

# Spectrum of Mitochondrial Genomic Variation and Associated Clinical Presentation of Prostate Cancer in South African Men

John P. McCrow,<sup>1^</sup> Desiree C. Petersen,<sup>1,2,3^</sup> Melanie Louw,<sup>4</sup> Eva K. F. Chan,<sup>2,3</sup> Katherine Harmeyer,<sup>1,2</sup> Stefano Vecchiarelli,<sup>2</sup> Ruth J. Lyons,<sup>2</sup> M. S. Riana Bornman,<sup>5</sup> and Vanessa M. Hayes<sup>1,2,3,5,6\*</sup>

<sup>1</sup>J. Craig Venter Institute, La Jolla, California

<sup>2</sup>Laboratory for Human Comparative and Prostate Cancer Genomics, Garvan Institute of Medical Research, Darlinghurst, Sydney, NSW, Australia

<sup>3</sup>Faculty of Medicine, University of New South, Wales Australia, Randwick, NSW, Australia

<sup>4</sup>Department of Pathology, University of Pretoria, South Africa

<sup>5</sup>School of Health Systems and Public Health, University of Pretoria, South Africa

<sup>6</sup>Central Clinical School, University of Sydney, Camperdown, Sydney NSW, Australia

**BACKGROUND.** Prostate cancer incidence and mortality rates are significantly increased in African–American men, but limited studies have been performed within Sub-Saharan African populations. As mitochondria control energy metabolism and apoptosis we speculate that somatic mutations within mitochondrial genomes are candidate drivers of aggressive prostate carcinogenesis.

**METHODS.** We used matched blood and prostate tissue samples from 87 South African men (77 with African ancestry) to perform deep sequencing of complete mitochondrial genomes. Clinical presentation was biased toward aggressive disease (Gleason score >7, 64%), and compared with men without prostate cancer either with or without benign prostatic hyperplasia.

**RESULTS.** We identified 144 somatic mtDNA single nucleotide variants (SNVs), of which 80 were observed in 39 men presenting with aggressive disease. Both the number and frequency of somatic mtDNA SNVs were associated with higher pathological stage.

**CONCLUSIONS.** Besides doubling the total number of somatic PCa-associated mitochondrial genome mutations identified to date, we associate mutational load with aggressive prostate cancer status in men of African ancestry. *Prostate* 76:349–358, 2016.

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**KEY WORDS:** African ancestry; mitochondria; outcomes; prostate cancer; variation

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Grant sponsor: NIH NCI Center to Reduce Cancer Health Disparities, USA; Grant number: NIH R21-CA170081; Grant sponsor: Australian Prostate Cancer Research Centre NSW (APCRC-NSW); Grant sponsor: The Petre Foundation, Australia; Grant sponsor: The Cancer Association of South Africa (CANSAs).

<sup>^</sup>John P. McCrow and Desiree C. Petersen contributed equally to this work.

Conflicts of interest: The authors have no conflict of interest related to this work.

\*Correspondence to: Prof. Vanessa M. Hayes, Laboratory for Human Comparative and Prostate Cancer Genomics, Division of Genomics and Epigenetics, Garvan Institute of Medical Research, The Kinghorn Cancer Centre, 370 Victoria Street, Darlinghurst, NSW 2010, Australia.

E-mail: v.hayes@garvan.org.au or vanessa.hayes@sydney.edu.au

Received 17 September 2015; Accepted 5 November 2015

DOI 10.1002/pros.23126

Published online 10 December 2015 in Wiley Online Library

(wileyonlinelibrary.com).

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## INTRODUCTION

Although prostate cancer (PCa) is the most commonly diagnosed male cancer and second highest contributor to cancer mortality in Western countries [1,2], the prevalence of PCa throughout Africa is largely unknown. The significance of African ancestry in PCa risk and outcomes has been driven by studies comparing incidence and mortality rates, as well as reported younger age at diagnosis, in African-Americans compared with European or Asian ancestral Americans [3,4]. We recently reported more aggressive histopathologically defined PCa and elevated prostate specific antigen (PSA) levels in Black men from South Africa [5], and postulate that genetics may be driving this ethnic-based disparity.

PCa is the most heritable of the adult cancers [6], with carcinogenesis often driven by a complex series of acquired genetic events [7]. Although genome-wide association studies (GWAS) have identified 76 variants associated with PCa risk in predominantly European ancestral populations [8], more than half of these variants failed validation within a Black South African cohort [9]. Additionally, GWAS and PCa genome profiling studies have been biased towards interrogation of the nuclear genome, largely ignoring the maternally inherited mitochondrial genome. Evidence for the significance of maternal inheritance in driving PCa risk and outcomes has been shown in a number of pivotal studies [10–12].

