epithelial barrier.

Systemic inflammation has been postulated to promote the development and progression of chronic disease in an age-independent manner [13, 14]. Increased inflammatory cell infiltration in BPH tissues has previously been reported by many groups [15-19], however it is not clear whether prostatic inflammation increases with age and contributes to the development of BPH, or if it is a consequence of BPH. In a recent report, we found that compared to young donor tissues, older NAP tissues displayed an increase in the infiltration of CD20 positive B-lymphocytes [20], suggesting that B-lymphocyte infiltration increased with age in the prostate. $CD45^+$ immune cells were positively correlated with age in the human prostate suggesting that increased prostatic inflammation is a consequence of aging [21], and increased infiltration of lymphocytes and macrophages with age has also been reported in the murine prostate [21, 22]. Increased inflammatory cell infiltration in BPH tissues has previously been reported by many groups. In a recent study, periglandular inflammation was observed in 94.7% of BPH patients, stromal inflammation was observed in 88.2% of patients and glandular inflammation was observed in 75.0% of a cohort of 76 BPH patients and the authors reported that 100% of BPH patients displayed infiltrating inflammatory cells [17]. The predominant infiltrating inflammatory cell type in BPH tissue was T-lymphocytes, which was identified in 96.1% of all specimens, followed by B-lymphocytes in 77.6% and macrophages in 52.6% [17]. Previous studies revealed that inflammatory infiltrates in BPH tissues predominantly consisted of T-lymphocytes and a smaller percentage of B-lymphocytes and macrophages [18, 19]. We recently reported that BPH tissues had an increased infiltration of CD4 and CD8 positive T-lymphocytes and CD68 macrophages compared to NAP [20].

Increased epithelial permeability and the presence of secretory proteins in the prostate stroma might induce stromal inflammation, proliferation or fibrosis, which are also frequently observed in BPH [23, 24]. In the rat model, formalin-induced prostatic inflammation resulted in activation of the transforming growth factor beta 1 (TGF-β1) signaling pathway, increased stromal COX-2 expression and down-regulation of E-cadherin immunostaining in the prostate, and subsequent bladder overactivity [25]. Furthermore, treatment of formalin-induced rats with cyclooxygenase-2 (COX-2) inhibitor reduced prostatic inflammation and improved bladder function [25, 26]. In benign prostate epithelial cell lines BHPrE1 and BPH-1, stimulation with TGF-β1 induced an increase in epithelial barrier permeability and a decrease in tight junction 'kiss points' [27, 28], suggesting that inflammation could contribute to the down-regulation of E-cadherin. Chronic prostatic inflammation and increased TGF-β1 have been associated with age-related prostatic disease and the onset of lower urinary tract symptoms (LUTS) (Reviewed in [29]). Decreased COX-2 expression in normal prostate compared to BPH has been previously reported [30]. Furthermore, BPH patients with inflammation were shown to be at increased risk of clinical progression to acute urinary retention or urinary incontinence [31]. Down-regulation of junction proteins such as E-cadherin and increased COX-2 may be associated with a decline in prostate epithelial barrier function and prostatic inflammation, thus contributing to the pathogenesis of BPH.

We recently reported that prostate luminal epithelial cell-specific homozygous deletion of E-cadherin in the murine prostate induced prostatic inflammation and subsequent bladder overactivity [32, 32]. Mice with prostate luminal epithelial cell-specific *Cdh1* deletion developed prostatic inflammation and hyperplasia, as well as changes in bladder voiding function consistent with lower urinary tract symptoms displayed by BPH/LUTS patients [32]. The finding that E-cadherin deletion could induce prostatic inflammation in a murine model suggests that E-cadherin down-regulation in the prostate could play a significant role in BPH pathogenesis. However, it is unclear what mechanisms are involved in E-cadherin down-regulation in the prostate. Thus, we sought to determine whether E-cadherin down-

Table 1. Demographics of human prostate tissue specimens

	Donor	NAP	BPH
No. patients	5	8	13
Mean age (range)	20.2 (15-26)	62 (50-77)	62.5 (50-77)
Race (%)			
African-American	0 (0%)	4 (50%)	7 (54%)
Caucasian	4 (80%)	4 (50%)	6 (46%)
Unknown	1 (20%)	0 (0%)	0 (0%)

NAP: normal adjacent prostate; BPH: benign prostatic hyperplasia; No.: number.

regulation and prostatic inflammation were associated with aging in the human prostate.

