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14. ABSTRACT (200words)

Mitochondrial-dysfunction is associated with prostate-cancer (PC). We hypothesized that differences in MDP-expression levels contributes to the elevated-risk of PC in Black men. Our project is testing the potential of MDPs as biomarkers for PC risk, both as circulating pre-diagnostic biomarkers and as surrogate mitochondrial genetic risk indicators in a group of Black and White men with and without PC. Our Specific Aims include 1) To confirm the association between SHLP2 levels and prostate cancer risk; 2) to determine the association between additional plasma MDPs and prostate cancer risk among Black and White men in a newly enrolled population; and 3) to determine the contribution of mitochondrial versus nuclear genetic-race, relative to self-reported-race, to the MDP-PC-risk connection. We have succeeded in enrolling substantial numbers of patients of both ethnicities. Our data confirms that MDP levels are generally lower in plasma of PC patients. We identified novel ethnic-specific mtDNA variants associated with PC. We showed that mtDNAcn is also associated with PC risk. This study is the first to study racial differences in MDP levels and their effect on PC. MDP levels and mitochondrial genetic origin may serve as diagnostic biomarkers of PC. Moreover, this study may set the stage for future mitochondrial-related interventions in minority populations.

15. SUBJECT TERMS
Mitochondria, mtDNA, mitochondrial derived peptides, prostate cancer risks, biomarkers

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Introduction

Incidence and death by cancer is highly disparate between racial and ethnic groups, with several possible causes such as differences in socioeconomic status, behavioral risk factors, and underlying genetics all playing a role. In particular, Black Americans are at higher risk of developing and dying from a number of cancers such as colorectal, lung, and prostate cancer. Prostate cancer is the second leading cause of cancer death in men in America and is one of the most racially disparate cancers. Blacks are 1.6 times more likely to be diagnosed with prostate cancer than Whites and 2.4 times more likely to die of the disease.

OUR AIMS for this project included:

Aim 1: Confirm the association between high serum SHLP2 levels and decreased prostate cancer risk and lower SHLP2 levels.

Aim-2: Determine the association between plasma MDPs and prostate cancer risk among Black and White men in a newly enrolled population.

Aim-3: Aim 3: Determine the contribution of mitochondrial versus nuclear genetic-race, relative to self-reported-race, to the MDP-PC-risk connection.

Keywords: PROSTATE CANCER, HEALTH DISPARITIES, MITOCHONDRIA, PEPTIDES, DIAGNOSTICS, HUMANIN MOTS-C, SHLP2.

Accomplishments

Major goals of the project

1: Confirm the association between high serum SHLP2 levels and decreased prostate cancer risk.
2: Determine the association between plasma MDPs and prostate cancer risk among Black and White men in a newly enrolled population.
3: Determine the contribution of mitochondrial versus nuclear genetic-race, relative to self-reported-race, to the MDP-PC-risk connection.

What was accomplished under these goals?

1) **Major Activities**

a. Patient and Sample Recruitment

Plasma samples have been collected from over 600 participants. Participants have been primarily Black as well as White, and a small percentage of Hispanics and Asian/Pacific Islander origin. Black men comprise 58% of the samples, and White men comprise the other 42% of the plasma samples. One third of Black men had negative biopsy results and two thirds had positive biopsy results. About 40% of White men had negative biopsy results and nearly 60% had positive biopsy results.

To achieve a comparable sample size, we analyzed a total of 400 samples (200 Whites - 100 each with negative and positive biopsy samples and 200 Blacks - also 100 each with negative and positive biopsy samples)

b. Conduct Humanin, MOTS-c, SHLP2, and SHLP6 assays on plasma samples

Our assay team ran SHLP2 serum assays on 400 men and an additional 800 assays on plasma from 200 patients (100 Black men and 100 White men; 50 each with and without positive biopsies for prostate cancer). Data is shown below.

c. mtDNA sequencing on 400 samples

We isolated DNA and PCR amplified the mitochondrial genome of 400 samples and followed this by sequencing of the entire mitochondrial genome for further analysis.

d. mtDNAcn on 400 samples

On the same samples as above, we performed mitochondrial copy number analysis on 400 samples and used this data to achieve objective 2e.

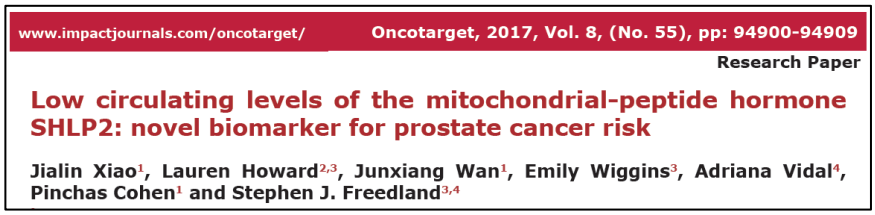
2) **Specific objectives that have been achieved**: We demonstrated as shown below that:

a. MDP levels are lower in Black men compared to White men
b. MDP levels are lower in biopsy positive cancer patients compared to negative ones.
c. mtDNA is highly correlated with self-reported ethnicity
d. mtDNA SNPs are predictive of prostate cancer risk
e. mtDNAcn is related to cancer diagnosis and MDPs

3) **Significant Results**

a. Several key papers have been published as a result of our work so far:

i. Xiao J, Howard L, Wan J, Wiggins E, Vidal A, Cohen P, Freedland SJ. Low circulating levels of the mitochondrial-peptide hormone SHLP2: novel biomarker for prostate cancer risk. *Oncotarget*. 2017; 8:94900-94909.


www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 55), pp: 94900-94909

Research Paper

Low circulating levels of the mitochondrial-peptide hormone SHLP2: novel biomarker for prostate cancer risk

Jialin Xiao[1], Lauren Howard[2,3], Junxiang Wan[1], Emily Wiggins[3], Adriana Vidal[4], Pinchas Cohen[1] and Stephen J. Freedland[3,4]

ii. Some of the key findings in these papers include:

