



# Peptides derived from small mitochondrial open reading frames: Genomic, biological, and therapeutic implications

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## ABSTRACT

Mitochondrial-derived peptides (MDPs) are a novel class of bioactive microproteins that modify cell metabolism. The eight MDPs that been characterized (e.g., humanin, MOTs-c, SHLPs1-6) attenuate disease pathology including Alzheimer's disease, prostate cancer, macular degeneration, cardiovascular disease, and diabetes. The association between disease and human genetic variation in MDPs is underexplored, although two polymorphisms in humanin and MOTs-c associate with cognitive decline and diabetes, respectively, suggesting a precise role for MDPs in disease-modification. There could be hundreds of additional MDPs that have yet to be discovered. Altogether, MDPs could explain unanswered biological and metabolic questions and are part of a growing field of novel microproteins encoded by small open reading frames. In this review, the current state of MDPs are summarized with an emphasis on biological and therapeutic implications.

## 1. Discovery of mitochondrial-derived peptides

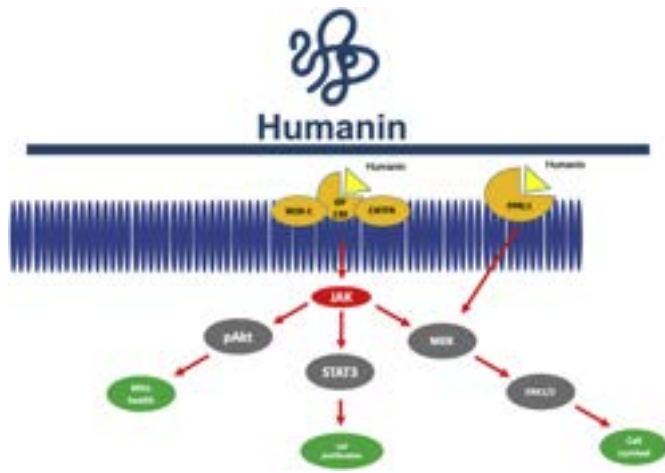
In order to reduce the high number of non-coding open reading frames (ORFs), ORFs greater than approximately 100 codons were exclusively used to annotate the human genome, as the likelihood that an ORF encodes a bona fide protein increases with its codon length [1]. However, high-throughput DNA sequencing, enrichment-based mass spectrometry, and immunological peptide detection have revealed over a thousand bioactive eukaryotic microproteins encoded by nuclear small open reading frames (sORFs) and several microproteins encoded by mitochondrial sORFs [2]. Moreover, there are millions of uncharacterized putative nuclear-encoded and hundreds of mitochondrial sORFs, but the detection and biology of these microproteins have yet to be corroborated [3]. Novel microproteins could answer crucial biological questions (e.g., mitochondrial-nuclear communication, metabolic dysfunction, etc.) that have mystified the field for decades. Microproteins serve as therapeutic candidates both in the form of the microprotein itself or sORF gene target. In particular, mitochondrial microproteins – originally termed mitochondrial-derived peptides (MDPs) – have could explain how the mitochondrion communicates intracellularly and intercellularly in disease-specific contexts.

### 1.1. Humanin

The first MDP discovered was from a sORF encoded within the mitochondrial *16S rRNA* and named humanin [4]. The detection of the 24-amino acid MDP humanin represented a paradigm shift in mitochondria biology and genetics because it was the first mitochondrial sORF-encoded microprotein found to have biological activity. Humanin was discovered by three independent laboratories in the early 2000s. First, Hashimoto et al. found humanin during a screen for genes that protected against amyloid beta (A $\beta$ ) toxicity [4,5]. These experiments revealed a cDNA fragment that mapped back to the mitochondrial *16S rRNA*. Ultimately, this mitochondrial-derived cDNA was named humanin because it was found to encode for a microprotein that displayed profound protection against AD-related neurotoxicity, an effect that the original authors though potentially could restore the “humanity” of patients suffering from dementia. Second, Ikonen et al. found that humanin bound IGFBP3 using an unbiased yeast two hybridization system and enhanced the protective effects of IGFBP3 against A $\beta$  toxicity [6]. Third, Guo et al. revealed humanin bounded the apoptotic protein BAX and mitigated cell apoptosis [7]. Since the discovery of humanin, hundreds of additional peer-reviewed reports have described humanin in the context of general biology, AD pathology attenuation, and chronic disease modification [8–21]. Humanin's precise mechanism of

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**Fig. 1. Humanin signaling cascade.** Humanin binds the trimeric WSX-1, GP130, and CNTFR receptor and the FRPL1 receptor to initiate intracellular cascades leading to mitochondrial maintenance and cell survival.