Here, we explored the potential association of E-cadherin, COX-2 and inflammation in prostate tissue specimens from young healthy donor and BPH patients. Immunostaining of E-cadherin, COX-2, CD4, CD8, CD20 and CD68 was performed and scored in serial sections from donor, normal adjacent prostate to BPH (NAP) and BPH tissues for comparison and correlation analyses.

Materials and methods

Patient cohort

The experimental cohort of prostate tissue specimens included 14 patients who received transurethral resection of the prostate or simple prostatectomy for symptomatic BPH, and 6 young healthy organ donors [20]. None of these patients had any prior history of chemo-, radio-, or hormone therapy. BPH specimens were composed of mixed hyperplastic nodules with both glandular and stromal expansion. Any case with the presence of moderate to severe prostatitis was excluded. The criteria used for defining moderate prostatitis was the presence of inflammatory cells in the prostate stroma infiltrating into prostate glands with or without the presence of crypt abscess formation. Two patients displayed prostatitis, one donor and one BPH, and were therefore not included in the analyses. The "deidentified" specimens were retrieved from the clinical files of UPMC by the University of Pittsburgh Biospecimen Core (PBC) with approval from the University of Pittsburgh Institutional Review Board for this research project under protocol #17010177. PBC also provided deidentified pathology reports for the patients constituting the study cohort through their IRB-approved Honest Broker System, HB015, to ensure research integrity. All participating patients or their next of kin provided informed consent for the banking protocol. Patient demographics for the analyzed cohort were listed in **Table 1**.

Histopathologic and immunohistochemistry analyses

Samples were fixed in 10% formalin for at least 24 hrs, then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Immunohistochemical staining was performed on five-micron sections of paraffin-embedded prostate tissue specimens as described [4]. Briefly, sections were deparaffinized and rehydrated through a graded series of ethanol. Heat-induced epitope retrieval was performed using a BioCare Decloaking Chamber (Biocare Medical, Pacheco, CA), followed by 5 minutes rinsing in TBS buffer. Primary antibodies for immunostaining of prostate tissue sections were rabbit monoclonal anti-E-cadherin (ab76319, EP913(2)Y, Abcam, Cambridge, MA, USA) and anti-rabbit polyclonal COX2/Cyclooxygenase 2 antibody (ab15191, abcam, Cambridge, UK), rabbit monoclonal anti-CD4 (EPR6855, ab133616, Abcam), CD8 mouse monoclonal anti-CD8 (144B, M7103, Dako), mouse monoclonal anti-CD20 (L26, 14-0202-82, Invitrogen, Carlsbad, CA, USA), and mouse monoclonal anti-CD68 (KP1, CM033, BioCare Medical). Serial sections were cut and stained using the following scheme: slide 1 was double stained with CD4 and CD8; slide 2 with CD20 and CD68, slide 3 with E-cadherin and COX-2, and slide 4 with H&E. Slides were then counterstained in hematoxylin and cover-slipped. Stained sections were imaged with a Leica DM LB microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped with an Imaging Source NII 770 camera (The Imaging Source Europe GmbH, Bremen, Germany) and NIS-Elements Documentation v 4.6 software (Nikon Instruments, Inc., Mellville, NY). All tissues were examined by a board-certified genitourinary pathologist (R.D.) using light microscopy. Scores for each antibody were determined for prostate glands in the same field for all slides by two independent investigators (LEP and CNH).