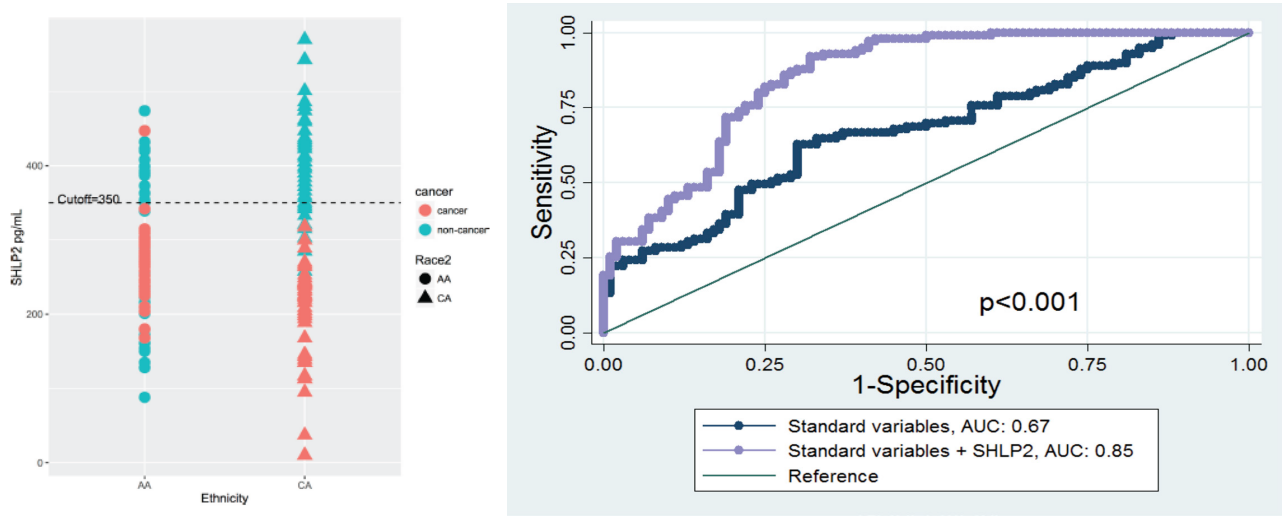


Figure 1. SHLP2 is a potential biomarker of prostate cancer. The left panel demonstrates that a cutoff of 350pg/mL can differentiate between cancer and non-cancer in both African-American (AA) and Caucasian-American (CA). The right panel graphs the improvement in sensitivity that adding SHLP2 to the model achieves.

Demonstrating the potential clinical utility of serum SHLP2, the distribution of SHLP2 levels with a cut-off at 350-pg/ml (Figure 1 Left panel) can differentiate between controls and PCa cases in both black and white men. Among men with SHLP2 > 350-pg/ml, 0/37 white (100% NPV) and only 1/20 black men had PCa (95% NPV). ROC curve and AUC statistics before and after adding SHLP2 in the model (Figure 1 Right panel). The true positive rate (sensitivity) is plotted in function of the false positive rate (1−specificity) for the model excluding or including SHLP2 levels. The AUC is a measure of how well a quantitative test can distinguish between subjects with and without prostate cancer. The AUC of the model including only age, DRE, race and PSA to predict PCa risk was 0.67. This improved to 0.85 when SHLP2 was added to the model ($p < 0.001$). Thus, lower SHLP2 was linked with increased PCa risk in white men, but no significant association was observed in black men. While SHLP2 > 350-pg/ml ruled out PCa in both races with high accuracy, SHLP2 was unrelated to PCa grade. These data suggest that the circulating mitochondrial-derived peptide hormone SHLP2 plays a key role in the development and racial disparity of prostate cancer.

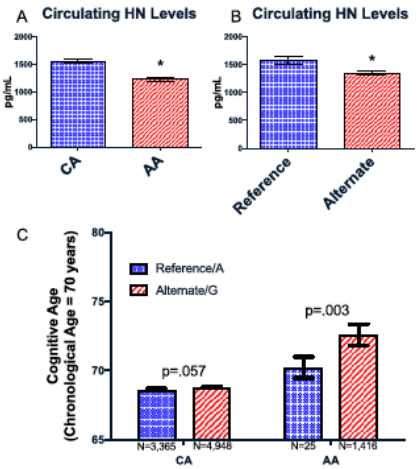


Figure 2. (A) African-Americans have lower circulating humanin levels than Caucasian-Americans. (B) Individuals with the mitochondrial SNP rs2854128 have a significant decrease in circulating humanin levels. (C) RS2854128 is associated with an increased cognitive age that is more pronounced in African-Americans. *Signifies $p < 0.05$.

Furthermore, in another publication (Yen et al. Humanin Prevents Age-Related Cognitive Decline in Mice and is Associated with Improved Cognitive Age in Humans. *Nature Scientific Reports*. 2018; 8:14212. doi: 10.1038/s41598-018-32616-7.), we identified a specific SNP (rs2854128) in the humanin-coding region of the mitochondrial genome that is associated with a decrease in circulating humanin levels (Figure

2B). In a large, independent cohort, consisting of a nationally representative sample of older adults, we found that this SNP is associated with accelerated cognitive aging, supporting the concept that humanin is an important factor in aging-related diseases in an ethnic-specific fashion (Figure 2C).

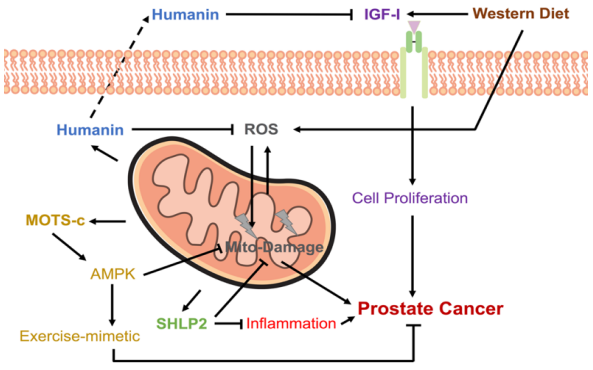


Figure 3. Mode of action of mitochondrial-derived peptides (MDPs) against prostate cancer

In a recent review, published in the journal *Carcinogenesis*: Xiao J, Cohen P, Stern MC, Odedina F, Carpten J, Reams R. Mitochondrial biology and prostate cancer ethnic disparity. *Carcinogenesis*. 2018; doi.org/10.1093/carcin/bgy133, we outlined the relationship between mitochondria, lifestyle and prostate cancer (Figure 3). Humanin, MOTS-c and SHLP2 are encoded in the mitochondrial genome and participate in various signaling events intracellularly or extracellularly. Humanin can suppress prostate cancer by inhibiting mitochondrial-generated ROS and reducing the levels of free circulating IGF-I. MOTS-c exerts anti-tumor effects via acting as an exercise-mimetic, while SHLP2 alleviates inflammation and promotes mitochondria health.

Additional data in submission related to Aims -1 and -2

<u>We performed extensive analysis of 4 different mitochondrial peptides in the enrolled population of Black and White men with and without prostate cancer</u>

As noted above, we ran our four novel MDP assays for humanin, SHLP2, SHLP6, and MOTS-c on 200 plasma samples collected over the last year (100 Blacks, 100 Whites, each set with 50 cancer and 50 controls).