Unlike nuclear DNA, multiple copies of the mitochondrial (mt)DNA are present within the 100–1,000 mitochondria per cell [13]. Lacking introns, over 93% of mtDNA codes for one of 37 genes including 13 proteins, 22 tRNAs, and 2 rRNAs, that are critical for energy production and apoptosis. Together with its high mutation rate [14], it is not surprising that mtDNA mutations have been implicated in human aging, disease, and cancer [15–17]. Heteroplasmy and mutational load of acquired mtDNA mutations, will further impact cancer progression [18] contributing to extreme clinical heterogeneity. An analysis of somatic mtDNA mutations in 1,675 cancers from 31 tissue types, including 80 examples of PCa, demonstrated that functionally deleterious mtDNA mutations were more likely to be heteroplasmic possibly as a result of negative selection [19].

The role of acquired functionally relevant mtDNA mutations driving carcinogenesis has been supported by the observation that cancer cells switch from aerobic glycolysis in mitochondria to lactic acid fermentation in the cytosol, known as the Warburg effect [20]. In PCa, acquired mtDNA mutations have been shown to; (i) accumulate in PCa tissue [21,22] at a rate around 55-times greater than nuclear DNA

mutations [23], (ii) are most frequent in PCa, after gastric and hepatocellular cancers, than any other human cancer [19], (iii) increase PCa tumorigenesis, including indications as early events [24–26], (iv) associate with biochemical indicators of aggressive PCa [27], and (v) enhance PCa bone metastasis [28].

The aim of this study was to determine the spectrum and associated clinical relevance of mitochondrial genome variation within a unique cohort of predominantly Black South African men presenting with PCa. Biased towards histopathologically aggressive disease, complete mitochondrial genomes were deep sequenced in a set of matched blood and prostate tissue derived DNA from 87 patients. Among the most diverse, earliest diverged, mitochondrial genomes [29,30], we identify a broad spectrum of somatic mutations associated with advanced PCa and elevated PSA levels at diagnosis.

## MATERIALS AND METHODS

### Study Design: Clinical and Pathological Data

A study sample of 87 men was recruited at the Steve Biko Academic Hospital in Pretoria, South Africa. Patients were consented as per the requirements of the University of Pretoria Faculty of Health Sciences Research Ethic Committee (#43/2010) and additional approvals obtained from the J. Craig Venter Institute Institutional Review Board (#2010–129) and Human Research Ethics Committee at the University of New South Wales (#HREC08244). Individuals self-identified ethno-linguistically as Black/Southern Bantu ( $n = 70$ ), Coloured ( $n = 7$ ), or European ( $n = 10$ ). Further discussion of ancestral history and terminology can be found in supplementary material. All tissue core biopsies were blindly rescored by a single histopathologist and confirmed as presenting with high-risk PCa defined as a Gleason score (GS)  $> 7$  ( $n = 39$ ), intermediate-risk PCa with a GS = 7 ( $n = 12$ ), low-risk PCa with a GS  $< 7$  ( $n = 10$ ), or no PCa ( $n = 26$ ) either with or without benign prostate hyperplasia (BPH). Matched blood and fresh tissue core biopsies were obtained, DNA isolated (Qiagen) and clinical information relating to age, serum PSA levels (ng/ml) and GS at diagnosis, were made available (Table I).

### Ancestral Contributions: Autosomal Genotyping

The 87 patients underwent genotyping using the Illumina Infinium HumanCore Beadchip ( $> 250$  K markers), with variant inclusion dependent on a GenTrain score  $\geq 0.5$  (Illumina GenomeStudio 1.9.4). The SNP and Variation Suite version 8.3.1 (Golden Helix; [www.goldenhelix.com](http://www.goldenhelix.com)) was used to

