Circulating Cell-Free Mitochondrial DNA: A Potential Blood-Based Biomarker for Sarcopenia in Patients Undergoing Maintenance Hemodialysis

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Background: Mitochondrial impairment and exaggerated inflammation are hallmarks of sarcopenia. Recently, cell-free mitochondrial DNA (cf-mtDNA) has been in the spotlight as an endogenous danger molecule that can potentially elicit inflammation. Yet, its actual impact on sarcopenia, especially in patients with maintenance hemodialysis (MHD), is still at an early stage of investigation.

Material/Methods: A total of 105 MHD patients were enrolled in this study. The subjects were classified into sarcopenia group (SP) and non-sarcopenia group (NSP) according to the DXA scan and grip strength. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. Circulating cf-mtDNA (ccf-mtDNA) was detected using Taq Man RT-qPCR. Cytosolic mtDNA and inflammation- and mitophagy-related genes in PBMCs were quantitated using SYBR Green RT-qPCR. ΔΨm was analyzed using the fluorescent probe JC-1.

Results: ccf-mtDNA content was significantly higher in SP group than in NSP group. Multivariate regression analysis showed a significant correlation of ccf-mtDNA with sarcopenia after adjusting for potential confounders. A similar trend of increased mtDNA was also observed in the mitochondria-free cytoplasm of PBMCs from SP patients, together with higher expression of TLR9 and IL-6 in this group. Next, using PBMCs as surrogates for mitochondria-rich cells, we found that ΔΨm was dramatically decreased in the SP group. In parallel, the mRNA levels of mitophagy-related genes Parkin and LAMP2 were increased in the SP group.

Conclusions: The results obtained demonstrated that cf-mtDNA, as a potential driver of inflammatory component, may be involved in the pathogenesis of the MHD-related sarcopenia.

Keywords: Inflammation • Mitochondria, Muscle • Renal Dialysis • Sarcopenia

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Background

Sarcopenia is a term derived from the Greek “sarx” – meat and “penia” – loss. This concept was first coined in 1988 by Rosenberg and denotes a syndrome characterized by progressive and generalized loss of muscle mass and strength [1]. Sarcopenia is common in chronic kidney disease (CKD) and end-stage renal disease (ESRD) patients, especially after long-term maintenance hemodialysis (MHD) [2,3]. MHD patients with concomitant sarcopenia not only worsen their quality of life, but also increase the risk of adverse health outcomes, including cardiovascular events and overall mortality [4]. Therefore, there is an urgent need to understand the underlying mechanisms of sarcopenia in MHD patients to design novel therapeutics.

Regardless of etiology, the presence of low-grade inflammation has been recognized as a characteristic pathological change in MHD and sarcopenia [5]. Mitochondria are endosymbiotic organelles that carry multiple copies of their own genomes (mitochondrial DNA, mtDNA), encoding proteins required for oxidative phosphorylation and respiratory metabolism [6]. Mitochondria are derived from early endosymbiotic bacteria capable of oxidative phosphorylation, and most of mtDNA contains inflammagenic unmethylated CpG motifs similar to those in bacterial DNA [7]. In this regard, if mtDNA is released out of the cell and becomes extracellular mtDNA, it can act as a damage-associated molecular pattern (DAMP) molecule and trigger an innate immune response [8].

Accumulated evidence suggests that mtDNA can be easily detected in the cell-free fraction of blood, and its levels are elevated under pathological conditions, such as trauma, sepsis, aging, caner, and immune-mediated disease, which are characterized by a chronic inflammatory status [9-13]. Chen et al. investigated the risk factors for ESRD and found that patients with ESRD exhibited significantly higher ccf-mtDNA contents than the healthy controls [14]. Another study reported that mtDNA release was significantly enhanced in MHD patients, and its level was positively correlated with inflammatory cytokines in vitro [15]. It has previously been demonstrated that high levels of plasma inflammatory substances are associated with sarcopenia, with chronic inflammation playing a role in this disorder [16]. However, the possible contribution of mitochondrial DAMPs to the inflammatory milieu that characterizes muscle wasting disorders has not yet well-explored. This question is worth being pursued as it could help identify novel biological targets for the management of muscle loss, especially in MHD patients, because of the high prevalence of MHD patients with this disease and the lack of effective medications.

Mitochondrial impairment occurs in multiple tissues under uremic conditions, such as muscle[17], heart [18], liver[19], lung[20], endothelial cells[21], and monocytes [22]. When mitochondria are damaged, mtDNA can be released into the extracellular spaces and then recognized by immune cells [23]. Peripheral blood mononuclear cells (PBMCs) are abundantly rich in mitochondria. Recently, tests of PBMCs have been proposed to offer valid information about “general” mitochondrial health [24-26]. Hence, we chose PBMCs as indicators of mitochondrial dysfunction in lieu of tissue biopsy collection to elucidate the release mechanism of ccf-mtDNA.