The majority of the data below is included in Ramirez-Torres A, Reagan AL, Howard LE, Wiggins E, Vidal AC, Wang J, Freedland SJ, Cohen P. Racial differences in circulating mitochondria-derived peptides may contribute to prostate cancer health disparities. Submitted. Lancet Oncology. 2021. Also presented as Abstract # MP09-13 and Selected as the BEST POSTER during the Moderated Poster Session MP09: Prostate Cancer: Markers I. AUA2020 Virtual Science. May 15, 2020

In this manuscript we tested whether humanin, SHLP2, and MOTS-c as measured in plasma correlated with PC risk by race in a separate sample of men undergoing prostate biopsy. There was a statistically significant p-interaction between all 3 MDPs and race for predicting PC (all p-interaction≤0.01). Among EA men, higher values of all MDPs were significantly associated with lower PC diagnosis (all p≤0.001). In contrast, in AA men, all MDPs were unrelated to PC diagnosis. Similarly, higher expression of all MDPs was associated with decreased risk of both low- and high-grade PC in EA men (all p≤0.005) but none were associated with low- or high-grade in AA men. AA controls had lower MOTS-c (p=0.026) and SHLP2 (p=0.001) values than EA controls (Table 1 and 2, Figure 4).

	AA Cases (N=50)	AA Controls (N=49)	p value	EA Cases (N=50)	EA Controls (N=50)	p value
Humanin (pg/ml)			0.015[1]			<0.001[1]
Median	654.7	815.9		546.6	798.7	
Q1, Q3	527.2, 793.6	587.0, 954.9		392.4, 702.0	694.0, 917.4	
SHLP2 (pg/ml)			0.774[1]			<0.001[1]
Median	1800.2	1798.5		1786.2	1835.5	
Q1, Q3	1757.6, 1832.2	1750.0, 1832.4		1722.5, 1814.0	1802.3, 1901.0	
MOTS-c (pg/ml)			0.392[1]			<0.001[1]
Median	356.8	360.4		338.9	372.5	
Q1, Q3	340.8, 379.4	348.0, 375.9		325.8, 359.1	362.1, 380.9	

[1]Wilcoxon rank sum

Table 1. Plasma levels of humanin, SHLP2, and MOTS-c levels by race and case/control status

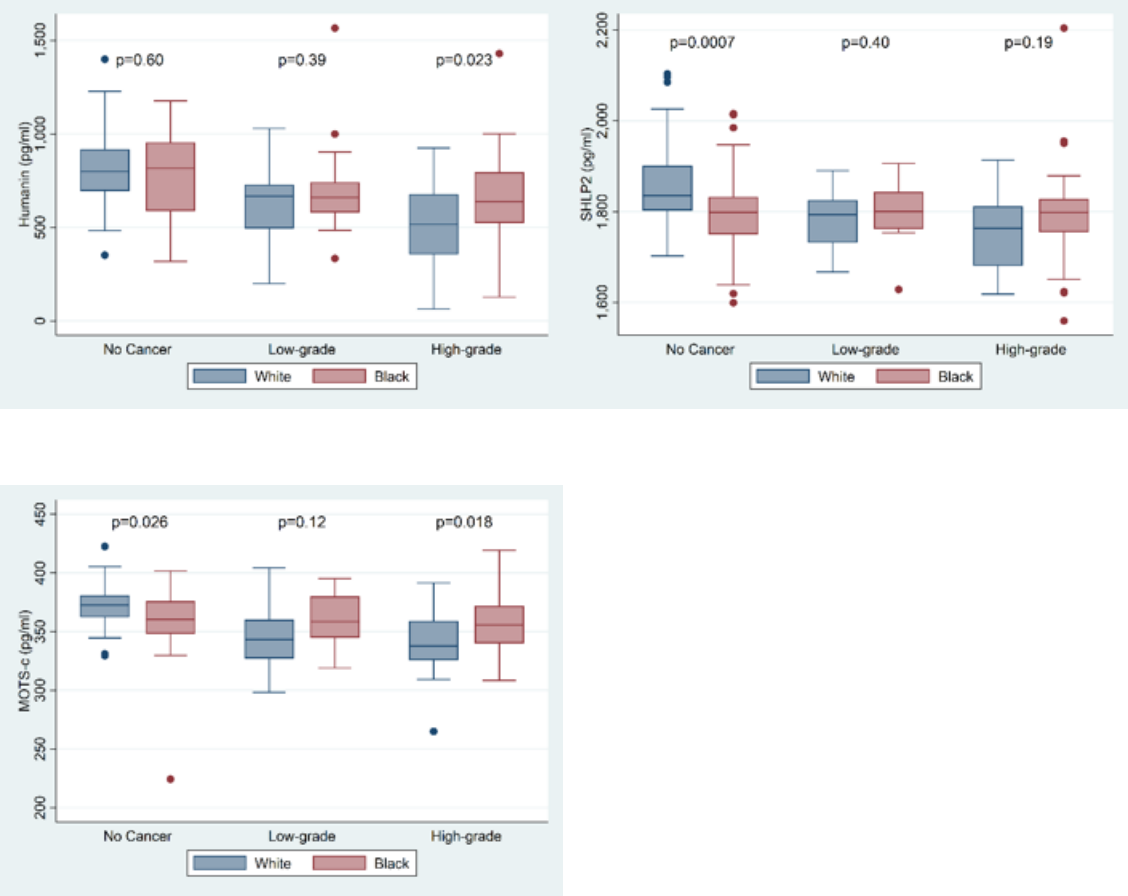


Figure 4. Distribution of humanin, SHLP2, and MOTS-c levels categorized by no cancer, low-and high-grade PC per race.

	EA		AA		
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**p****
Humanin*					
Unadjusted					
Overall PC (vs. none)	0.94 (0.92-0.97)	<.0001	0.98 (0.97-0.999)	0.043	
No PC	Ref.		Ref.		
Low-grade PC	0.96 (0.93-0.98)	0.003	0.99 (0.97-1.01)	0.37	
High-grade PC	0.93 (0.90-0.96)	<.0001	0.98 (0.96-0.998)	0.029	
Adjusted					
Overall PC (vs. none)	0.95 (0.92-0.97)	<.0001	0.98 (0.97-1.00)	0.054	0.014
No PC	Ref.		Ref.		0.048
Low-grade PC	0.96 (0.93-0.99)	0.004	0.99 (0.96-1.01)	0.21	
High-grade PC	0.94 (0.91-0.97)	<.0001	0.98 (0.96-1.00)	0.065	
SHLP2*					
Unadjusted					
Overall PC (vs. none)	0.86 (0.80-0.92)	<.0001	1.00 (0.96-1.05)	0.94	
No PC	Ref.		Ref.		
Low-grade PC	0.89 (0.81-0.96)	0.005	1.01 (0.95-1.07)	0.76	
High-grade PC	0.84 (0.77-0.91)	<.0001	1.00 (0.95-1.05)	0.92	
Adjusted					
Overall PC (vs. none)	0.85 (0.78-0.93)	0.0002	1.01 (0.96-1.06)	0.75	0.0004
No PC	Ref.		Ref.		0.001
Low-grade PC	0.87 (0.79-0.96)	0.005	1.01 (0.95-1.07)	0.75	
High-grade PC	0.83 (0.75-0.91)	0.0002	1.01 (0.95-1.07)	0.79	
MOTS-c*					
Unadjusted					
Overall PC (vs. none)	0.54 (0.42-0.70)	<.0001	0.97 (0.84-1.13)	0.72	
No PC	Ref.		Ref.		
Low-grade PC	0.58 (0.43-0.77)	0.0002	0.97 (0.79-1.19)	0.80	
High-grade PC	0.52 (0.40-0.69)	<.0001	0.97 (0.83-1.15)	0.75	
Adjusted					
Overall PC (vs. none)	0.57 (0.44-0.73)	<.0001	0.97 (0.83-1.14)	0.73	0.0003
No PC	Ref.		Ref.		0.001
Low-grade PC	0.58 (0.43-0.77)	0.0002	0.94 (0.77-1.15)	0.57	
High-grade PC	0.55 (0.41-0.73)	<.0001	1.00 (0.82-1.21)	0.99	