**TABLE I. Clinical and Pathological Characteristics**

	Total, n = 87	Southern Bantu, n = 70	Coloured, n = 7	White, n = 10
Patient characteristic at presentation	median (range)			
Age (years)	70 (50–99)	70 (50–99)	67 (54–76)	73.5 (63–80)
Serum PSA (ng/ml)	26 (0.34–3459)	26.7 (4–2194)	59 (5.5–3459)	7.85 (0.34–1210)
Mitochondrial haplogroup	n (%)			
L0	37 (43%)	32 (46%)	5 (71%)	0
L1	7 (8%)	7 (10%)	0	0
L2	19 (22%)	17 (24%)	2 (29%)	0
L3	14 (16%)	14 (20%)	0	0
R/L4	10 (11%)	0	0	10 (100%)
Diagnosis and PCa grade group	n (%)			
PCa GG = 5 (GS > 8)	20 (23%)	17 (24%)	2 (29%)	1 (10%)
PCa GG = 4 (GS = 8)	19 (22%)	16 (23%)	2 (29%)	1 (10%)
PCa GG = 3 (GS = 4 + 3)	7 (8%)	6 (9%)	0	1 (10%)
PCa GG = 2 (GS = 3 + 4)	5 (6%)	4 (6%)	1 (14%)	0
PCa GG = 1 (GS < 7)	10 (11%)	8 (11%)	1 (14%)	1 (10%)
No PCa, possible BPH	26 (30%)	19 (27%)	1 (14%)	6 (60%)

facilitate merging of genotype data of study participants with ancestral populations with (i) a previously published dataset [31] that includes 19 Ju/'hoan representing the earliest-diverged population and (ii) the Illumina iControl database ([www.illumina.com](http://www.illumina.com)) that includes the more recent-diverged Yoruba ( $n = 20$ ), Han Chinese ( $n = 20$ ), and European ( $n = 20$ ) populations. Markers were excluded that were monomorphic, indels, or had linkage disequilibrium (LD) with  $r^2 \geq 0.2$  within a 50-variant sliding window. A total of 103,670 SNP markers were used to determine ancestral substructure using STRUCTURE 2.3.3 [32], with the most appropriate  $K = 4$  determined by the  $\Delta K$  method [33].

#### Data Generation: Mitochondrial Genome Sequencing

The complete mitochondrial genome was captured via long-range PCR amplification using the Platinum<sup>®</sup> Taq DNA Polymerase High Fidelity kit (Invitrogen) and previously published primer sets creating two overlapping amplicons of  $\sim 7.2$  kilobases (kb) and  $\sim 9.7$  kb. Pre-amplification allowed for mtDNA specificity, eliminating mtDNA-derived pseudogenes in the nuclear genome (NuMTs). Amplified products were quantified using the Quant-iT<sup>™</sup> PicoGreen<sup>®</sup> dsDNA Assay Kit (Invitrogen) prior to library preparation using the Ion Xpress<sup>™</sup> Plus Fragment Library Kit. The 200 bp libraries were barcoded (Ion Xpress<sup>™</sup> Barcode Adapters 1–16 Kit), quantified and sized (Agilent High Sensitivity DNA Kit), template prepared (Ion OneTouch<sup>™</sup> 2 System), and sequenced on the Ion

Personal Genome Machine (PGM<sup>™</sup>) System using the Ion 318<sup>™</sup> Chip. Each chip was run with eight matched blood and prostate tissue samples generating at least 600 Mb of data with an average coverage depth of almost  $3,000 \times$  per sample.

#### Data Inferences: Statistical Analyses

All Ion Torrent sequence data were quality trimmed (Phred-scale Q20, see Supplementary Table SII). Reads were aligned to the rCRS mtDNA reference genome using BWA MEM alignment [34]. Variant analysis of somatic mutations was done using VarScan 2.0 [35] from read pileups generated by Samtools [36] based on the BWA alignments. The VarScan somatic program was run with strand bias filtering, and tumor purity allowed as low as 10%. SNVs from matched blood and prostate tissue samples were further filtered for a difference of tumor above blood frequency exceeding 2.0%.

Statistical analyses were performed using R ([www.R-project.org](http://www.R-project.org)). Linear regression (lm) and Generalized Linear Models (glm) were used for linear or categorical regression of PCa status. PCa categories were defined by the new grading group (GG) system [37] as follows: 1.  $GS \leq 6$ , 2.  $GS = 3 + 4$ , 3.  $GS = 4 + 3$ , 4.  $GS = 8$ , 5.  $GS \geq 9$ . The Welsh  $t$ -test ( $t$ -test) was used to compare means between two classes of data. The binomial exact test (binom.test) was used to compare observed to expected somatic mutation coverage of mtDNA genes and PCa categories. One-way ANOVA (anova) was used to evaluate the significance of multi-term linear models for association with GS.