Statistical analysis

Summary statistics of donor, NAP, and BPH are presented. Due to the small sample size in all three groups, the group comparisons between donor vs. NAP and BPH vs. NAP for all outcomes was presented with median and inter-quartile range values, and the significance was tested using the Wilcoxon signed-rank test. The Pearson correlation coefficient was used to determine the correlation between E-cadherin and COX-2 immunostaining with age and infiltration of inflammatory cells in the combined samples from groups donor vs. NAP. A simple linear regression analysis was used between the dependent variables (outcomes) and the group of the independent variable. A multiple regression method was used to include the predictor variable 'Age' along with the group. Several regression models were built to analyze the significance of the primary predictor group and age. A type I error or a p value < 0.05 was used as a threshold to identify statistical significance. Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). GraphPad Prism version 9 was used for graphics (GraphPad Software, San Diego, CA, USA). Values were expressed as means ± S.D.

Results

Expression of adherens junction protein E-cadherin and COX-2 in the aging prostate and in BPH

Previously, we and others have reported that E-cadherin was down-regulated in BPH compared to NAP [3, 4]. However, no previous reports identified in the literature have examined the impact of aging on E-cadherin or COX-2 expression in the prostate. An initial cohort of six donor (aged 15-26 yrs) and 14 BPH patients (aged 50-77 yrs) was selected for this study (**Table 1**). Two patients displayed prostatitis (one donor and one BPH patient) and were removed from the dataset, and a total of five donor and 13 BPH patient specimens were analyzed. Of the 13 BPH patients, eight had normal adjacent prostate tissues within the specimen which were analyzed and included as NAP.

In young donor prostate tissues, intense membrane E-cadherin immunostaining was observed in the basal and luminal epithelial cells of prostate glands (**Figure 1A**, left panels). NAP glands in BPH patients aged 50-77 years displayed a similar staining pattern with decreased intensity compared to donor tissues (**Figure 1A**, center panels), while E-cadherin immunostaining intensity in BPH glands was decreased even further (**Figure 1A**, center and right panels). Quantification of the H-Score for each tissue type confirmed that E-cadherin expression was decreased with age in normal tissues and was further decreased in BPH tissues (**Figure 1B**).

Basal epithelial and stromal COX-2 immunostaining was previously reported in benign tissues of prostate cancer specimens [33]. In a separate study, COX-2 immunostaining in BPH specimens was observed in luminal epithelial cells and associated with the infiltration of macrophages in the surrounding stroma [34]. Here, we scored stromal and epithelial COX-2 staining in the same areas that were scored for E-cadherin. Healthy donor tissues displayed a small percentage of COX-2-positive epithelial cells, with a non-significant increase in COX-2-positive epithelial cells in NAP and BPH specimens (**Figure 1C**). Stromal COX-2 staining was rare in donor specimens and was increased in NAP stroma. There was no significant increase in stromal COX-2 staining observed in BPH compared to donor (P = 0.10) or NAP (P = 0.24) (**Figure 1D**).

Correlation of E-cadherin and COX-2 with age and inflammation in prostate

Infiltration of inflammatory T-lymphocytes, B-lymphocytes and macrophages was determined in areas of serial sections corresponding to those scored for E-cadherin and COX-2. Compared to donor tissue, there was an overall increase in CD20 positive B-lymphocytes in NAP, and an increase in CD4 and CD8-positive T-lymphocytes, CD20-positive B-lymphocytes and CD68-positive macrophages in BPH compared to NAP (**Figure 2**). In order to determine whether the down-regulation of E-cadherin and increased stromal COX-2 immunostaining were correlated with aging in the prostate, the healthy young donor cohort was compared to older NAP tissues using a Wilcoxon Signed Rank Test due to the smaller sample size (**Table 2**). E-cadherin was significantly down-regulated in the NAP tissues compared to donor

