

Case Study of Noni Extract in Men with Very Low-Risk or Low-Risk Prostate Cancer

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Abstract

The optimal treatment strategy for patients with early prostate cancer (PCa) is unknown. We explored the feasibility of administering noni supplementation to modify gene expression of a relevant clinical signature in the prostate of men on active surveillance for PCa. A total of 6 participants with low-risk ($n=5$) to very low-risk ($n=1$) PCa who were candidates for active surveillance received 6200 mg/day of noni in capsule form for 1 year; median age was 65.5 years (range, 58–75 years). Participants were tested for serum prostate-specific antigen (PSA) levels every 3 months. At 12 months, they underwent a repeat transrectal ultrasound-guided prostate biopsy. These biopsy samples were queried for expressing 12 key genes and rates of apoptosis, angiogenesis, and proliferation. The primary outcome was the change in expression of the 12 genes that comprise the Oncotype DX prostate cancer test from baseline to 12 months of noni supplementation. Noni was well tolerated, with only 1 participant reporting side effects of grade 2 diarrhea, requiring a drug holiday of 7 days. Median serum PSA slightly increased from 7.1 ng/mL (4.4–9.7 ng/mL) prior to therapy to 7.9 ng/mL (5.7–10.2 ng/mL) on therapy. Changes were observed in the expression levels of several genes, including *FAM13C*, *KLK2* (associated with the androgen pathway), and *GSTM2* (associated with cellular organization) at 12 months. Noni supplementation was associated with favorable clinical parameters, including stable serum PSA among most patients and no evidence of tumor on repeat biopsy, and correlated with modulation of numerous genes and proteins.

Keywords

Prostate Cancer, Active Surveillance, Noni

Abbreviations and Acronyms

AE = adverse events
AP-1 = activating protein-1
EGF = epidermal growth factor
MVD = microvessel density
NCCN = National Comprehensive Cancer Network
PIRADS = Prostate Imaging–Reporting and Data System
PSA = prostate-specific antigen
RT-PCR = reverse transcription polymerase chain reaction
TPA = tumor-promoting phorbol ester
TSA = tyramide signal amplification

Introduction

Prostate cancer (PCa) is the most common malignancy affecting men, with an estimated 191 930 cases per year in the United States.¹ Despite the high incidence, disease-related mortality statistics are quite favorable compared to other cancers. For example, fewer than one-third of men diagnosed with PCa will die of the disease, making it considerably less lethal than lung

and colorectal cancers, which rank first and second respectively in terms of cancer-related deaths.¹ Prostate-specific antigen (PSA) is a serum-based biomarker used for PCa screening and detection. The high proportion of PSA-detected cancers in current clinical practice is mainly responsible for the stage migration since patients with early PCa are now diagnosed at a lower tumor stage and grade compared to PCa patients 1 or 2 decades ago.² Although PSA testing may allow for earlier curative therapy of potentially life-threatening disease; it also identifies a large group of patients with a relatively low risk of disease progression or relapse in whom radical therapy may be unnecessary and harmful due to its associated morbidity and costs.³ Thus, the optimal treatment strategy for these patients with early PCa (ie, very low-risk and low-risk disease) is unknown.

Morinda citrifolia (noni), a traditional medicine of Native Hawaiians, Other Pacific Islanders, and Asian populations, has been used to treat various diseases for centuries.⁴ Previously, noni has been reported to have significant antioxidant activity due to asperulosidic acid (an iridoid glycoside), damnacanthal (an anthraquinone), and scopoletin (a coumadin).⁵ These compounds have been shown *in vitro* cellular and molecular biologic studies to inhibit cancer development and progression. Specifically, asperuloside was reported to suppress tumor-promoting phorbol ester (TPA)- or epidermal growth factor (EGF)-induced cell transformation and associated activating protein-1 (AP-1) transcription factor activity⁶; damnacanthal, which has strong tyrosine kinase-inhibitory effects, can support apoptosis, and has been shown to induce normal phenotypes in ras-transformed cells⁷; and scopoletin possesses antiproliferative effects.⁸

Therefore, based on the above data, we set out to conduct a case study in which we administered oral noni to test the hypothesis that noni supplementation would modify gene expression of a relevant clinical signature in the prostate of men on active surveillance for PCa.