**Fig. 2. Biological effects of MOTS-c.** MOTS-c has been shown to act on muscle, white fat, brown fat, and circulate in the periphery.

action includes signaling through a trimeric receptor consisting of WSX-1, GP130 and CNTFR as well as a separate FRPL1 receptor [22,23] (Fig. 1). Downstream of humanin receptor activation are STAT3 and ERK1/2, which modified mitochondrial biology, cell proliferation, and cell survival [24]. In essence, humanin helped initiate the novel field of mitochondrial sORF-encoded microproteins (see Fig. 2).

### 1.2. MOTS-c and SHLPs

In addition to humanin, seven more MDPs have been characterized: MOTS-c and six humanin-like peptides (SHLPs 1–6). MOTS-c was the second MDP discovered and is a 51 bp sORF within the 12S rRNA that is highly conserved and translated in the cytosol [25]. The effects of the 16 amino-acid MOTS-c are vast but mediated through AMPK activity. MOTS-c has been reported to act profoundly on mouse skeletal muscle and has also been detected in skeletal muscle [26]. Administering MOTS-c to mice on a high-fat diet improved insulin resistance, attenuated high-fat diet induced weight gain, and minimized fat accumulation in the liver [27]. Lu et al. demonstrated that MOTS-c enhanced lipid catabolism in white adipose tissue, activated brown adipose tissue, and prevented ovariectomy-induced obesity and insulin resistance

through AMPK [28]. An unbiased metabolomics approach demonstrated that MOTS-c administration to high-fat diet fed mice regulated sphingolipid metabolism, monoacylglycerol metabolism, and dicarboxylate metabolism [29]. MOTS-c has been described as an “exercise mimetic” that systematically regulates glucose and insulin action [25].

SHLPs are encoded by sORFs within the 16S rRNA and have many similar effects as humanin [9]. Five of the six SHLPs (SHLPs1-4 and 6) that were initially reported by Cobb et al. were detected at varying levels across tissues, and detection of these MDPs was validated by comparing levels in cells void of mitochondrial DNA (i.e.,  $\rho$ 0 cells) that produced no detectable SHLPs. The tissue specificity of certain SHLPs suggest precise mechanisms of action. Of the six SHLPs Cobb et al. characterized, SHLP2, SHLP3, and SHLP6 had the most profound effects on cellular biology. Specifically, SHLP2 and SHLP3 increased mitochondrial oxygen consumption rate and ATP while reducing reactive oxygen species. In the same report, SHLP2 protected cells against nervous system insults such as A $\beta$  toxicity and macular degeneration. The original authors found SHLP2 in mouse and also in prostate at lower levels, whereas SHLP4 was detected in prostate at high levels. Although SHLP2 levels are lower in prostate tissue, follow-up studies on SHLP2 levels have predicted prostate cancer, an observation that hints at potential communication among MDPs such as SHLPs [30]. SHLPs have been characterized in a cytoprotective context, but SHLP6 has been reported to primarily induce apoptosis and is found in much lower quantity across all tissues [9]. The field is just beginning to understand the tissue specificity of MDPs.

### 1.3. Human genetic variation in MDPs

The degree of human genetic variation in MDPs is unclear. Most large-scale genome-wide association study (GWAS) analytic plans do not include the mitochondrial genome due to its haploid nature. If mitochondrial DNA variants are included in the GWAS analytic plan, potentially meaningful variants fail to be detected due to the conservative false-discovery Bonferroni correction typically used for nuclear DNA variants. Nevertheless, there have been reports on the associative effects of mitochondrial genetic variation on disease, although few interpret findings with careful consideration to the mitochondrial sORFome [31–36].