Table 2. Association between humanin, SHLP2 and MOTS-c and overall risk of cancer and risk of cancer grade, stratified by race

	PC vs. no PC			High-grade PC vs. low-grade or no PC		
	MDP alone	Base model	Base model + MDP	MDP alone	Base model	Base model + MDP
EA Men						
Humanin	0.80	0.65	0.82	0.78	0.77	0.86
SHLP2	0.77	0.65	0.81	0.74	0.77	0.84
MOTS-c	0.83	0.65	0.84	0.76	0.77	0.82
AA Men						
Humanin	0.64	0.65	0.69	0.63	0.72	0.73
SHLP2	0.52	0.65	0.65	0.52	0.72	0.72
MOTS-c	0.55	0.65	0.66	0.55	0.72	0.72

Table 3. Area under the curve (AUC) for humanin, SHLP2 and MOTS-c models of single MDPs for PC and high-grade PC diagnosis.

	PC vs. no PC			High-grade
	MDPs alone	Base model	Base model + MDPs	MDPs alone
EA Men				
Humanin + SHLP2	0.83	0.65	0.85	0.80
Humanin + MOTS-c	0.84	0.65	0.85	0.80
SHLP2 + MOTS-c	0.84	0.65	0.86	0.79
Humanin + SHLP2 + MOTS-c	0.85	0.65	0.86	0.80
AA Men				
Humanin + SHLP2	0.65	0.65	0.68	0.63
Humanin + MOTS-c	0.65	0.65	0.69	0.64
SHLP2 + MOTS-c	0.55	0.65	0.66	0.53
Humanin + SHLP2 + MOTS-c	0.65	0.65	0.68	0.64

Table 4. Area under the curve (AUC) for humanin, SHLP2, and MOTS-c combination models for PC and high-grade PC diagnosis

Comparison of MDP levels by race

When comparing across races (Fig 4), CA controls had significantly higher levels of SHLP2 ($p=0.0007$) and MOTS-c ($p=0.026$) vs. AA controls, but humanin was similar between groups ($p=0.60$). In low-grade comparison, humanin, SHLP2 and MOTS-c had similar levels between groups and didn't show differences across races. Interestingly, CA high-grade had significantly lower levels of humanin ($p=0.023$) and MOTS-c ($p=0.018$) vs. AA high-grade

The levels of all 4 MDPs are significantly lower in White patients with Cancer compared with White subjects with negative biopsies (Figure 5). In comparison, only plasma humanin levels are lower in Black patients with Cancer compared with Black subjects with negative biopsies. Of note is that the degree of reduction (while very highly significant) varies from 10-30%. In comparison, the reductions in

	White	Black
	p**	p**
SHLP2		
Overall PC (vs. none)	0.53	0.70
Humanin		
Overall PC (vs. none)	0.96	0.079
MOTS-c		
Overall PC (vs. none)	0.47	0.059

Table 5. Biomarker and bmi interactions
Multivariable models are adjusted for age at biopsy, BMI, DRE, and PSA at biopsy. p** = p-value for biomarker and BMI interaction.

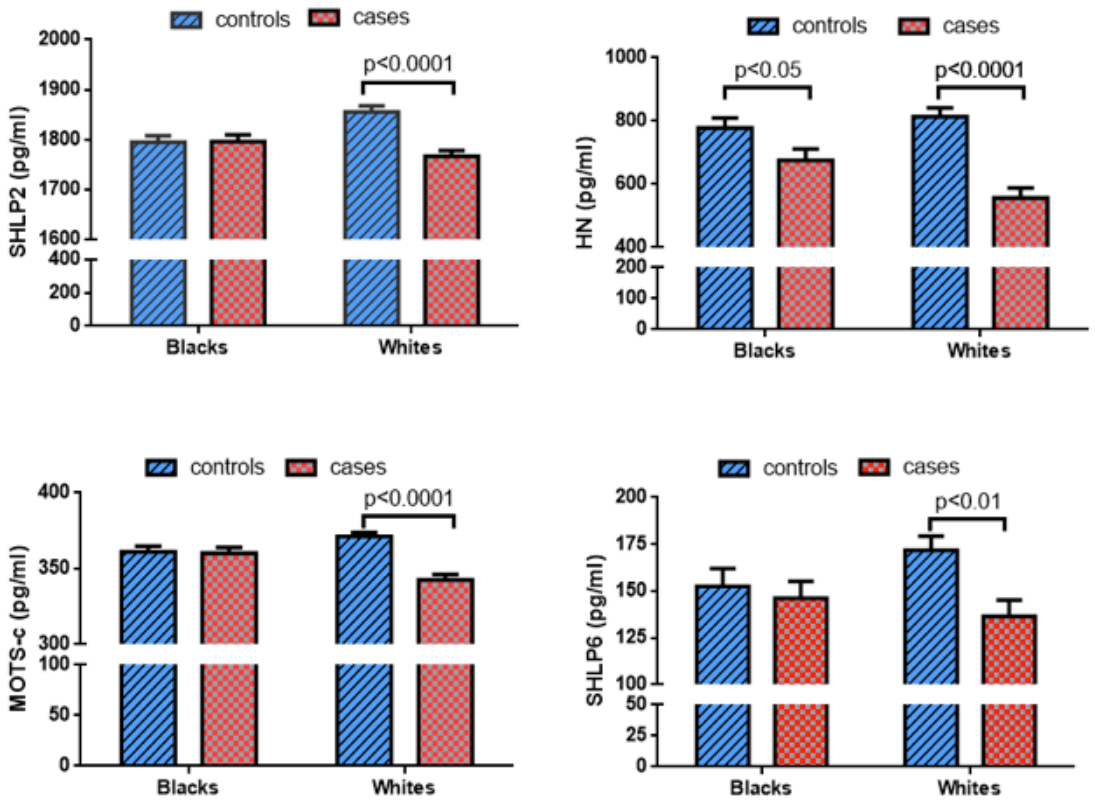


Figure 5. Plasma MDP levels in Black and White patients with or without PC

serum SHLP2 were more prominent (close to 50%). The reasons for this are unclear and we will evaluate this in the next year. These results also suggest a more general issue related to OVERALL mitochondrial function in patients that may predispose them to cancer. We will also evaluate the potential demographic and lifestyle aspects of this observation.