### Large Deletions: Targeted Sanger Sequencing

A large 3,379 bp mitochondrial genome deletion (junction 10744:14124) has previously been reported as a potential PCa biomarker [38]. As detection of large deletions is problematic using short read next generation sequencing technology, together with the large long-range amplicons described, we designed a targeted approach including amplification of a 3.8 kb region using primers 10534F: 5'-AGGAGTATCCT-GAGGCATGG-3' and 14447R: 5'-ATCGCTCACACCT-CATATCC-3'. Deletion bands identified during agarose gel electrophoresis were excised from the gel and purified, followed by Sanger sequencing using both forward and reverse primers.

### Sequence Data Validation: Illumina MiSeq

Data validation using an alternative sequencing platform was performed for a random subset of 12 tissue and matched blood samples from 12/87 men. Library preparation using the Illumina Nextera XT DNA Library Preparation Kit involved simultaneous fragmentation and adapter tagging, followed by the addition of sequencing indexes to the amplified tagged DNA. A total of 24 barcoded samples were pooled for a single  $2 \times 250$  bp paired-end sequencing run on the Illumina MiSeq system. Quality trimming was done at Q33 (Phred scale) resulting in 18M reads and 4Gb of sequence. The same procedure was used to quantify somatic SNVs from MiSeq data as were used for Ion Torrent data to validate the results across the two sequencing platforms. Although a number of somatic indels were found in the Ion Torrent data, all indels were invalidated by MiSeq data, yet all SNVs were validated. Only SNVs were used in subsequent mtDNA somatic mutation analyses.

### Previous Disease Associations: Database Interrogation

All 113 somatic SNVs identified in this study that were associated with PCa were researched for previous association to diseases. The list of somatic mutations was downloaded from Mitomap ([www.mitomap.org/mitomap](http://www.mitomap.org/mitomap)) on May 15, 2015 and merged with results from searches of each mutation on Pubmed ([www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)). See Table SI for the full list of SNVs associated with PCa in the 77 ancestral African men from this study. There were 17/113 SNVs found to be previously associated with a disease in Mitomap, and an additional three found in Pubmed searches. A single SNV, (m.G3357A)

of the 113 presented here was previously implicated in PCa [24].

## RESULTS

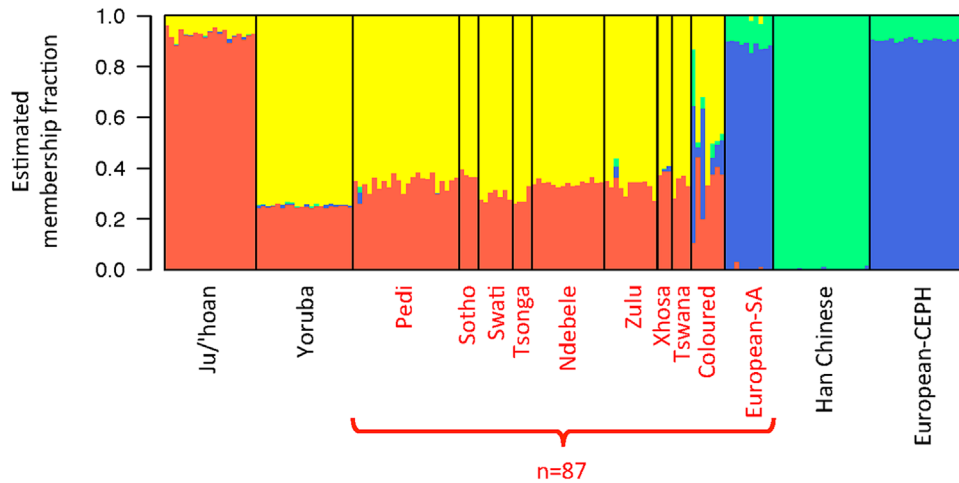
### Ancestral Contributions of Study Participants

In order to clarify the extent of ancestral African versus non-African contribution to the study participants, STRUCTURE ( $K=4$ ) analysis was performed for over 100,000 randomly distributed autosomal markers. In contrast to African-Americans with on average ~20% reported European ancestral contribution [39], Black South Africans have negligible non-African ancestral contributions (Fig. 1). The Coloured participants, however, show varying contributions of both African and non-African ancestral fractions, concurring with previous population-based studies [31,40]. In concordance with previous reports on South African populations [5,31], the African ancestral contribution to Black and Coloured South Africans can be further classified as African-Bantu and African-Khoesan. We confirm a lack of African ancestral contribution to the 10 European South Africans.