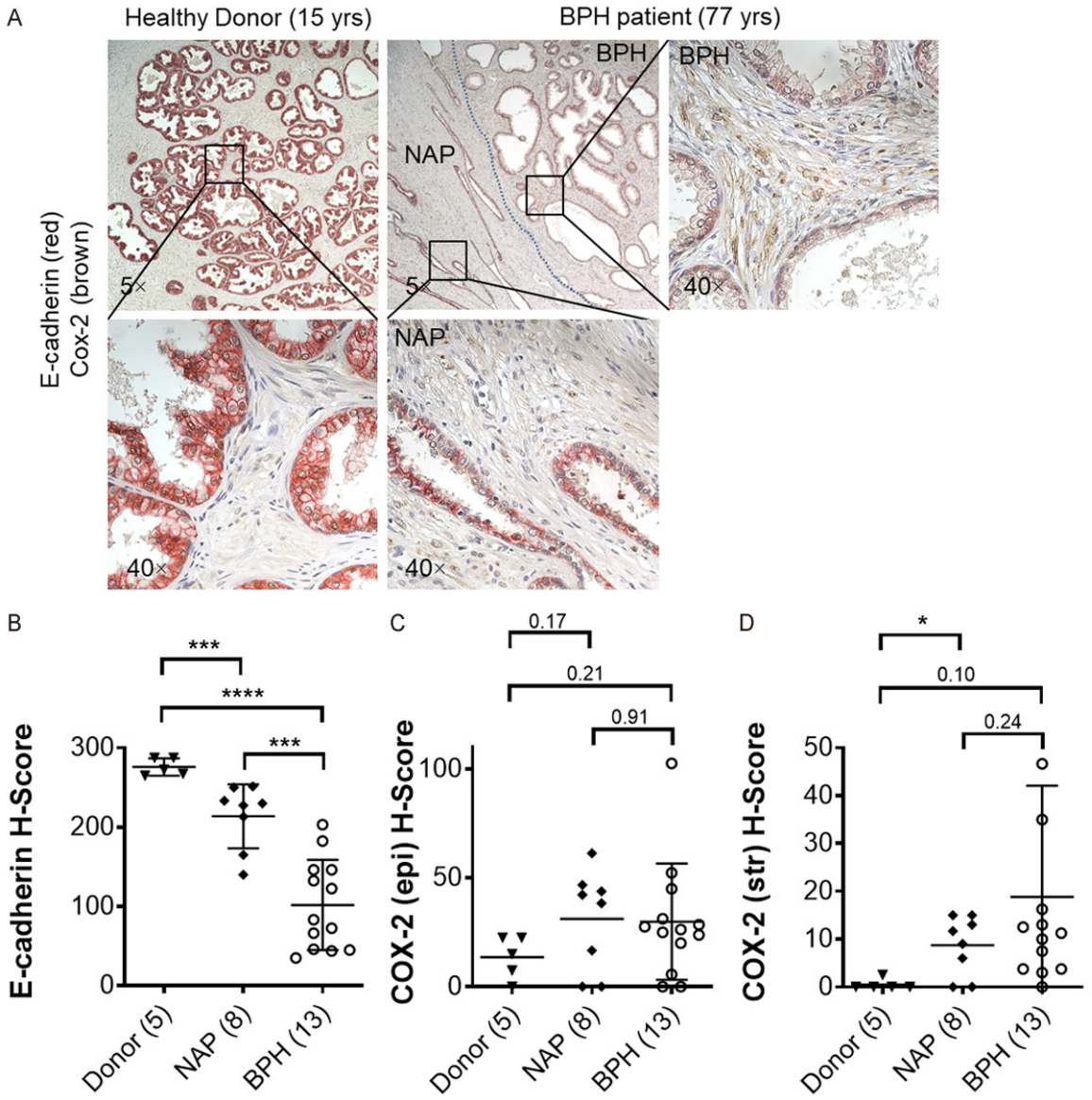

Figure 1. Expression of E-cadherin and COX-2 in the prostate. A. Representative immunostaining of E-cadherin (red) and COX-2 (brown) expression in young healthy donor, and normal adjacent prostate (NAP) and BPH specimens. B. Quantification of mean E-cadherin staining intensity H-score. C. Quantification of mean COX-2 staining intensity H-Score in prostate epithelial cells. D. Quantification of mean COX-2 staining intensity in prostate stromal cells. Number of patients in parentheses. Data represent mean ± S.D.; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

prostate suggesting that aging in the prostate is associated with a decrease in E-cadherin. BPH tissues were then compared to NAP to determine if these same outcomes were also altered in BPH. E-cadherin was further down-regulated and inflammatory cell infiltration included CD4 and CD8 T-lymphocytes and CD68 macrophages were increased, suggesting that inflammation in BPH is different than that observed in NAP. Correlation analyses identified a significant negative correlation between age and E-cadherin in the prostate (-0.56, $P = 0.0483$). A simple linear regression model comparing donor versus NAP revealed a significant association between E-cadherin down-regulation and increasing age, and between stromal COX-2 up-regulation and age (**Table 3**). Aging was also associated with an increase in prostate mass and volume (**Table 3**).