Furthermore, when we compared the levels of these MDPs in biopsy negative men between Black and Whites, the following picture emerged (Figure 6):

Plasma SHLP2 levels were lower in Black men compared to White men, however, the degree of difference was smaller than we observed for serum SHLP2. Interestingly, MOTS-c levels were also lower in Blacks, while humanin and SHLP6 levels were not significantly different.

Higher expression of all three MDPs studied (plasma SHLP2, humanin, and MOTS-c) were associated with lower PC risk in CA men but not in AA men. However, in general, AA controls had lower MDP levels than CA controls. These data support the importance of MDPs and perhaps mitochondrial dysfunction in PC and suggest greater dysfunction in AA men (lower MDP levels in controls), which may contribute to excess PC risk in AA men, though this requires confirmation in future larger studies.

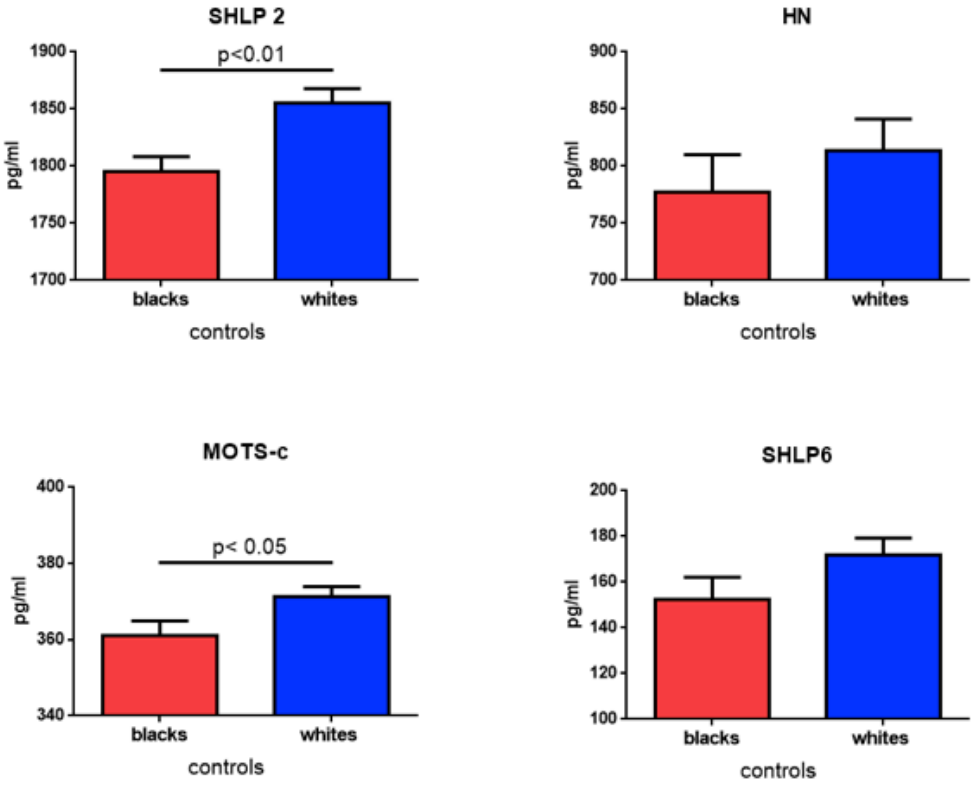


Figure 6. Plasma levels of MDPs in Black and White patients.

Additional data related to aim-3

We published a paper: Miller B, Arpawong TE, Jiao H, Kim SJ, Yen K, Mehta HM, Wan J, Carpten JC, Cohen P. Comparing Nuclear and Mitochondrial DNA from a Multi-Ethnic Cohort to Adjust for Genetic Ancestry in Association Studies. *Cells*. 2019. 8(4), 306; doi: 10.3390/cells8040306.

In this work we showed that health disparities are explained by mtDNA**.** Aging and ethnicity are two of the greatest risk factors for prostate cancer. Among U.S ethnic groups of individuals over 75 years, African Americans exhibit the highest risk. It is unclear why ethnicity is a risk factor, although several studies illustrate that lifestyle, education, geographical location, and nuclear genetics play a multi-factorial role. Notably, in our recent work published in the mitochondrial genetics special issue of *Cells*, we showed that array-captured mtSNPs model genetic ancestral admixture within sub-ethnic samples in the Health and Retirement Study (HRS) (Figure 7). This mtDNA admixture could partially explain disparities between and within ethnicities, but the field has lacked comprehensive analytic plans to address the relationship between mtDNA and disease. In fact, general genomic analyses

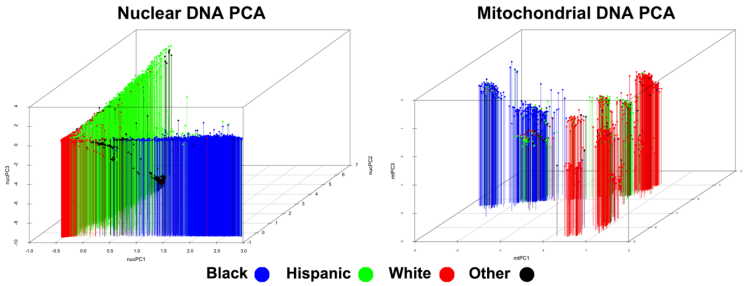


Figure 7. Multi-ethnic principal component analysis on nuclear and mitochondrial SNPs. Array-captured mtSNPs model heterogeneity within sub-ethnic samples in the Health and Retirement Study. (Miller et al., 2019)

ignore the mitochondrial genome due to its non-nuclear behavior (i.e., haploid genome, faster mutation rate, non-Hardy-Weinberg, etc.), and most multi-ethnic cohorts lack ample power and longitudinal designs needed to estimate the effects of mitochondrial genes on disease. Ultimately, mitochondrial genetic variation among and within ethnicities could represent therapeutic targets for PCa.

We further analyzed mtDNA through whole mitochondrial sequencing on 400 patients (200 Blacks and 200 Whites) to a) compare the ability of mitochondrial sequencing to correlate with self-reported ethnicity (and our data demonstrated it performed better than the nuclear data previously reported, and most importantly, identify novel mtDNA SNPs that predict prostate cancer risk in Black versus Whites.