Methods

Participants

Patients from 2 healthcare facilities (in Honolulu and Hilo, HI) with very low risk (<5% risk of disease relapse after primary treatment, criteria; cT1c, Gleason ≤ 6 , PSA <10 ng/mL, fewer than 3 positive biopsy cores $\leq 50\%$ cancer in any biopsy core, PSA density <0.15 ng/mL/g) or low risk (10% risk of disease

relapse after primary treatment, criteria; cT1-2a, Gleason ≤ 6 , PSA < 10 ng/mL)⁹ prostate cancer, who were considering active surveillance were approached for study enrollment. Gleason score is the most common prostate cancer grading system used and is interpreted from a core biopsy, which is a procedure where a needle is passed through the skin to take a sample of tissue from a mass or lump. If a core biopsy is noted to harbor cancer, it is considered a positive core for cancer. Also, 2 key pre-study criteria must have been met. First, the commercial Oncotype DX prostate cancer test (Genomic Health, Redwood City, CA) had to have been performed and confirmed very low-risk or low-risk disease. Second, pre-study prostate multiparametric MRI must have demonstrated no extraprostatic disease and be devoid of PIRADS > 3 lesions. All participants signed an institutional review board approved informed consent. The study (NCT02648919) was approved by Western IRB (WIRB #20151535) and received an Investigational New Drug application approval from the Food and Drug Administration.

Treatment

Participants in the study were enrolled at a single dose level of noni (620 mg tablets, Healing Noni, Hilo, HI) administered 3 times a day as 3 capsules with breakfast, 3 capsules with lunch, and 4 capsules with dinner, for a total daily dose of 6200 mg/day. This dose was determined by a previous phase 1 study in cancer participants to be safe.^{10,11} All participants were evaluated for adverse events (AEs). AEs and laboratory values were graded using the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 5.0. Dose de-escalation was not allowed. If an AE was reported, noni was held until resolution of the AE. If the hold of noni was > 21 days, the participant was removed from the study. Also, noni was also permanently discontinued for grade 3 and 4 AEs, unless not related to therapy.

During the study, participants were asked to self-administer the study medication, avoid consumption of additional noni, and complete daily drug logs to record study agent intake and concomitant medications. Participants were expected to maintain $\geq 85\%$ compliance with study agent intake; comply with dietary, medication, and supplement restrictions; and complete drug logs to the best of their ability. Participants were interviewed at quarterly clinic visits, and pill counts were performed to verify compliance with study requirements.

Follow-up

Participants were seen quarterly in urology clinics, and serum PSA levels were measured. Serum for blood chemical, hematological, and clotting analyses were obtained at baseline and study conclusion (month 12). Furthermore, at 12 months, the participants underwent a repeat transrectal ultrasound-guided prostate biopsy (minimal 10 cores), similar to the original diagnostic biopsy. Disease progression was defined as an in-

crease in Gleason score, increased number of positive cores, or increased tumor volume.

Gene Expression

Gene expression allows the measurement of expression of certain genes associated with cancers ability to grow. Briefly, histologic evaluation of hematoxylin and eosin (H&E) pathology sections confirmed that the core prostate biopsy tissue used to assess gene expression contained viable tumor. Cores between 3 and 10 mg from each participant were homogenized with a QIAGEN TissueRuptor before total RNA was extracted with the QIAGEN RNeasy Mini kit. The resulting RNA was quality checked with an Agilent Bioanalyzer. From the RNA, cDNA was synthesized using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR was performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers for the following 12 targets: androgen pathway (*AZGP1*, *KLK2*, *SRD5A2*, and *FAM13C*), cellular organization (*FLNC*, *GSN*, *TPM2*, and *GSTM2*), proliferation (*TPX2*), and stromal response (*BGN*, *COL1A1*, and *SFRP4*) along with 5 reference genes¹² for normalization are reported in Supplemental Methods.

Cytokine and Serum Scopoletin Levels

Cytokines, small secreted proteins released by cells allowing interactions and communications with other cells, were quantified in the serum of study participants. Many cytokines are related to inflammation, which is common in cancer, while noni is known to have anti-inflammatory properties. Whole blood was collected in sodium heparin tubes at baseline, 3, 6, 9, and 12 months. Within 2 hours, the blood was processed, separating buffy coat from serum. Both were snapped frozen and stored at -80°C until further analysis. Then, 2 aliquots of serum from each participant at each time point were thawed, 1 aliquot was used for cytokine testing, and the other for scopoletin testing. Serum samples were profiled for 22 cytokines using a customized Luminex assay (Cat # FCSTM18-22, R&D Systems, Minneapolis, MN) as per assay instructions. Measurements were performed in duplicate using a Luminex 200 instrument (Luminex Corporation, Austin, TX) and were analyzed using a standard curve for each molecule (xPONENT® software, Luminex Corporation). Serum scopoletin, a metabolite of noni and a surrogate to quantitatively assess drug compliance, was measured as previously reported.¹¹ Briefly, serum samples collected at baseline and 9 months were subjected to High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ECD) as an objective marker of compliance to noni therapy.