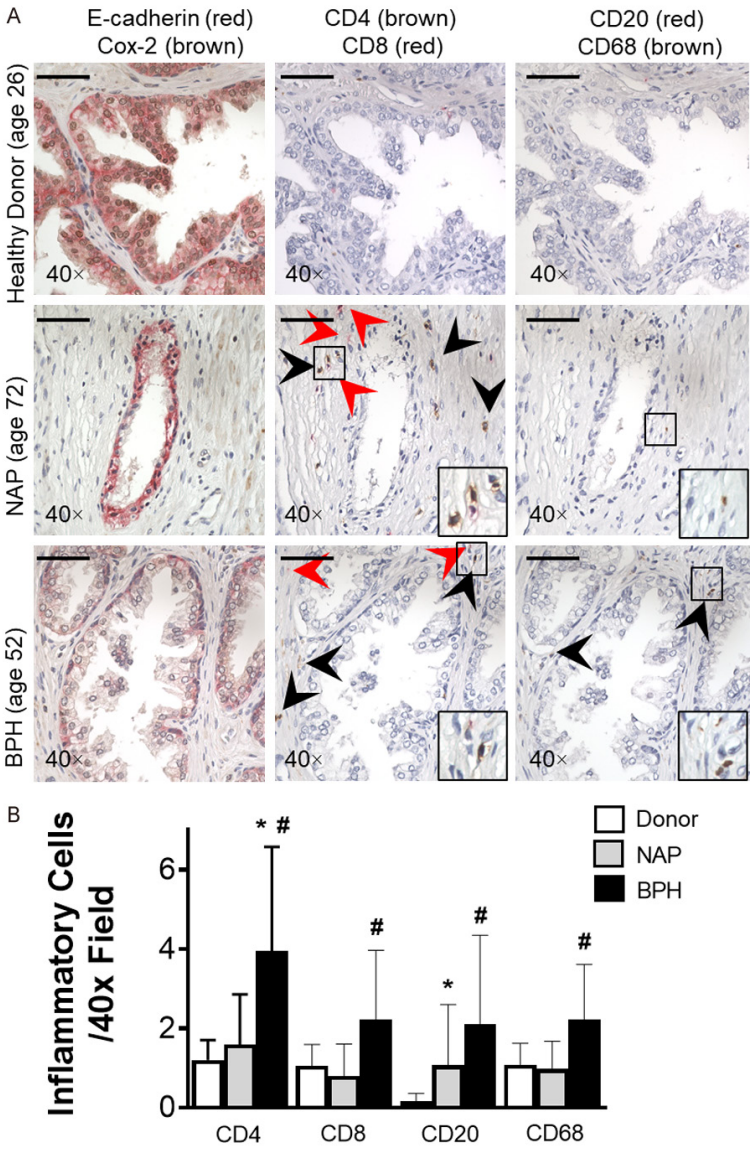

Figure 2. E-cadherin, COX-2 and infiltrating inflammatory cells in prostate. A. Immunostaining of E-cadherin (red), COX-2 (brown), CD4 (brown), CD8 (red), CD20 (red) and CD68 (brown) in serial sections of young healthy donor, normal adjacent prostate (NAP) and BPH specimens. Age of patient in parentheses. Scale bars indicate 50 μm. B. Quantification of the number of infiltrating inflammatory cells in donor, NAP and BPH specimens per 40× field [20]. Data represent mean ± S.D.; *, $P < 0.05$ compared to donor; #, $P < 0.05$ compared to NAP.