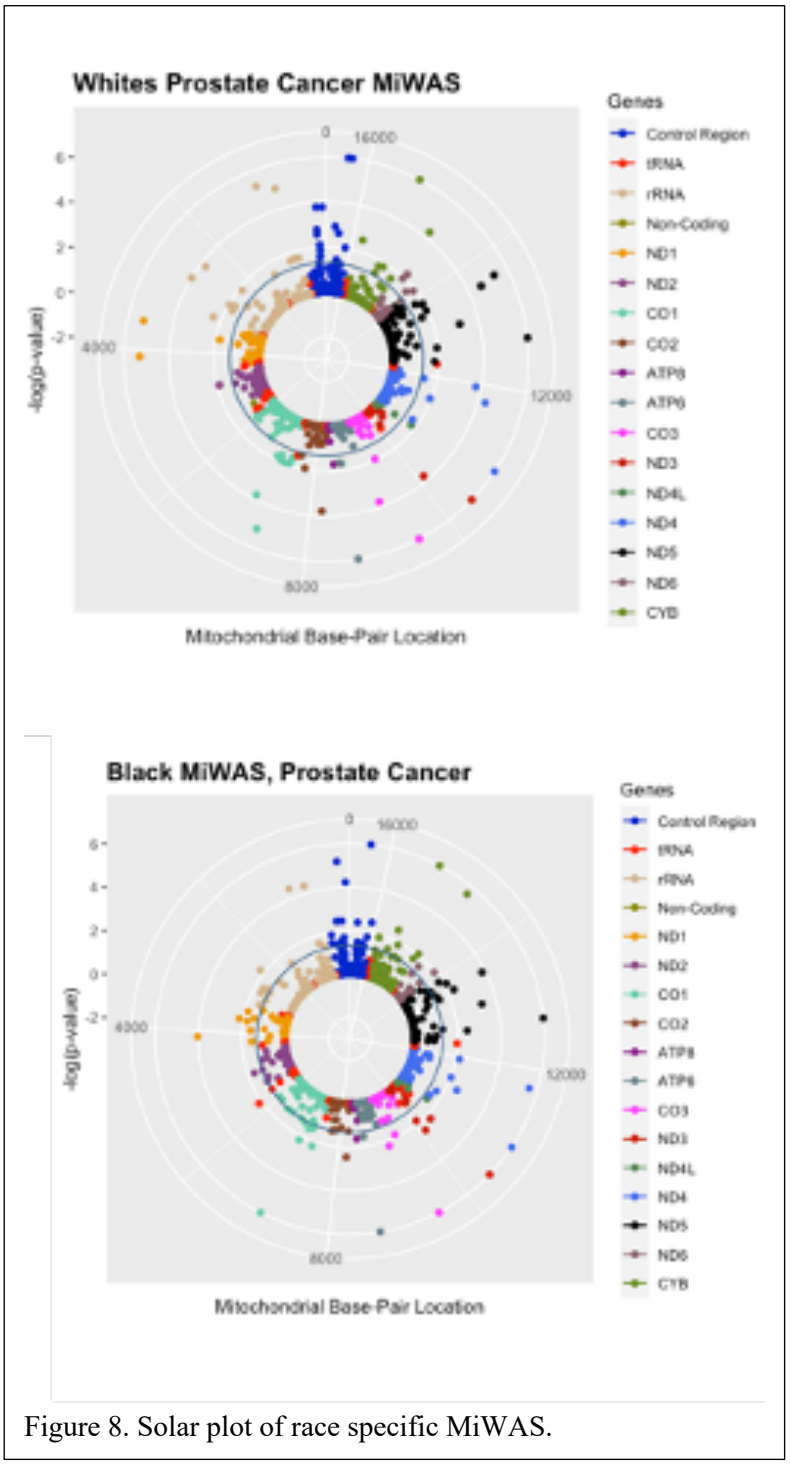


Figure 8. Solar plot of race specific MiWAS.

Methods: Our methods involved two phases: (1) sequencing processing and (2) Mitochondrial-Wide Association Study – called MiWAS. For the processing of sequencing files, raw fastq files from mitochondrial whole genome sequencing data were downloaded into computing clusters at USC's Center for High-Performance Computing. Fastq files were generated by Fulgent Genetics, a commercial sequencing service that generated sequencing data from saliva-derived DNA. Fastq files were processed through FastQC. Mitochondrial variants were called using STAR, SAMtools and BCFtools, tabix, and GenomeAnalysisTK (GATK) for use with FastAlternateReferenceMaker. We mapped the fastq files to the human reference mitochondrial genomes using STAR alignment. Variant calling was performed using SAMtools mpileup. The files were converted to VCF format and indexed with tabix. By looping all samples through the GATK FastaAlternateReferenceMaker, individual fasta files for each sample were generated. We then converted the VCF file to PLINK formatted binary data files (e.g., bam, bim, and fam). PLINK is a widely-used open-source tool to conduct genetic analyses. All PLINK files have undergone strict quality control standards applied to SNPs (e.g., removal of duplicates and SNPs with missing call rates >2%, or sex difference in allelic frequency greater than 0.2) and samples (e.g., applying minor allele frequency thresholds of 0.01, removal of individuals missing >=10% of genotypes).For MiWAS, we implemented adaptive permutation chi-square haploid allelic test (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4070098/) in PLINK. This

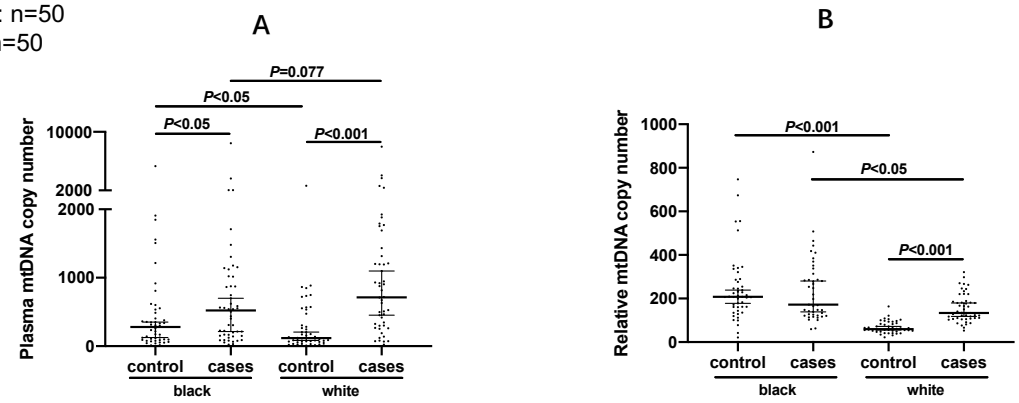
method compares each observed test statistics against all permuted maximum statistics over all mitochondrial SNPs for each replicate. This permutation scheme sustains the correlation structure between mitochondrial SNPs. The corrected p value (i.e., empirical p value) is considered genome significant under 0.05. We conducted each MiWAS by ethnicity (i.e., white and black).