Multiplex Immunofluorescence Staining

Multiplex immunofluorescence staining allows the analysis of several key molecules, in this case, CD31, cleaved caspase-3,

and Ki-67, which are surrogates for angiogenesis, apoptosis, and cellular proliferation, respectively, in a single biopsy sample from a participant. Multiplex immunofluorescence staining was performed automatically in the DISCOVERY ULTRA system (Ventana Medical Systems, Inc., Tucson, AZ) using individual tyramide signal amplification (TSA)-conjugated fluorophores to detect various targets. Paraffin-embedded tissue samples were stained with a prediluted mouse monoclonal anti-CD31 (Cat # 760-4378, Roche Diagnostics, Indianapolis, IN), a 1:2000 dilution of rabbit monoclonal anti-cleaved caspase-3 antibody (Cat # 9661, Cell Signaling, Danvers, MA), a prediluted rabbit monoclonal anti-Ki-67 antibody (Cat # 790-4286, Roche Diagnostics) and a prediluted mouse anti-cytokeratin 8/18 antibody (Cat # 760-4344, Roche Diagnostics) as previously reported.¹³ Secondary OmniMap antibodies were purchased from Roche Diagnostics. The tyramide signal amplification (TSA) was purchased from Perkin Elmer, and the polymer amplification system was obtained from VECTOR labs. The immunofluorescent staining was performed with DISCOVERY RED610 kit (cleaved caspase-3: #760-245, Ventana Medical Systems, Inc.), DISCOVERY FAM kit (cytokeratin 8/18: #760-243, Ventana Medical Systems, Inc.), DISCOVERY DCC Kit (Ki-67: # 760-240, Ventana Medical Systems, Inc.), and DISCOVERY Rhodamine 6G Kit (CD31: # 760-244, Ventana Medical Systems, Inc.). Lastly, the slides were mounted in ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA). The slides were digitized on the high-resolution Tissue-FAXS 200 scanner system (Tissuegnostics, Vienna, Austria) with 20x magnification, and images were sequentially analyzed with NIH ImageJ.

Power Analysis

The study was designed to test feasibility (ie, whether subjects could be identified, recruited, followed, and samples collected

for correlative studies); therefore, a single-arm design was chosen over a 2-arm with placebo control. The primary outcome was the change in expression of the 12 genes that comprise the Oncotype DX prostate cancer test from baseline to 12 months of noni supplementation. This prospective case study was to generate high-quality preliminary data and demonstrate feasibility. Pearson correlation coefficient tests with a 5% significance level (2-sided) and 80% power allows sample sizes of 6 participants to detect a difference between the null hypothesis correlation of 0 and the alternative hypothesis correlation of 0.76.¹⁴ The maximum width of the 95% confidence interval for proportions associated with sample sizes of 6 is 0.63.¹⁵

Results

From January 1, 2016, to December 2017, 6 of 10 participants who were screened were enrolled. The mean age was 66.6 years, 83% (n=5) had low-risk cancer, and 17% (n=1) had very low-risk cancer. All 6 participants completed the 12-month intervention period. Baseline characteristics of the participants are depicted in Table 1. Noni supplement was well tolerated with only 1 participant (participant #5) experiencing a treatment-related AE (grade 2 diarrhea), which improved with a 7-day drug discontinuance (data not shown). Compliance with the daily administration of 6200 mg of noni was generally excellent based on the drug logs and returned drug vials, with a mean of <3% of doses missed per month per participant. Though we collected serum throughout the study for analysis, the 9-month serum was more representative of drug compliance as a noncompliant participant may begin to take the drug before the 12-month final visit, and thus reported. As such, the median serum scopoletin was 5.1 ng/mL (range, <0.5–15.0 ng/mL) at 9 months, up from a median of 2.1 ng/mL (range, <0.5–3.2 ng/mL) at baseline. One participant (participant #6) may have been noncompliant despite the drug logs and visual inspection of drug vials.

Subject ID	Age	Race	Serum PSA	Clinical T Stage ^a	Risk Category	MRI ^b	GPS ^c
Ppt 1	62	White	4.4	T2a	Low	PIRADS 3, max 13 mm without ECE	37
Ppt 2	74	Asian	5.4	T1c	Low	PIRADS 3, max 11 mm without ECE	34
Ppt 3	75	Native Hawaiian/ Other Pacific Islander	7.3	T1c	Very low	PIRADS 3, max 14 mm without ECE	17
Ppt 4	62	White	9.7	T1c	Low	PIRADS 3, max 10 mm without ECE	36
Ppt 5	58	Unknown	6.9	T1c	Low	PIRADS 3, max 12 mm without ECE	38
Ppt 6	69	Asian	7.9	T1c	Low	PIRADS 3, max 19 mm without ECE	39

Abbreviations: ECE, extracapsular extension; GPS, genomic prostate score; MRI, magnetic resonance imaging; PIRADS, Prostate Imaging-Reporting and Data System; PSA, prostate-specific antigen. ^a Based on the results of the digital rectal exam. ^b PIRADS score based on size of lesion(s) and whether ECE is noted on MRI. ^c Analysis of initial prostate biopsy.