When comparing BPH to NAP, E-cadherin was significantly down-regulated in BPH, while COX-2 expression was not different between BPH and NAP (**Table 4**). Pearson correlation analysis comparing E-cadherin and stromal COX-2 to age, inflammatory cells, prostate mass and volume in all prostate tissues analyzed showed that E-cadherin expression was negatively correlated with age, CD4, and CD8 (**Table 5**). COX-2 expression was positively correlated with age and with CD8-positive T-lymphocytes.

Discussion

Here we show that immunostaining of E-cadherin was significantly down-regulated, and COX-2 immunostaining in the stromal compartment was significantly up-regulated with age in the prostate. E-cadherin was also further decreased in BPH tissue compared to NAP, while COX-2 expression was not significantly different in NAP vs. BPH. Correlation analyses identified an inverse correlation between E-cadherin and the number of infiltrating CD4- and CD8-positive T-lymphocytes. Increased COX-2 expression in the stroma was associated with increased CD8-positive T-lymphocytes. These results suggest that E-cadherin expression is decreased with age, potentially contributing to a decline in epithelial barrier function in the prostate, while COX-2 expression in the stroma is increased with age. E-cadherin is also down-regulated in age-related BPH, suggesting that decreased E-cadherin could contribute to BPH pathogenesis and progression.

Declining epithelial barrier function as a consequence of aging has been observed in skin, lung, kidney and intestine [1]. E-cadherin was down-regulated by photoaging in skin, resulting in increased permeability and inflammation [35]. Here, we show that E-cadherin expression is decreased in aging prostate compared to young donor prostate. COX-2 expression is increased, and infiltration of B-lymphocytes is increased with age in the prostate. Taken together, these findings suggest that epithelial barrier function declines with age in the prostate, and inflam-

Table 2. Comparing E-cadherin and COX-2 H-Scores and number of inflammatory cells per field in young healthy donor (Donor), normal adjacent prostate to BPH (NAP) and BPH tissues. Donor vs. NAP and BPH vs. NAP

Outcome	Donor (n = 5) vs. NAP (n = 8)		*p* value[1]	BPH (n = 13) vs. NAP (n = 8)		*p* value[1]
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
E-cadherin H-Score average for tissue type	272.5 (20)	228.8 (52.5)	0.0043*	83.3 (100.3)	228.8 (52.5)	0.0010*
COX-2 staining H-score average in epithelial cells	15 (15)	40.3 (36.9)	0.2102	26.3 (11.3)	40.3 (36.9)	0.6894
COX-2 staining H-Score average in stromal cells	0 (0)	10.3 (11)	0.0374*	11.3 (12.5)	10.3 (11)	0.5615
Weight of Prostate in grams	36 (9)	49.1 (47.3)	0.3142	55.4 (43.1)	49.1 (47.3)	0.4810
Volume of prostate	19.1 (2.6)	33.5 (30.2)	0.0135*	45.4 (40.7)	33.5 (30.2)	0.6053

IQR: Inter-Quartile range (difference between third quartile-First Quartile); [1]*p* value based non-parametric test using Wilcoxon Signed Rank Test due to smaller sample size; *indicates statistically significant difference between groups. NAP: Normal adjacent prostate to BPH; BPH: Benign prostatic hyperplasia.

Table 3. Generalized linear model in young healthy donor and normal adjacent prostate to BPH (Donor vs. NAP)

Outcome Model	Simple Regression Model			Adjusted with Covariate		
	Estimate (SE)	*p* value	R-square	Estimate (SE)	*p* value	R-square
E-cadherin H-Score average for tissue type			0.31			0.57
Intercept	287.58 (24.86)	---		247.92 (26.08)	---	
Age	-1.08 (0.49)	0.0483*		1.39 (1.08)	0.2272	
Group (ref. = Donor)	---	---		-120.25 (48.69)	0.0331*	
COX-2 staining H-score average in epithelial cells			0.17			0.19
Intercept	7.68 (12.49)	---		13.47 (16.37)	---	
Age	0.36 (0.24)	0.1677		0.001 (0.68)	0.9984	
Group (ref. = Donor)	---	---		17.55 (30.56)	0.5784	
COX-2 staining H-Score average in stromal cells			0.55			0.55
Intercept	-3.85 (2.85)	---		-4.66 (3.77)	---	
Age	0.20 (0.06)	0.0038*		0.25 (0.16)	0.1334	
Group (ref. = Donor)	---	---		-2.47 (7.05)	0.7331	

R-Square: Square of correlation; *indicates statistically significant difference between groups. The unadjusted and covariate adjusted model with intercept, estimate, standard error (SE) of both and its significance.