Two studies have examined human MDP genetic variation in large-scale population cohorts. First, Yen et al. found that the mitochondrial SNP rs2854128 associated with lower circulating humanin levels and cognitive age in the Health and Retirement Study ( $n = \sim 16,000$ ) [37]. Specifically, humanin levels were 15% lower in reference allele carriers and especially low in those of African-American ancestry. rs2854128 represented the first known SNP to associate with a circulating, detectable MDP. Second, the mitochondrial SNP rs111033358 was found to increase risk of type 2 diabetes (T2D) in males of Asian ancestry in a comprehensive meta-analysis of over 27,000 individuals enrolled in the J-MICC, MEC, and TMM cohorts [38]. rs111033358 changes the 14th amino acid of MOTS-c from lysine to glutamine (K14Q), and administration of the mutant MOTS-c K14Q peptide failed to attenuate weight gain and improve insulin sensitivity compared to MOTS-c wild type *in vivo* [38]. Since human mitochondrial genetic variation varies substantially by ethnicity, it is plausible that ethnic-specific mitochondrial DNA mutations affect MDPs and, as a result, modify disease risk. For instance, mitochondrial genetic variation, captured by data reduction techniques, predicted height within and between self-reported ethnicities in the Health and Retirement study [39].

## 2. Classification of the putative mitochondrial sORFome

The 16,569 bp mitochondrial genome encodes for 13 large proteins involved in oxidative phosphorylation: ATP6, ATP8, CO1, CO2, CO3, CYB, ND1, ND2, ND3, ND4L, ND4, ND5, and ND6 [40]. Re-annotating

Genetic Code	Strand	Median Size (Amino-Acid)	Distribution (Amino-Acid)
Standard	Heavy	19	
Standard	Light	20	
Mitochondrial	Heavy	20	
Mitochondrial	Light	17	

**Fig. 3. Classes of MDPs.** Putative MDPs can be classified into four categories based on genetic code and strand specificity. The distribution graph on the far right illustrates putative MDP length (i.e., number of amino acids).

the mitochondrial genome to include sORFs between 9 and 40 amino acids revealed hundreds putative MDP sORFs that can be separated into four classification categories based on the strand of origin (heavy or light) and genetic code (i.e., standard or mitochondrial-specific genetic code). There are several differences between the mitochondrial-specific genetic code and standard genetic code. The standard genetic code uses AGA and AGG codons for arginine, whereas the mitochondrial genetic code uses these codons for termination [41]. The UGA codon is used for termination in the standard genetic code, but the mitochondrial genetic code uses UGA for tryptophan [42]. Mitochondrial-specific genetic code also uses two alternative initiation codons (ATA and ATT) in addition to the standard initiation codon of ATG [43].

The largest class of MDP sORFs is derived from the mitochondrial-specific genetic code light-strand class (Fig. 3). The median amino-acid length of the mitochondrial-specific codon light-strand class is 17, and the distribution of these putative MDPs are skewed towards lengths around 10 amino acids. No mitochondrial-specific codon light-strand MDPs have been discovered yet. The mitochondrial-specific heavy-strand class encodes for putative MDPs with a larger median amino-acid length of 20. No MDPs from this class have been identified, either. MDP amino acid length distribution of standard genetic code class is not as positively skewed as the mitochondrial-specific class, and the median amino-acid lengths of the standard genetic code ORFome are 19 and 20 on the heavy and light strand, respectively. As the microprotein field continues to grow, it is possible that MDPs from these classes will be validated.

The eight MDPs that have been discovered are encoded by the standard genetic code class. The MDPs humanin, MOTS-c, and SHLP6 are encoded by the standard genetic code heavy-strand class, and the MDPs SHLP2-5 are encoded by the standard genetic code light-strand class. MOTS-c can only be translated in the cytosol because its sORF has tandem start and stop codons that are specific to the mitochondrial genetic code. The MOTS-c transcript, therefore, must be exported out of the mitochondrion into the cytosol. It has indeed been reported that mitochondrial RNA is exported out of the mitochondrion to the cytosol via unclear mechanisms [44–46]. Of the eight MDPs that have been characterized, humanin, MOTS-c, SHLP2, and SHLP6 have notable associations with several human diseases and lifespan.

### 3. Associative and experimental effects of MDP levels and diseases

Immunological detection methods for humanin, MOTS-c, SHLP2, and SHLP6 can be used on a variety of biological samples (e.g., blood, cell extracts, and tissue extracts). These techniques (e.g., ELISA, Western blot, etc.) detect low levels of both endogenous and synthetic MDPs and can be easily applied to measure MDP levels in diseased patients, various animal models, cellular experiments, and pharmacokinetic studies [26]. Several reports have quantified MDP levels in biological tissues in cross-sectional human association studies, *in vitro* experiments, and *in vivo* paradigms.