Only 1 of 6 participants (participant #1) experienced an overall increase in PSA levels of >30%, though his total PSA was still below 10 ng/ml, the other participants showed stable PSA levels (+/-15% from baseline; Figure 1). Though rising serum PSA levels are problematic, serum PSA is not used as a marker of treatment response except when definitive therapy (eg, prostatectomy, radiation therapy) is rendered.¹⁶ No abnormalities were found in chemical, hematological, and clotting analyses in the 6 participants (data not shown).

All 6 participants underwent a repeat biopsy at 12 months, noted as post-therapy biopsy (Figure 2). At the time of biopsy, the palpable disease was not appreciated in any participant. Two participants did not have histologic evidence of cancer on biopsy, while 1 participant showed atypia. It has been reported that over 35% of men on active surveillance may have a negative prostate biopsy when a 12-month prostate biopsy is performed.¹⁷ However, in the current study, 2 participants had increases in their Gleason score (6 to 7). Both of these participants also showed an increase in the absolute number of positive cores and an increase in the percentage of positive cores (Figure 2).

Furthermore, though underpowered, there was no significant difference between pre-treatment and post-treatment levels of markers of microvessel density (MVD also termed “angiogenesis”; CD31), apoptosis (cleaved caspase-3), and proliferation (Ki-67) (data not shown). Similarly, immunofluorescent staining of the pre-treatment and post-treatment biopsies depicts the spatial relationship between MVD, apoptosis, and proliferation (Figure 2).

Using RT-PCR, the gene expression of 12 transcripts, which comprise the Oncotype DX prostate cancer test, was queried. The alteration of androgen, cellular organization, proliferation, and stromal response pathways after noni therapy is illustrated with a heatmap (Figure 3). Two participants showed increased expression of *FAM13C*, *KLK2* (associated with androgen pathway), and *GSTM2* (associated with cellular organization) over the study period. However, no participants had reductions in common genes. Furthermore, serum analysis showed increases in IL6 and CXCL10 in 2 participants while on treatment, while most other cytokines were reduced while on treatment (Figure 4).

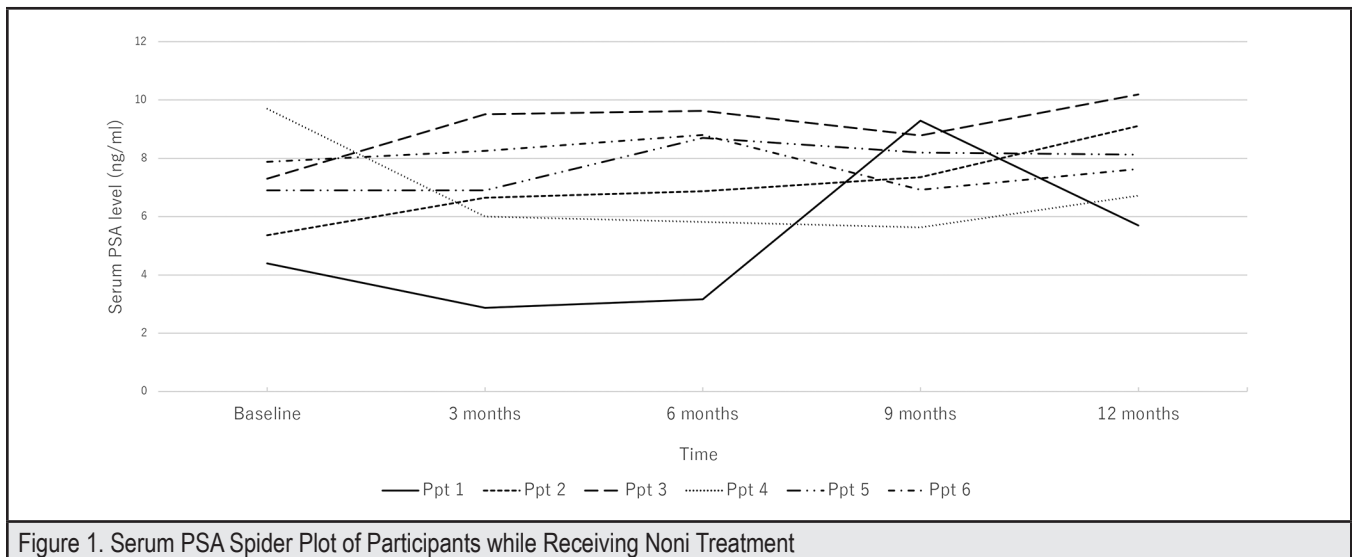


Figure 1. Serum PSA Spider Plot of Participants while Receiving Noni Treatment

Subject ID	Biopsy Status	Gleason Score	Number of Positive Cores	Percentage Positive Core
Ppt 1	Pre-therapy	3+3	1	12.5
	Post-therapy	3+3	6	75
Ppt 2	Pre-therapy	3+3	2	14.3
	Post-therapy	4+3	5	35.7
Ppt 3	Pre-therapy	3+3	1	12.5
	Post-therapy	4+3	3	33.3
Ppt 4	Pre-therapy	3+3	1	9.1
	Post-therapy	Atypia	0	0
Ppt 5	Pre-therapy	3+3	1	11.1
	Post-therapy	N/A	0	0
Ppt 6	Pre-therapy	3+3	1	8.3
	Post-therapy	N/A	0	0