In the current study, we first sought to determine the association between circulating mtDNA and the risk of sarcopenia in MHD patients. Furthermore, we attempted to decipher the potential molecular mechanisms underlying its release. These findings provide novel mechanistic insights into the linkage between ccf-mtDNA and MHD-related sarcopenia and enable the identification of new therapeutic targets of this disease.

Material and Methods

Participants

A cross-sectional study design was conducted in this research. All subjects were recruited from Hemodialysis Center of First Affiliated Hospital of Chengdu Medical College between June 2020 and October 2020. Inclusion criteria for the MHD patients were as follows: (1) age above 40; and (2) patients undergoing regular hemodialysis prescription, 3 times a week, for 4 h per session at least 6 months. The age- and sex-matched healthy controls (HC) were recruited from the same region who took routine health examinations and did not have any type of systemic diseases. The exclusion criteria for all subjects were as follows: (1) active inflammatory diseases within the last 3 months; (2) malignant tumors; (3) immune system diseases; (4) active liver disease; (5) acute cardiovascular and cerebrovascular disease; and (6) inability to complete the measurement of body composition and grip strength due to physical disability. A total of 105 MHD patients were enrolled in our examination program. Referring to the diagnostic criteria established by the Asian Working Group for Sarcopenia (AWGS) [27], participants were divided into 2 groups: MHD patients without sarcopenia (NSP, n=82) and MHD patients with sarcopenia (SP, n=23). The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Chengdu Medical College and adhered to the principles outlined in the Declaration of Helsinki. All participants gave informed written consent.

Sample Size Calculation

Since no previous studies have investigated the role of ccf-mtDNA in kidney disease-related sarcopenia, there was a lack of data to predict the difference in ccf-mtDNA between sarcopenia and no-sarcopenia groups and hence no power
calculation was performed prior to the study. The sample size of the validation study was calculated using Power Analysis and Sample Size software (PASS, v 11.0.10). Group sample sizes of 23 (Sarcopenia) and 82 (No-sarcopenia) achieved 95.69% power to reject the null hypothesis of equal means when the ccf-mtDNA mean difference is μ1 (No-sarcopenia) – μ2 (Sarcopenia)=3.72-6.19=−2.5 with standard deviations of 2.5 for No-sarcopenia and 3.7 for Sarcopenia, and with a significance level (alpha) of 0.050 using a two-sided two-sample unequal-variance t-test. Power analyses indicated that the sample size in this study was appropriate.

Assessment of Sarcopenia

Muscle strength was assessed by grip strength, which was measured by a dynamometer (KYTO Digital Hand Dynamometer, Model EH101, China), and low grip strength was defined as <26 kg in men and <18 kg in women. Physical performance was assessed by usual gait speed (m/s) on a 6-m course, and a slow walking speed was defined as slower than 1.0 m/s. Muscle mass was measured through dual-energy X-ray absorptiometry (DXA) (enCORE, General Electric, USA). Skeletal muscle mass index (SMI) was defined by dividing appendicular skeletal muscle mass by height in meters squared (kg/m²). Low muscle mass was defined as SMI <7.0 kg/m² in men and <5.4 kg/m² in women. The diagnosis of sarcopenia was based on simultaneous satisfaction of both low muscle strength and mass according to the 2019 AWGS criteria.

Sample Collection

Venous blood samples were drawn from each patient immediately prior to initiation of dialysis. Whole blood was collected into vacutainers and either aliquoted for storage at -80°C or processed into separate cellular and cell-free fractions with 2 hours. Whole blood was collected from purified PBMCs using RNeasy (Baifeite, Chengdu, China) according to the manufacturer’s recommendation. RNA was extracted from purified PBMCs using RNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA). RT-qPCR was performed to detect mitochondrial DNA Cytb in the cytoplasmic fraction using the following primers:

- Cytb: sense primer, 5'-CGCTACCTTACAGCGCAATG-3',
- antisense primer, 5'-CGATGTGATAGGGAGGCAATA-3',
- FAM-labeled TAMRA quenched probes, 5'-CGCTTCAATTC-3'.

The linearity of the quantitative assay was assessed using the template cloned into plasmid DNA and serially diluted to prepare a series of calibrators with known concentrations. Then, the absolute values of the mtDNA were determined by calculating from this standard curve, as previously described [28]. Results were presented as mtDNA×10⁵ copies per µl.