#### 3.1. Humanin levels and cardiovascular function, cognition, and lifespan

Humanin has been measured in several biological contexts. Widmer et al. reported that humanin levels were lower in human patients with poor endothelial function versus individuals with healthy endothelial function [47]. Yen et al. observed that cognitive age and ethnicity associated with circulating levels of humanin [37]. Lee et al. observed that humanin positively correlated with lifespan and modified by the GH/IGF-1 axis in mice. In Lee et al. studies, transgenic GH mice displayed nearly 75% less circulating humanin compared to wild type mice. They also showed that humanin levels were over 30% higher in the Ames mouse model (i.e., a mouse model in which GH is deficient). Deleting *IGF1* in mice also increased humanin levels over 15% and, conversely, knocking down *IGFBP3* reduced humanin by 70% [48].

The precise pharmacokinetics and tissue distribution of humanin and analogues has been characterized using a humanin ELISA by Chen et al. [49]. The half-life of humanin is relatively fast, as about 80% of baseline humanin injected intraperitoneal (IP) is cleared from the circulatory system in 30 min. Notably, in the same report, Chen et al. reported that *IGFBP3* knockout mice displayed a remarkably higher half-life of IP-injected humanin. Rather than the 80% of IP humanin clearance rate observed in wild type mice, *IGFBP3* knockout only cleared 25% of IP-injected humanin after 30 min. In these wild type mice injected IP with humanin, circulating levels of *IGFBP3* dropped by 20% after 2 h, suggesting a tightly regulated relationship between *IGFBP3*. Moreover, humanin levels were substantially higher (measured by ELISA) in a novel mouse model that overexpresses humanin via human sORF transgene [50].

#### 3.2. MOTS-c levels and cardiovascular function and senescence

MOTS-c has been measured in tissues from mice, rats, and humans. Lee et al. reported that circulating MOTS-c levels were similar across species. In humans with coronary endothelial dysfunction, circulating MOTS-c was nearly 20% lower in diseased patients, which was similar to the decrease in circulating humanin in the same diseased patients [25]. In these same patients, improving coronary blood flow and epicardial function led to increased levels of MOTS-c. Levels of MOTS-c can also be detected in cellular extracts by using ELISA [51]. In fact, when cells undergo replicative senescence, MOTS-c levels dropped by ~20% [52]. It is expected that many biological conditions will present differences in MOTS-c levels that will be studied in the future.

#### 3.3. SHLP2 and SHLP6 levels in prostate cancer and senescence

SHLP2 levels have been quantified in young and old male and female wild type mice. For example, all older mice regardless of gender displayed lower circulating SHLP2 levels, and female mice additionally displayed slightly lower levels of SHLP2 [9]. In addition, circulating SHLP2 levels have been used as a biomarker for prostate cancer [30]. That is, SHLP2 levels were nearly halved in prostate cancer patients, and SHLP2 levels were lower in black individuals compared to white individuals without prostate cancer, suggesting an ethnic-specific regulation that needs more follow-up. In the same report, adding SHLP2 levels to a standard predictive model for prostate cancer improved accuracy by nearly 30%. While the utility of the SHLP2 ELISA has been demonstrated, no observable differences in SHLP6 levels have been reported, although the SHLP6 ELISA has been used in senescence contexts [52]. Altogether, humanin, MOTS-c, SHLP2, and SHLP6 levels have potential to explain ethnic health disparities and disease pathology.

## 4. Conclusions and future research

Technological advancements in DNA sequencing, peptide detection, and computational power will undoubtedly reveal functions of current

and soon-to-be discovered microproteins. In regards to MDPs, several unanswered questions will be answered as technology continues to evolve.

#### 4.1. MDP translation

How MDPs are translated remain a crucial question. It is traditionally thought that the small size of the mitochondria encodes for 13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs, but transcriptomics analyses suggests the mitochondria genome is more complex. Hallmark work from Mercer et al. revealed that the mitochondrial transcriptome included potentially 80 canonical and non-canonical cleavage sites [40]. That the mitochondria transcriptome possibly contains 80 cleavage sites is curious, and even more intriguing is the observation that the mitochondria also contains independently regulated small transcripts around the size of 15–30 nucleotides with unclear functions. Perhaps these small mitochondrial RNA fragments behave to modify translation of mitochondrial transcripts, but more work is needed to determine their function. Mercer et al. also reported that mitochondrial DNA contains numerous binding motifs, and RNaseA and PARE data suggest protein footprints are associated with sites of transcript termination and cleavage downstream of the light strand promoter. In addition, whether MDPs are translated in the cytosol or mitochondrion – or in both cytoplasmic and mitochondrial ribosomes – remains unclear. The ability to sequence nucleotides embedded within ribosomes might reveal the main site of translation. Mitochondrial RNA processing is complex and future work will elucidate the precise mechanisms of MDP translation.