Results: For the white-specific MiWAS, 100 controls and 92 cases were examined. 432 mitochondrial SNPs passed quality assurance and a 1% minor allele threshold. 82 mitochondrial SNPs were considered significantly associated with the incidence of prostate cancer ($p < 0.05$; Figure 8). For the black-specific MiWAS, 95 controls and 99 cases were examined. 441 mitochondrial SNPs passed quality assurance and the 1% minor allele threshold. 77 mitochondrial SNPs were significantly associated with prostate cancer. We are in the process of contextualizing these SNPs with mitochondrial-derived peptides.
This preliminary data represents an important opportunity to curate novel mitochondrial peptides that are related to prostate cancer risk and may be useful for a) genetic risk assessment, b) assay development for improving diagnosis, and c) drug development for replacing MDPs that are mutated in prostate cancer.

We are currently collaborating with the group overseeing the Multiethnic Cohort study that recently analyzed prostate cancer risk: Conti DV, ..., Haiman CA. Trans-ancestry genome-wide association meta-analysis of prostate cancer identifies new susceptibility loci and informs genetic risk prediction. Nat Genet. 2021 Jan;53(1):65-75. doi: 10.1038/s41588-020-00748-0. Epub 2021 Jan 4. PMID: 33398198.
We hope to validate our findings using this larger cohort.

Comparing circulating cell-free mtDNAcn with WBC mtDNA copy number

- **Samples: Prostate cancer plasma (n=199)**
  black controls: n=49
  black cases: n=50
  white controls: n=50
  white cases: n=50



A, Plasma mtDNA copy number. DNA were extracted from plasma.
B, WBC mtDNA copy number, ND1 gene expressions were normalized by β-actin.

Exact value of Fig. A
1. Black control: median=280.3
2. Black cases: median=521.9
3. White control: median=116.9
4. White case: median=714.2

Exact value of Fig. B
1. Black control: median=207.8
2. Black cases: median=172.3
3. White control: median=59.74
4. White case: median=134.1

Figure 9. mtDNA copy number and prostate cancer

mtDNA copy number (mtDNAcn) has been previously linked to prostate cancer (Tu H, Gu J, Meng QH, Kim J, Davis JW, He Y, Wagar EA, Thompson TC, Logothetis CJ, Wu X. Mitochondrial DNA copy number in peripheral blood leukocytes and the aggressiveness of localized prostate cancer. Oncotarget. 2015 Dec 8;6(39):41988-96) and other cancers (Yang K, Li X, Forman MR, Monahan PO, Graham BH, Joshi A, Song M, Hang D, Ogino S, Giovannucci EL, De Vivo I, Chan AT, Nan H. Pre-diagnostic Leukocyte Mitochondrial DNA Copy Number and Colorectal Cancer Risk. Carcinogenesis. 2019 Sep 26. pii: bgz159. doi: 10.1093/carcin/bgz159). Our preliminary data demonstrate specificity of cell free mtDNAcn relative to WBC mtDNAcn, in terms of cancer diagnosis and also the first time it has been shown to display ethnic differences. This data is important in the emerging spectrum of mtDNAcn analysis: (Mitochondrial DNA copy number in human disease: the more the better? Filograna R, Mennuni M, Alsina D, Larsson NG. FEBS Lett. 2020 Dec 12. doi: 10.1002/1873-3468.14021. Online ahead of print. PMID: 33314045)

Opportunities for training and professional development
Dr. Cohen has a track record in training underrepresented minority students and is involved in a program with the Historically Black Colleges Howard University and FAMU that sends two undergraduate students to his lab for three months every summer since 2017 that has resulted in a highly successful research experience for these URM students.

Dissemination of the results to communities of interest
Dr. Cohen has presented this work in various meetings. Some data has also been published by Drs. Cohen and Freedland (see list of publications below)

Impact

Ethnic disparity in health outcomes is one of the greatest challenges facing our nation. Black men are at greater risk for aggressive Prostate Cancer, and recent data from our laboratory has identified that black men have lower levels of the mitochondrial peptide humanin and the small humanin-like peptide SHLP2. These peptides are encoded within the mitochondrial DNA (mtDNA) and gene variants of these novel mitochondrial small open reading frames (sORF) are maternally inherited and are strongly associated with ethnicity. Our recent data shows that low levels of SHLP2 are a potent predictor of prostate cancer risk, with higher levels offering apparent protection from the disease, in an ethno-dependent fashion. Thus, we believe that black men may be at greater risk for the clinical consequences of mitochondrial altered function and peptide secretion, leading to various diseases including prostate cancer.

Short-Term Impact - Biomarker Discovery: We are testing the novel hypothesis that the levels of the four recently described mitochondrial peptides (humanin, MOTS-c, SHLP-2 and SHLP-6) could serve as biomarkers for PC risk. If successful, these findings will establish the mitochondria as a key player in understanding prostate cancer pathogenesis and will initiate further studies to test the possible clinical utility of using mitochondrial derived peptide (MDP) levels in the screening and prognostication of patients with high risk for developing prostate cancer, particularly African Americans. Most men diagnosed with PC will not die from their cancer, and there is need for new methods to differentiate those men who need treatment from those with a non-lethal phenotype. Future clinical trials will confirm the extent to which

MDP levels alters tumor characteristics or provides an independent biomarker for PC prognosis in black and white men.

Mid-Term Impact - Target Populations for Intervention: With evidence that MDPs affect PC risk or prognosis in high-risk populations through an inflammatory pathway, we lay the groundwork for interventions to increase the levels of these peptide hormones in black and white men who are at risk for PC progression or recurrence. As our recent data indicates that dietary interventions modify mitochondrial peptide levels, we see a possible direction in providing lifestyle advice based on these diagnostic markers that could improve with appropriate recommendations.

Long-term Impact - Feasible Change in Clinical Care: Mitochondrial-based therapeutics of various kinds are being tested for a number of diseases (including some MDPs). It is entirely possible that such therapies will directly or indirectly raise MDP levels and thus treat the primary disease or delay the development of recurrence or metastasis (which we have already shown in xenograft studies in mice treated with MDP)s. Our study will provide important information toward establishing effective blood MDP levels to target in order to improve PC outcomes.

Impact on other disciplines: This work will inform other health disparity related diseases that may be affected by mitochondrial peptides including Alzheimer's Disease.

Impact on Technology Transfer: none

Impact on Society: As noted above, we believe this work is foundational in developing better strategies to screen for prostate cancer risk in minority men and potentially could lead to novel therapies.