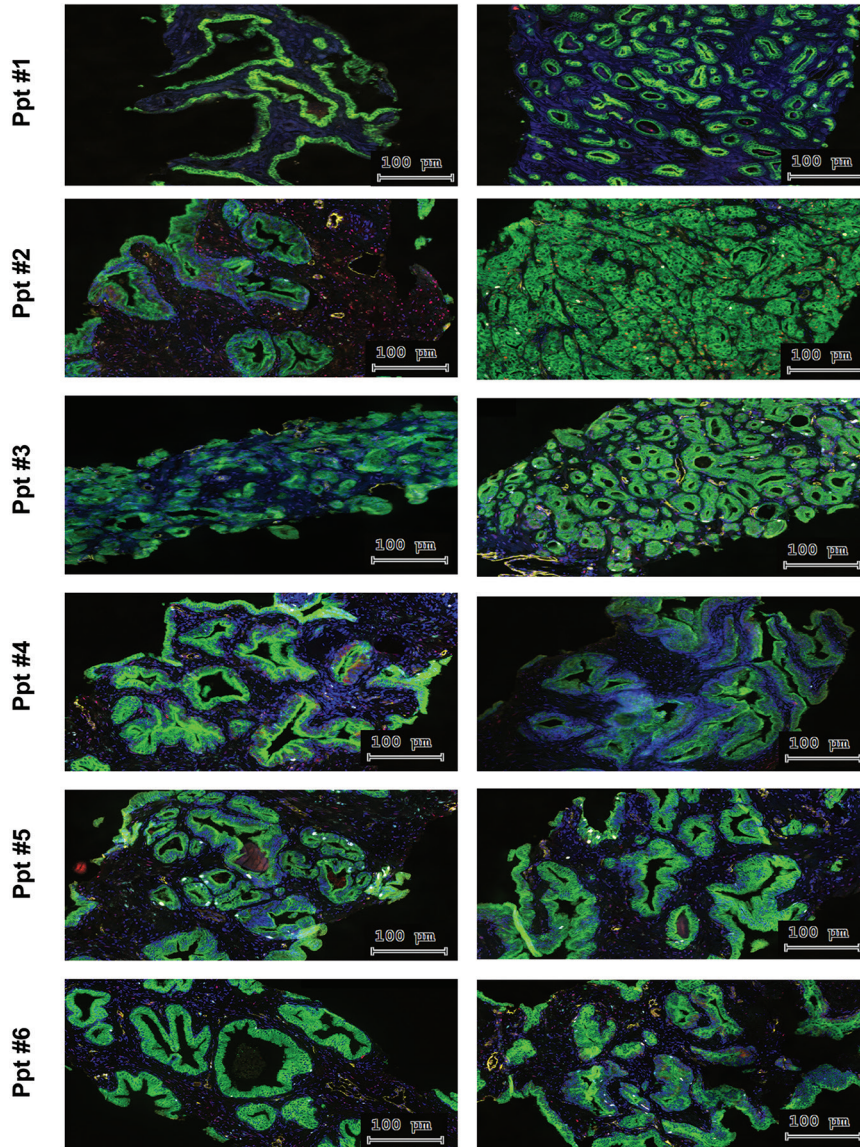


Figure 2. Histopathological Changes of Prostate Tissue Associated with Noni Treatment. The slides were digitized on the high-resolution TissueFAXS 200 scanner system (Tissuegnostics, Vienna, Austria). Right column, pre-therapy biopsy and left column post-therapy biopsy. 20x magnification immunofluorescence images of prostate tissues show staining with DAPI (blue), CD31 (angiogenesis marker, yellow), Ki67 (proliferation marker, sky blue), cleaved caspase 3 (apoptosis marker, red), and CK8/18 (epithelial marker, green)