#### 4.2. Complete MDP human genetic variation

The degree of human mitochondrial genetic variation within mitochondrial sORFs and its associated effects on disease are still dramatically underexplored due to several reasons. One limitation of mitochondrial genome wide association studies (MiWAS) is the varying number of mitochondrial single nucleotide polymorphisms (SNPs) across sequencing chips. Large population genotyping data that used the Illumina HumanHap550 Genotyping BeadChip and Affymetrix Genome-Wide SNP Array 6.0 do not even contain mitochondrial SNPs. It is estimated that only 64 SNPs are necessary to tag mitochondrial SNPs with a frequency greater than 1% in European populations with an r-squared value of 0.8 [53]. In the Health and Retirement Study, Miller et al. showed that genetic principal components derived from nuclear or mitochondrial genotyping arrays explained a similar amount of the variance in Whites, Blacks, and Hispanics [39]. While a limited number of SNPs are necessary to tag certain populations confidently, the ideal analytic plan includes whole mitochondrial DNA sequencing, which will reveal rare mitochondrial DNA variants and the degree of heteroplasmy in certain mitochondrial genetic regions that contain sORFs. Large human population cohorts are beginning to conduct whole genome sequencing. These data sets will reflect comprehensive mitochondrial genetic architecture that haplogroup or mtSNP tagging analyses attempt to recapitulate. Whole mitochondrial DNA data could unravel the effects of certain mitochondrial genetic variants, many of which might change the function of MDPs.

#### 4.3. Nuclear-encoded sORF microproteins that act on mitochondria

It is expected that ongoing research will reveal more microproteins not only derived from mitochondrial DNA but also nuclear DNA. For instance, Chu et al. characterized a novel microprotein derived from nuclear DNA, called PIGBOS, that localized to the mitochondrion and regulated uncoupling protein responses [54]. The same laboratory found a separate microprotein called MIEF1 that regulated mitochondrial translation [55]. Moreover, three independent, separate laboratories discovered the same microprotein encoded by a lncRNA that has

been called mitoregulin, MPM, and MOXI. This novel microprotein supported super complexes and modified mitochondrial respiratory efficiency [56]. Separate reports noted that MOXI (mitoregulin or MPM) modified muscle and fat metabolism by acting on the mitochondria [57,58]. Since these newly discovered microproteins act on the mitochondria, future studies might focus on the interaction between nuclear-encoded microproteins and MDPs (e.g., effect of MIEF1 on MDP expression). Vice versa, how MDPs regulate nuclear-encoded microproteins, such as the mitochondrial MIEF1, could explain unclear biological processes. MOTS-c, for example, has been shown to regulate expression of NRF2 target genes by translocating to the nucleus [59]. Although NRF2 is not a microprotein, that MOTS-c regulates target nuclear genes suggests that MOTS-c and perhaps additional MDPs have the capacity to regulate nuclear-encoded genes. Whether MDPs modify expression of nuclear-encoded peptides is unclear, but future analyses might consider the effect of MDPs on nuclear-encoded peptides via RNASeq, ribosome profiling, and peptidomics.

#### 4.4. Future directions

While technological improvements at the nucleotide and protein level have revealed several bioactive microproteins, there is still room for improvement. In particular, enhancing mass-spec enrichment analysis could yield several more MDPs and complement MDP ELISAs. Additionally, the advent of long-read sequencing could provide greater depth into MDP transcripts. Finally, the degree to which MDP genetic variation associates with human disease could become clear with whole mitochondrial genome sequencing data derived from large-scale human populations. Over the last two decades, eight MDPs have been characterized and their biology will continue to be explored, but it is expected that additional MDPs will have biological and therapeutic relevance.

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#### CRediT authorship contribution statement

**Brendan Miller:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Su-Jeong Kim:** Conceptualization, Formal analysis, Writing - review & editing. **Hiroshi Kumagai:** Writing - original draft. **Hemal H. Mehta:** Project administration, Writing - review & editing. **Wang Xiang:** Writing - original draft. **Jiali Liu:** Writing - original draft. **Kelvin Yen:** Conceptualization, Formal analysis, Writing - review & editing. **Pinchas Cohen:** Supervision, Conceptualization, Resources, Writing - review & editing.

#### Declaration of competing interest

Pinchas Cohen is a consultant and stockholder of CohBar Inc.

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