Products
none

Patents:
none

List of publications since DOD grant funding

1. Xiao J, Howard L, Wan J, Wiggins E, Vidal A, Cohen P, Freedland SJ. Low circulating levels of the mitochondrial-peptide hormone SHLP2: novel biomarker for prostate cancer risk. *Oncotarget*. 2017; 8:94900-94909.
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3. Kim SJ, Wan J, Cohen P, Yen K. Mitochondrial derived peptides as novel regulators of metabolism. *J Physiol*. 2017 595:6613-6621.
4. Xiao J, Cohen P, Stern MC, Odedina F, Carpten J, Reams R. Mitochondrial biology and prostate cancer ethnic disparity. *Carcinogenesis*. 2018; doi.org/10.1093/carcin/bgy133.
5. Yen K, Wan J, Mehta HH, Miller B, Christensen A, Levine ME, Salomon MP, Brandhorst S, Xiao J, Kim SJ, Navarrete G, Campo D, Harry GJ, Longo V, Pike CJ, Mack WJ, Hodis HN, Crimmins EM, Cohen P. Humanin Prevents Age-Related Cognitive Decline in Mice and is Associated with Improved Cognitive Age in Humans. *Nature Scientific Reports*. 2018; 8:14212. doi: 10.1038/s41598-018-32616-7.
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8. Miller B, Arpawong TE, Jiao H, Kim SJ, Yen K, Mehta HM, Wan J, Carpten JC, Cohen P. Comparing Nuclear and Mitochondrial DNA from a Multi-Ethnic Cohort to Adjust for Genetic Ancestry in Association Studies. *Cells*. 2019. 8(4), 306; doi: 10.3390/cells8040306
9. Howard LE, Zhang J, Fishbane N, Hoedt AM, Klaassen Z, Spratt DE, Vidal AC, Lin D, Hitchins MP, You S, Freeman MR, Yamoah K, Davicioni E, Freedland SJ. Validation of a genomic classifier for prediction of metastasis and prostate cancer-specific mortality in African-American men following radical prostatectomy in an equal access healthcare setting. Prostate Cancer Prostatic Dis. 2020 Sep;23(3):419-428. doi: 10.1038/s41391-019-0197-3. Epub 2019 Dec 16. PMID: 31844180
10. D'Souza RF, Woodhead JST, Hedges CP, Zeng N, Wan J, Kumagai H, Lee C, Cohen P, Cameron-Smith D, Mitchell CJ, Merry TL. Increased expression of the mitochondrial derived peptide, MOTS-c, in skeletal muscle of healthy aging men is associated with myofiber composition. Aging (Albany NY). 2020 Mar 17;12. doi: 10.18632/aging.102944. Online ahead of print. PMID: 32182209.
11. Woodhead JST, D'Souza RF, Hedges CP, Wan J, Berridge MV, Cameron-Smith D, Cohen P, Hickey AJR, Mitchell CJ, Merry TL. High-intensity interval exercise increases humanin, a mitochondrial encoded peptide, in the plasma and muscle of men. J Appl Physiol. 2020 Apr 9. doi: 10.1152/japplphysiol.00032.2020. Online ahead of print. PMID: 32271093.
12. Yen K, Mehta HH, Kim SJ, Lue Y, Hoang J, Guerrero N, Port J, Bi Q, Navarrete G, Brandhorst S,

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13. Miller B, Silverstein A, Flores M, Cao K, Kumagai H, Mehta HH, Yen K, Kim SJ, Cohen P. Host mitochondrial transcriptome response to SARS-CoV-2 in multiple cell models and clinical samples. Sci Rep. 2021 Jan 8;11(1):3. doi: 10.1038/s41598-020-79552-z. PMID: 33420163.
14. Ramirez-Torres A, Reagan AL, Howard LE, Wiggins E, Vidal AC, Wang J, Freedland SJ, Cohen P. Racial differences in circulating mitochondria-derived peptides may contribute to prostate cancer health disparities. Submitted. Lancet Oncology. 2021. Also presented as Abstract # MP09-13 and Selected as the BEST POSTER during Moderated Poster Session MP09: Prostate Cancer: Markers I. AUA2020 Virtual Science. May 15, 2020
15. Miller B, Kim SJ, Kumagai H, Mehta HH, Xiang W, Liu J, Yen K, Cohen P. Peptides derived from small mitochondrial open reading frames: Genomic, biological, and therapeutic implications. Exp Cell Res. 2020 May 5:112056. doi: 10.1016/j.yexcr.2020.112056. [Epub ahead of print] PMID: 32387288.
16. Silverstein AR, Flores MK, Miller B, Kim SJ, Yen K, Mehta HH, Cohen P. Mito-Omics and immune function: Applying novel mitochondrial omic techniques to the context of the aging immune system. Transl Med Aging. 2020;4:132-140. doi: 10.1016/j.tma.2020.08.001. Epub 2020 Aug 21. PMID: 32844137
17. Cohen P. COVID-19 and the Future of Aging: Prospects for Geroscience. Next-Avenue. December 2020. https://www.nextavenue.org/covid-19-and-the-future-of-aging-prospects-for-geroscience/

Participants and Other Collaborating Organizations
Site-1: University of Southern California
Individuals have worked on the project:

Name:	Pinchas Cohen
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0035-8366
Nearest person month worked:	1.2
Contribution to Project:	He was responsible for coordinating all aspects of the project including experimental design and data analysis. Dr. Cohen supervised all personnel at USC and insure communications with Dr. Freedland.
Funding Support:	1PO1AG34906,USCSchoolFund,RF1AG061834,U54CA233465,R01EY027363,R56AG062693

Name:	Junxiang Wan
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-2638-5258
Nearest person month worked:	5
Contribution to Project:	She performed Humanin, SHLP2 SHLP6, MOTS-c ELISA assays which she has developed on 200 subjects.
Funding Support:	1P01AG034906,USCschoolaccount,RF1AG061834,U54CA233465,R01EY027363

Name:	Hemal Mehta
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	0000-0002-8439-278X
Nearest person month worked:	4
Contribution to Project:	She helped Dr. Wan in MDP extraction procedure from the plasma.
Funding Support:	1P01AG034906;BIGAFARawardUSCschool accountU54CA233465,R56AG062693

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

R01AG069698-01 Metformin-Regulated Mitochondrial Peptides and their Effects on Aging
R01AG068405 Ethnic-Specific Mitochondrial DNA Variations that Contribute to Dementia
P30AG068345 The University of Southern California and Buck Institute Nathan Shock Center

Site 2: Cedars Sinai Medical Center, Los Angeles, CA
& Institute for Medical Research/Durham Veteran's Administration, Durham, NC

Name:	Stephen Freedland
Project Role:	Collaborating-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	Dr. Freedland oversaw patient recruitment and plasma collection at Cedars and UNC
Funding Support:	Cedars accounts and various NIH and DOD grants

Name:	Adriana Vidal
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.35
Contribution to Project:	Dr. Vidal along collected and archived plasma samples and shipped those to USC (Cohen Lab) for MDP analysis.
Funding Support:	Cedars accounts

Partner's Contribution to the Project: Dr. Freedland was in charge of patient recruitment and sample collection. He sent Dr. Cohen 200 plasma samples from controls and cases to analyze the mitochondrial derived peptides (MDP) levels by ELISA.

- **Financial Support –** Nothing to report
- **In-Kind Support –** Nothing to report
- **Facilities –** Nothing to report
- **Collaboration –** Nothing to report
- **Personnel exchanges** – Nothing to report
- **Others –** Nothing to report

Special Reporting Requirements

We are not aware of any special reporting requirements for this project.

Appendices

None