Table 4. Generalized linear model in normal adjacent to BPH and BPH (NAP vs. BPH). The unadjusted and covariate adjusted model with intercept, estimate, standard error (SE) of both and its significance

Outcome Model	Simple Regression Model			Adjusted with Covariate		
	Estimate (SE)	*p* value	R-square	Estimate (SE)	*p* value	R-square
E-cadherin H-Score average for tissue type			0.55			0.55
Intercept	213.85 (18.17)	---		220.24 (78.69)	---	
Group (ref. = NAP)	-111.92 (23.09)	0.0001*		-111.86 (23.73)	0.0002*	
Age	---	---		-0.10 (1.23)	0.9343	
COX-2 staining H-score average in epithelial cells			0.001			0.02
Intercept	31.11 (8.95)	---		51.28 (38.47)	---	
Group (ref. = NAP)	-1.29 (11.38)	0.9107		-1.12 (11.60)	0.9243	
Age	---	---		-0.33 (0.60)	0.5964	
COX-2 staining H-Score average in stromal cells			0.07			0.13
Intercept	8.71 (6.67)	---		-20.75 (27.99)	---	
Group (ref. = NAP)	10.15 (8.48)	0.2458		9.89 (8.44)	0.2564	
Age	---	---		0.47 (0.44)	0.2929	

R-Square: Square of correlation; *indicates statistically significant difference between groups. NAP: Normal adjacent prostate to BPH; BPH: Benign prostatic hyperplasia; SE: standard error.

mation is increased. *In vitro* cell line studies demonstrated that knockdown of E-cadherin increased monolayer permeability [4]; and, TGF-β1 stimulation inhibited E-cadherin expression and increased monolayer permeability in benign prostatic epithelial cell lines [27, 28].

Table 5. Pearson correlation of E-cadherin and stromal COX-2 (str) immunostaining with age and inflammatory cells in prostate

E-cadherin *vs.*	Age	COX-2 (epi)	COX-2 (str)	CD8	CD4	CD20	CD68
Pearson r							
r	-0.56	-0.28	-0.14	-0.36	-0.29	-0.45	-0.40
95% CI	-0.78 to -0.23	-0.60 to 0.13	-0.50 to 0.26	-0.66 to 0.030	-0.61 to 0.11	-0.71 to -0.073	-0.68 to -0.015
R squared	0.32	0.076	0.020	0.13	0.083	0.20	0.16
p value							
p (two-tailed)	0.0027*	0.070	0.15	0.022*	0.043*	0.19	0.082
COX-2 (str) *vs.*	Age	COX-2 (epi)	E-cadherin	CD8	CD4	CD20	CD68
Pearson r							
r	0.39	0.39	-0.36	0.48	0.26	0.012	0.035
95% CI	0.0033 to 0.68	0.0087 to 0.68	-0.66 to 0.030	0.11 to 0.73	-0.14 to 0.59	-0.38 to 0.40	-0.36 to 0.42
R squared	0.15	0.16	0.13	0.23	0.069	0.00015	0.0012
p value							
p (two-tailed)	0.049*	0.046*	0.070	0.014*	0.19	0.95	0.87

CI: confidence interval; *indicates statistically significant difference between groups.

E-cadherin deletion in the murine prostate epithelium induced chronic prostatic inflammation [32]. In the murine knockout mouse, deletion of E-cadherin resulted in an increased infiltration of T-cells, B-lymphocytes and macrophages. Taken together these results suggest that declining prostate epithelial barrier function and related chronic inflammation occur with aging in the prostate, and these alterations could contribute to the development and progression of BPH.

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Disclosure of conflict of interest

None.

Abbreviations

BPH, benign prostatic hyperplasia; NAP, normal adjacent prostate to BPH.

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