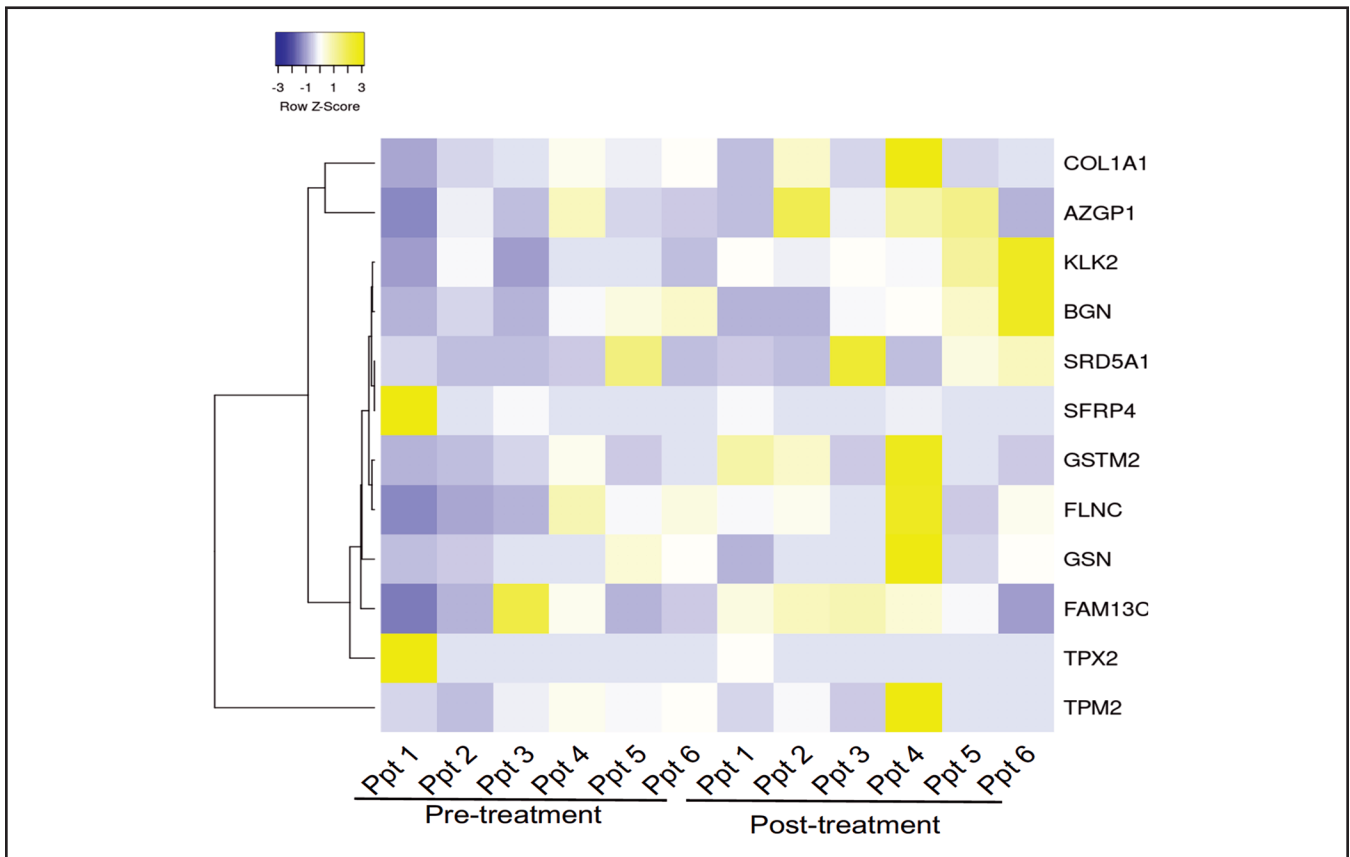


Figure 3. Heatmap Illustrating Differences in the 12-Transcript Expression Among the Six Participants. Cluster analysis of RT-PCR data profiling in six participants. Each row represents one of the 12 transcripts present in Oncotype Dx Prostate Cancer test and each column a participant. The scale represents standard deviations from the mean after a Z-transformation of signal values of a gene across all samples. Yellow represents a higher level of gene expression and blue a lower level, relative to the mean across all samples for each gene.