### Association of mtDNA Haplogroup With Clinical Presentation

Significant maternal African-Khoesan contribution to the Southern Bantu and Coloured of South Africa is reflected in the notable representation of ancient modern human maternal lineages, specifically the L0 mitochondrial haplogroups (See Supplementary Fig. S1). Among the 87 patients, 37 (32 Black and 5 Coloured) carried L0 (Table I), including almost 50% with the earliest diverging L0d haplogroup recently shown to have emerged ~172 thousand years ago (kya) (95%CI:149–199 kya) [30]. Interestingly, these individuals presented with more aggressive PCa, or higher average GS (mean = 6.3), than those carrying recently diverging (non-L0) haplogroups (mean = 4.9),  $P=0.049$ , (Fig. 2A). We also observed a general trend towards elevated serum PSA levels at the time of clinical presentation (mean = 190 ng/ml) in individuals with earlier diverged maternal lineages (mean = 116 ng/ml), although these differences were not significant at the  $P=0.05$  Level, (Fig. 2B). Although it is important to note the potential susceptibility of earlier diverging haplogroups to PCa and extreme PSA levels, we must take caution as the effects of population structure and culture on the likelihood of an individual seeking clinical diagnosis are not always possible to mitigate.

We find no significant association between PCa status and age at presentation (Fig. 2C). With a mean age of



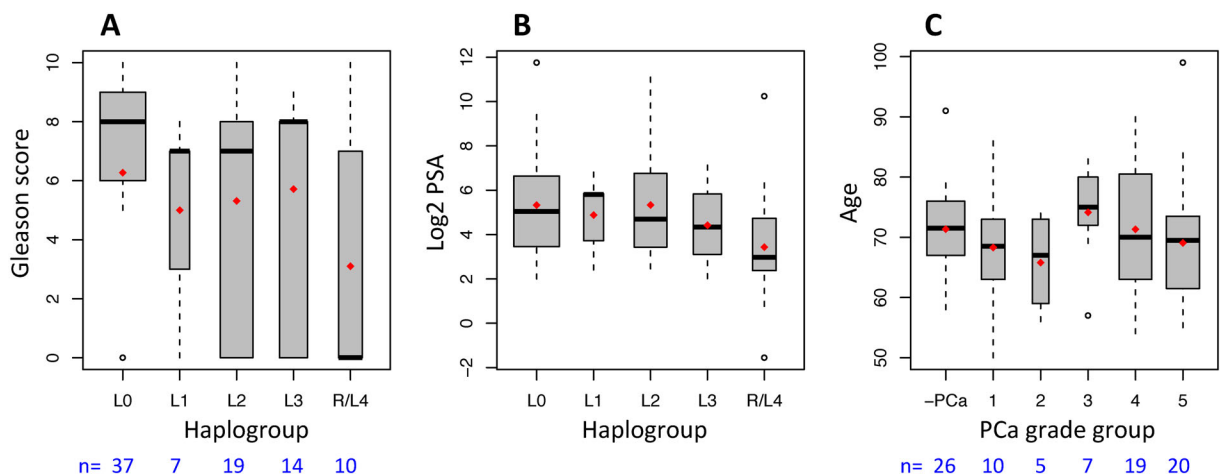
**Fig. 1.** Cluster ancestral fractions for PCa participants in this study (red label) and reference populations (black label) for comparison. Ancestral fractions estimated by STRUCTURE with  $K = 4$  clusters. The Orange cluster corresponds to African-Khoesan ancestry, Yellow to African-Bantu, Blue to European, and Green to Asian.

70.4 years the ages of subjects are more evenly distributed among PCa risk categories for this study than may be present in general in the South African population. Age ranges also were not significantly different between ancestral haplogroups (Supplementary Fig. S2), minimizing confounding effects due to age.

### Spectrum of mtDNA Somatic Mutations and Associated Clinical Presentation

Sequencing of matched blood and prostate tissue mtDNA samples were used to identify 144 somatic mutations. With a focus on African ancestry, we

assessed the distribution of 134 somatic mutations identified in 53 of 77 patients with African ancestry (70 Southern Bantu, 7 Coloured), in 128 different positions across the mitochondrial genome. There were 34 in NADH dehydrogenase subunits, 33 in rRNAs, 27 in Cytochrome c oxidases, 26 in the control region, 7 in Cytochrome b, 5 in ATP synthase, and 2 in tRNAs. Although somatic mutations were distributed across the entire mitochondrial genome (Fig. 3, we observe significantly more than expected in the control region under a purely stochastic process (binomial  $P = 1.2e-6$ ). The rRNA regions were also enriched for somatic mutations, though to a lesser