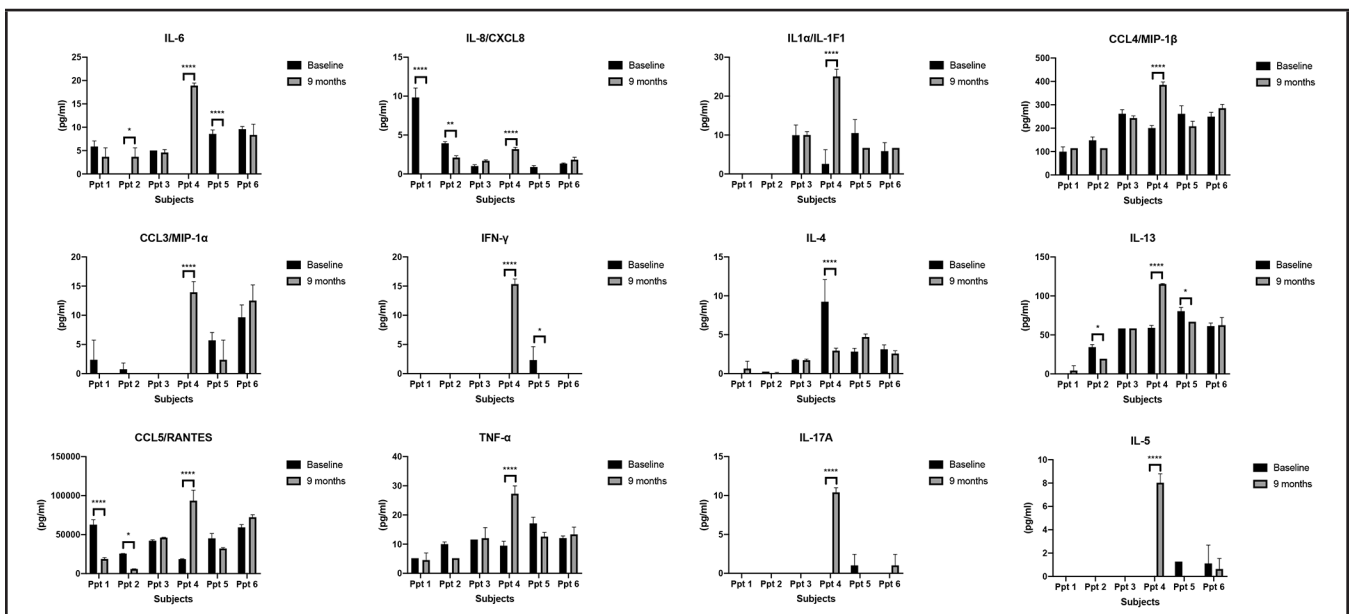


Figure 4. Changes in Serum Cytokine Profiles following Noni Treatment. A panel of cytokines were queried in serum samples collected at 9 months and compared to samples collected at baseline using the 22-plex customized human cytokine Luminex assay (R&D Systems).

Discussion

Identification of the suitable patient population is fundamental to the development of an appropriate treatment strategy. For patients with PCa, the outcome after therapy has been shown to be dependent on tumor stage, serum PSA, and Gleason score (grade).¹⁸⁻²⁰ Because of this association, PCa can be stratified as very low risk (<5% risk of disease relapse after primary therapy, criteria; cT1c, Gleason ≤ 6 , PSA <10 ng/mL, fewer than 3 positive biopsy cores $\leq 50\%$ cancer in any core, PSA density <0.15 ng/mL/g); low risk (10% risk of disease relapse after primary therapy, criteria; cT1-2, Gleason ≤ 6 , PSA <10 ng/mL); intermediate risk (25% risk of disease relapse after primary therapy, criteria; cT2b-2c, Gleason 7, PSA 10–20 ng/mL) and high risk (>50% risk of disease relapse after primary therapy, criteria; cT3a, Gleason 8–10, PSA ≥ 20 ng/mL). Thus, it is important to tell patients with very low-risk and low-risk PCa that long-term survival is possible with conservative management. Despite this information, 56% to 91% of very low-risk or low-risk PCa are reluctant only to surveil their PCa.²¹ Thus, an opportunity exists to incorporate a therapeutic option, with minimal side effects, into the armamentarium of physicians caring for patients with very low-risk and low-risk PCa.

We report that the administration of noni in men with very low-risk or low-risk PCa was feasible and well tolerated in most patients, which is aligned with previous studies from our group.^{10,11} Critical molecular modulation associated with treatment includes reduced tumor vasculature (MVD) in Ppt 3 ($P = .013$) and 5 ($P = .00011$), reduced cellular proliferation in post-treatment decreased in Ppt 2 ($P = .070$) and 3 ($P = .0036$) and increased apoptosis, program cell death in Ppt 2 ($P = .074$). Furthermore, we noted modification in gene expression profile while on treatment. Specifically, *FAM13C* and *KLK2* (markers of androgen metabolism) and *GSTM2* (a marker of cellular organization) were increased over time in 2 participants, which may hint at noni-induced changes. Identifying a difference in gene expression with the initiation of noni will support the design of future studies with adequate power.

Diet has long been associated with PCa etiology.²² The most provocative data supporting the influence of dietary factors on the incidence of PCa come from international studies and studies of immigrant populations in the United States. For example, historically, the incidence of PCa in Japan has been extremely low. However, as Japanese men migrate to Hawai'i or the US mainland and subsequently adopt western culture, their incidence of PCa rapidly approaches that of whites.^{23,24} The diets of native Japanese and other Asians are rich in fiber and low in saturated fat. These cultural differences may contribute to the lower rates of clinical PCa in Asia compared to Northern Europe and North America.

Furthermore, the influence of dietary interventions and their role in prevention and treatment have been highly speculated.

Recently, the Men's Eating and Living (MEAL) Study (CALGB 70807 [Alliance]), a randomized clinical trial conducted at 91 US urology and medical oncology clinics that enrolled 478 men aged 50 to 80 years with biopsy-proven very low-risk or low-risk PCa, was reported. Patients were randomized to a counseling behavioral intervention promoting 7 or more daily vegetable servings or a control group. Among 478 patients randomized (mean age [SD], 64 [7] years; mean PSA level [SD], 4.9 [2.1] ng/mL), 443 (93%) eligible patients were included in the primary analysis. There were 245 progression events (intervention: 124; control: 121) with no significant difference in time to progression (unadjusted hazards ratio, 0.96 [95% confidence interval {CI}, 0.75–1.24]; adjusted hazard ratio, 0.97 [95% CI, 0.76–1.25]) between the 2 groups.²⁵ We anxiously await novel correlative analysis from this study as it could shed light on the association between diet and cancers.