**Fig. 2.** Correlations between (A) ancestral mtDNA-haplogroups and clinical presentation by Gleason score, (B) haplogroups and serum PSA levels, and (C) PCa grade group categories of risk associated with age at presentation, including no PCa (-PCa). The earliest lineage (L0) presented with significantly ( $P < 0.05$ ) higher PCa risk than later diverging lineages. There was no significant association between age and PCa grade group. Red dots on box-plots indicate mean values.

extent ( $P = 5.2e-3$ ), while the NADH dehydrogenases and tRNAs had fewer somatic mutations than expected ( $P = 1.8e-3$  and  $P = 7.0e-4$ , respectively).

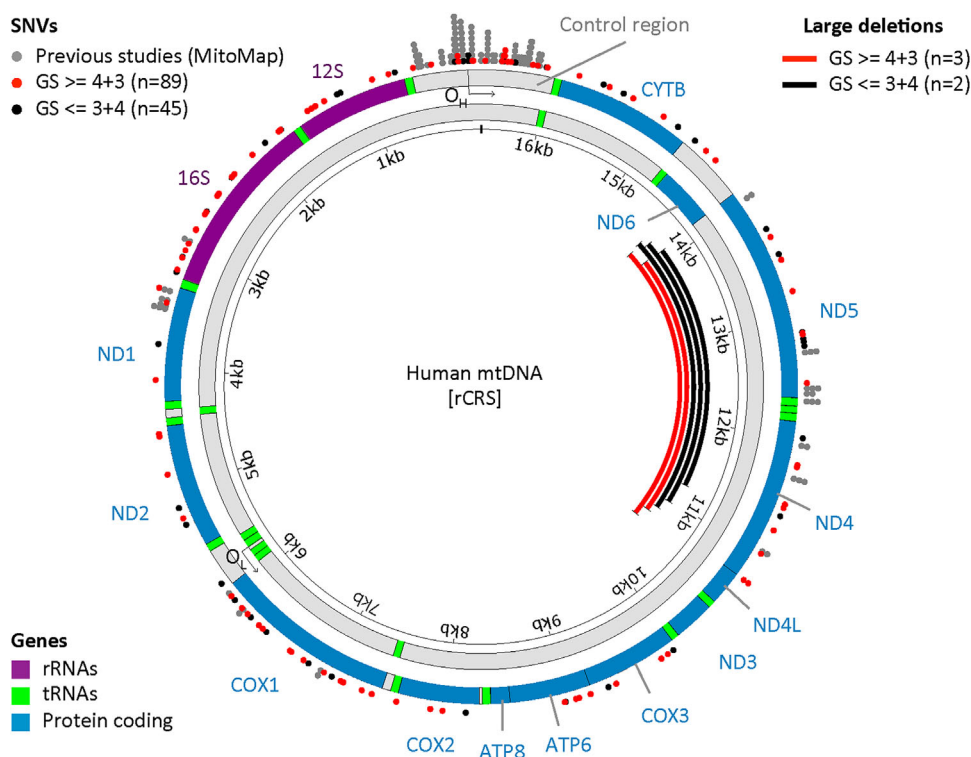
The low number of somatic mutations found in tRNAs, is in contrast to the findings of Kloss-Brandstatter, 2010 [27], where tRNA somatic mutations were found to be abundant in patients with PCa. The only tRNA mutations found here were in a patient presenting with indolent PCa (GG = 1, GS < 7) and one with no PCa. Of the 61 African-ancestral patients with PCa, a total of 113 different SNVs were observed, of which 95 did not appear to have any previous PCa association (see Supplementary Table SI).

Unlike, the previous association of a large 3,379 bp mtDNA deletion mutation (34 mtΔ) identified in a European population as a significant biomarker for PCa [38], this mutation was absent within our study. However, analysis of the entire mitochondrial genome found this region to be the most likely to acquire a deletion variant at sufficient detectable frequency. Four patients (4/87, 4.6%) presented with a large deletion mutation including a single individual presenting with two. Deletions varied in length (from

2,879 to 3,769 bp), involved a repeat sequence at the donor and recipient junction (2–10 bases) removing most of ND4 and ND5, and the three intervening tRNAs (Supplementary Table SIII). These four patients represented all three population identifiers and carried one or multiple somatic SNVs, suggesting patient specific genomic instability.

We find only five mutations shared between multiple individuals (Fig. 3). Four of the five are in the control region, with two G->A transitions m.G103A and m.G16390A, a A->G transition m.A189G, shared between two individuals, and a C->T transition m.C16380T shared by three individuals. The fifth shared mutation is a synonymous T->C transition found in the ND4L subunit, m.T10756C, and both patients have aggressive high-risk PCa (GG > 3, GS > 7). Most of these shared mutations involve at least one patient with aggressive PCa, with the exception of m.G103A, which is found in two men both with no PCa.

There is also a trend among these shared mutations that the individual with an appreciably larger variant frequency of the mutant allele also has the higher-risk aggressive PCa. We examine further this association between PCa progression and the number of somatic



**Fig. 3.** Distribution of somatic SNVs in aggressive PCa patients (red, this study GS >= 4 + 3), and no-PCa or indolent PCa (black, this study GS <= 3 + 4), overlaid with previously reported PCa associated SNVs from Mitomap (gray). Large deletions detected by targeted amplification are depicted as red (GS >= 4 + 3) and black (GS <= 3 + 4) lines. The human mitochondrial genome is depicted as the heavy strand (outer) and light strand (inner), with location of genes, and origins of replication for each strand. The control region and rRNA genes contain a significant enrichment of SNVs compared to other regions.

mutations or the cumulative total of variant frequency in the population of heteroplasmic mitochondria.

Associating the extent of mutational load in mtDNA and African ancestral PCa clinical presentation, we make the following observations. There are 80/134 somatic mutations among the highest risk PCa patients ( $GS > 7$ ), which is higher than would be expected at random ( $P = 7.3e-3$ ), and only 17/134 among individuals with no PCa, which is far fewer than we would expect given the number of subjects with no PCa ( $P = 2.4e-4$ ). As with serum PSA levels (Fig. 4A), we observe an accumulation of both the number of somatic mutations (Fig. 4B) and the frequency of these mutations (Fig. 4C) within the heteroplasmic mtDNA population that are associated with increased PCa risk.

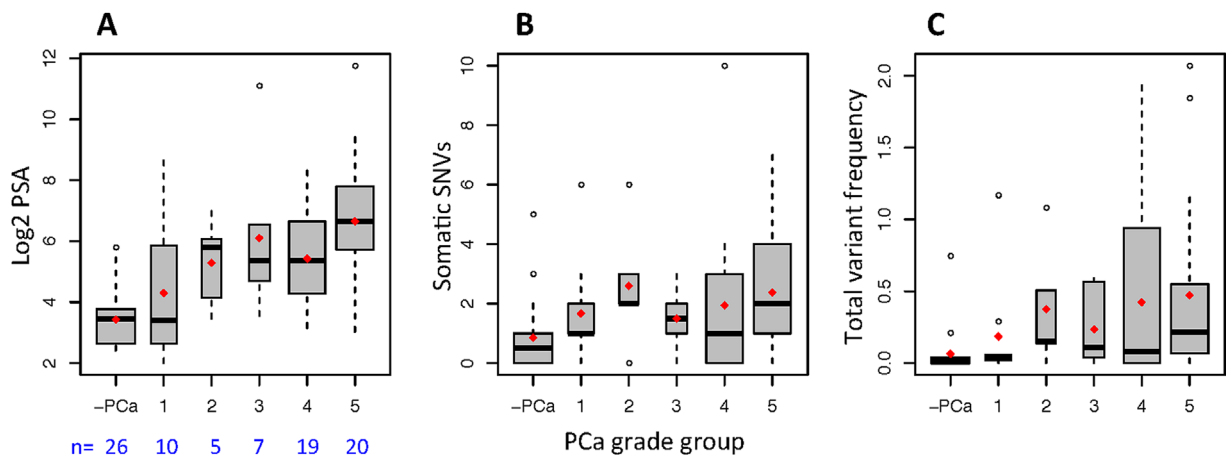
Factors correlated with increasing GS and PCa risk include serum PSA levels, number of somatic variants (SNV), and the cumulative variant frequency of somatic mutations (VF). We use the  $\log_2$  transform of PSA for correlations due to the extensive range of PSA values measured among the patients. We find the strongest association to increasing GS with  $\log_2$ -PSA (linear regression,  $P = 8.8e-7$ ), and categorically between PCa ( $GG \geq 3$  or  $GS \geq 4 + 3$ , generalized linear model  $P = 9.6e-5$ ). Significant associations with GS were also apparent from linear regression with VF and SNVs, ( $P = 2.5e-3$  and  $P = 1.3e-2$ , respectively). All three factors are positively correlated with each other and increasing GS, and provide predictive power for determining PCa aggression (Fig. 5). Multi-term linear models were compared using ANOVA but no significant increase was found above the single term  $\log_2$ -PSA model. The strong interdependence of

these factors limits the effectiveness of combining multiple predictors.