In our case study, we explored a well-known medicinal fruit from the Pacific, *Morinda citrifolia* or noni, in men with very low-risk or low-risk PCa. We treated 6 men with 6200 mg per day for 12 months. Our dose regimen was based on a previous phase 1 study, which we conducted.^{10,11} Overall, the treatment was well tolerated. Serum PSA levels at study entry were not significantly different after 12 months of therapy (median, 7.1 ng/mL, [range, 4.4–9.7] versus median, 7.9 ng/mL [range, 5.7–10.23], respectively). Furthermore, 50% of participants had a negative prostate biopsy at 12 months.

Currently, gene expression CLIA assays (Oncotype DX[®], Genomic Health, Redwood City, CA and Prolaris[®], Myriad, Salt Lake City, UT) are available to assist clinicians in determining the likelihood of more aggressive cancer^{26,27} or determining the likelihood of having metastatic disease²³ and have been incorporated into current National Comprehensive Cancer Network (NCCN) guidelines.⁹ Thus, we sought to study the ability of noni supplementation to modify mRNA expression levels of the genes related to determining the likelihood of aggressive disease. Initially, Genomic Health was to perform the analysis on baseline tissue and then tissue obtained at 12-month biopsy. Unfortunately, Genomic Health could not run the assay on 12-month prostate biopsy samples in which active cancer was not identified. Even though Genomic Health performed GPS assay on all baseline prostate biopsies, our laboratory performed RT-PCR on 12-month and baseline (comparator) tissue. As evident in Figure 3, noni treatment was associated with increases in expression of *FAM13C* (Ppt 1 and 4), *KLK2* (androgen signaling, Ppt 2 and 4), and *GSTM2* (cellular structure, Ppt 1 and 3), and increases in serum cytokines IL6 and CXCL10.

Scopoletin is 1 of many metabolites of noni. Perhaps its presence in the blood occurs in patients who can better metabolize noni into its active components. A similar phenomenon has been reported with isoflavone supplements, with higher levels of free serum equol reported in patients who responded to isoflavone therapy.²⁸ If effective metabolism is the key, then

serum scopoletin levels may be a valuable surrogate to monitor molecular changes associates with noni.

There are several weaknesses of this study. First, the study is only a single-arm, pilot study, demonstrating the feasibility of deploying a dietary intervention to this population. Second, we could not verify any positive or negative clinical or molecular trends due to noni ingestion, mainly due to small sample size and interpatient variability.

We report in a case study that long-term noni treatment in men with very low-risk or low-risk PCa was feasible to perform and was associated with favorable gene expression changes. Consideration should be given for additional testing of noni in human clinical trials involving men with very low-risk and low-risk PCa.

Supplementary Method

Preamplification

The cDNA was preamplified using SsoAdvanced™ PreAmp Supermix (Bio-Rad Laboratories, Hercules, CA) and PrimeP-CR™ PreAmp for SYBR® Green Assays for each target gene according to the manufacturer's instructions (Supplementary Table 1). The preamplification was performed in a thermal cycler under the following conditions: 95°C for 10 min followed by 8 cycles of 95°C for 15 sec and 60°C for 4 min.

Quantitative Reverse Transcriptase-PCR

The amplified product was mixed with iTaq Universal SYBR Green Supermix, and 20 µl of the PCR reaction mix was applied into each well. qPCR was performed for 45 cycles in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following conditions: enzyme activation (95°C, 15 min), amplification (95°C for 20 sec and 60°C for 45 sec; 45 cycles in total), and cooling (40°C, 5 sec). The level of expression was calculated with the crossing point (Cp) method. All gene assays were measured in duplicate.

Conflict of Interest

None of the authors identify any conflict of interest.

Disclosure Statement

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Supplementary Table 1. List of Primers	
Genes	Unique Assay ID
Target genes	
AZGP1	qHsaCED0056827
BGN	qHsaCID0014636
COL1A1	qHsaCED0043248
FAM13C	qHsaCID0010924
FLNC	qHsaCID0012034
GSN	qHsaCID0021416
GSTM2	qHsaCED0038361
KLK2	qHsaCID0014394
SFRP4	qHsaCID0022180
SRD5A1	qHsaCID0012570
TPM2	qHsaCED0002140
TPX2	qHsaCID0016024
Reference genes	
ARF1	qHsaCED0002471
ATP5E	qHsaCID0038009
CLTC	qHsaCID0017813
GPS1	qHsaCED0038152
PGK1	qHsaCED0042912

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