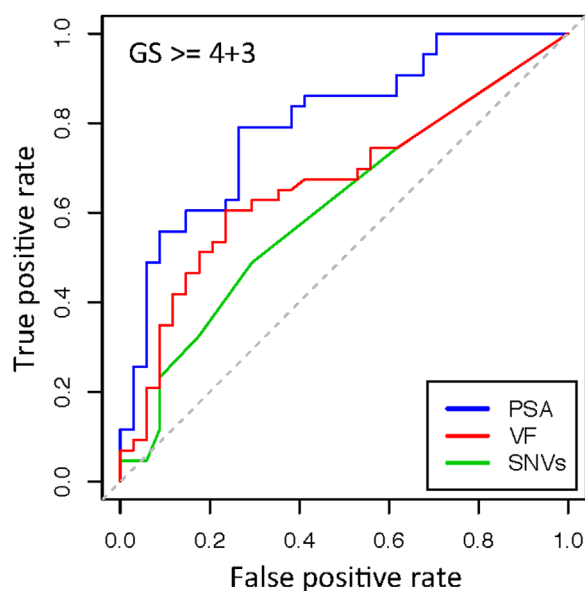
## DISCUSSION

In this study, we more than double the number of somatic PCa-associated mitochondrial genome mutations identified to date by focusing on men with African ancestry biased towards more aggressive disease. Data presented here suggest elevated risk associated with somatic mtDNA mutations in the control region and rRNA subunits, and no correlation between synonymous and non-synonymous exonic mutations. Other studies have found more risk-associated mutations in tRNAs or with non-synonymous mutations in specific genes. While it is possible our findings are unique to an ancestral African cohort, the limited concordance of previous studies continues to suggest that specific mutations are not as important as a non-specific mutational load to the presentation and aggression of PCa in general.

Although the increase in mtDNA mutations is a factor associated with PCa aggression, there must be a limitation on how many mutations a cell can withstand among mitochondrial populations before it is no longer viable. We expect the cancer phenotype to be more apparent with increasing mutations and frequency of mitochondria with these mutations, but with upper limits on both. Our findings are consistent with this hypothesis of limited mutation range. There is also the possibility that mitochondrial mutations are elevated simply due to a background of higher mutagenesis or reduction in repair, and that they are not causal but rather are indicators of such a cellular



**Fig. 4.** PCa grade groups, including no PCa (-PCa), associated with (A) serum PSA levels, and mtDNA mutational load measured by (B) number of somatic SNVs, and (C) total variant frequency of somatic SNVs in South African men of African ancestry. Red dots on box-plots indicate mean values.



**Fig. 5.** Receiver Operator Characteristic (ROC) curve for three factors associated with PCa aggression. Serum PSA levels, number of SNVs, and cumulative variant frequency (VF) calculated as the sum of variant frequency differences between tumor and blood samples that are above 2.0%. VF thus accounts for both the breadth and depth of mutation load within each population of heteroplasmic mtDNA.

environment. In either case, mutational load in mtDNA is an important indicator of PCa risk.

## CONCLUSIONS

Among men with African ancestry presenting with extreme serum PSA levels and a bias towards histopathologically defined aggressive PCa, we find the breadth and depth of mutational load, measured as the number, and frequency of mtDNA somatic mutations respectively, to be associated with PCa aggression. We confirm that PSA screening is the most important indicator for determining PCa risk in ancestral African populations. These results provide evidence that PCa progression is more likely characterized by the accumulation of mitochondrial mutations across the entire genome and in greater frequency within heteroplasmic cellular populations than it is by specific mutations. However, it remains important to continue cataloging common PCa-associated mutations in different populations as the effects of specific mutations, especially within varied genetic backgrounds, cannot be assumed to be consistent. The mutations provided here will further help with the quantification of risk across the mitochondrial genome, while with increasing precision and decreasing cost of next-generation sequencing we hope more studies will follow that focus on PCa within understudied populations.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the following for their help and funding of this study: NIH R21-CA170081, Australian Prostate Cancer Research Centre NSW, the J. Craig Venter Institute, the Garvan Institute, the Petre Foundation, Australia, the Cancer Association of South Africa (CANSAs), and all the study participants.

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