Measurement of Mitochondria-Free Cytosolic mtDNA in PBMCs Using SYBR Green RT-qPCR

Mitochondria-free cytosol was prepared first using mitochondrial isolation kits (Thermo Fisher Scientific, Waltham, MA, USA). Cytosolic DNA was collected using a DNasey Blood & Tissue Kit (Qiagen, Germantown, MD, USA). RT-qPCR was performed to detect mitochondrial DNA Cytb in the cytoplasmic fraction using the following primers:

- F-5'-ATGACCGCAATACGCAAAT-3' and R-5'-CGAAGTTTCATCATGCGGAG-3'.
- Cytb: sense primer, 5'-CGCTACCTTACAGCGCAATG-3',
- antisense primer, 5'-CGATGTGATAGGGAGGCAATA-3',
- FAM-labeled TAMRA quenched probes, 5'-CGCTTCAATTC-3'.

The results were normalized using nDNA-encoded 18S-RNA as an internal control and are expressed as relative variation with respect to the control sample, arbitrarily set as 1.

Measurement of Inflammation- and Mitophagy-Related Genes in PBMCs Using SYBR Green RT-qPCR

RNA was extracted from purified PBMCs using RNeasy (Baifeite, Chengdu, China) according to the manufacturer’s recommendation. The following primers were used for qPCR amplification:

- F-5'-GAGGAACCTCTCGGAGGATGAGAT-3' and R-5'-TGGGAGTCTACAGGGTGGAA-3',
- Cytosolic cyclic GMP-AMP synthase (cGAS):
- F-5'-GAAGCAACTACGACTAAAGCCATT-3' and R-5'-TTCGATGTGAGAGAAGGATAGCC-3',
- Nucleotide-binding oligomerization domain (NOD)-like receptor 3 (NLRP3):
- F-5'-GGACCTCGAGTGTGAAGCAT-3' and R-5'-TGGGAGTCTACAGGGTGGAA-3',
- Tumor necrosis factor alpha (TNF-α):
- F-5'-CAACCTCCTCTCCTGCAATTG-3' and R-5'-ACGGGCAATGATCCCAAAGTA-3',
- Interleukin-1 beta (IL-1β):
- F-5'-GCACGATGCACTCTTGGATTGAT-3' and R-5'-GGCCAAAGGGCCAGGTATTTT-3',
- Interleukin-6 (IL-6):
- F-5'-TGACAACACTCTTCTAGGCTCAG-3' and
Fisher’s exact test. Logistic regression was used to describe

and explain the relationship between dependent binary vari-
ables and independent variables. Values of \( p < 0.05 \) were con-
sidered statistically significant. All statistical analyses were conducted using SPSS 18.0.

**Results**

**Characteristics and Laboratory Data of MHD Patients with and without Sarcopenia**

The study recruited 105 subjects with MHD that were cate-
gorized into sarcopenia group (SP, \( n=23 \)) and non-sarcopenia
group (NSP, \( n=82 \)). The general characteristics and laboratory
data of the 2 groups with respect to their sarcopenia status are presented in Table 1. SP patients had a longer duration of
dialysis, higher ccf-mtDNA content, and lower BMI, HS, SMi,
VitD, and P than NSP patients. There was no difference with
respect to age, sex, Cr, SBP, DBP, TC, TG, LDL-C, FBS, Ab, Hb,
Ca or hs-CRP between the 2 groups.

**Association Between ccf-mtDNA and the Occurrence of Sarcopenia in MHD Patients**

MtDNA copy number was quantified by amplification of a high-
ly conserved region of mtDNA-encoded CytB gene. As shown in
Table 1, the level of ccf-mtDNA was significantly higher in
SP group than in NSP group. However, we wondered if the
higher levels of ccf-mtDNA in subjects with sarcopenia were
a consequence of other parameters, as dialysis duration, BMI,
HS, SMi, VitD and P were also significantly different between
the 2 groups. Statistically significant variables were included
in the multivariate logistic regression model. As the diagnostic
criteria for sarcopenia include HS and SMI, these 2 variables
were excluded from further analyses. Of note, after adjusting
for these confounders, dialysis duration, BMI, and ccf-mtDNA
remained predictive of sarcopenia; however, VitD and P were
no longer associated with sarcopenia (Table 2). As reduced
BMI is well known to be a risk factor for sarcopenia in CKD pa-
tients [29], our data strongly suggest that longer dialysis du-
ration and higher ccf-mtDNA levels represent newly identified
risk factors for the MHD-related sarcopenia.

**A Similar Trend Of Increased mtDNA Was Also Observed in the Mitochondria-Free Cytoplasm of PBMCs From SP Patients**

As a DAMP molecule, circulatory mtDNA can be easily engulfed
by immune cells, thus fueling the secretion of proinflamma-


**Measurement of Mitochondrial Membrane Potential (\( \Delta \Psi \text{m} \)) Using Flow Cytometry**

PBMCs were isolated from whole blood using the Ficoll-Hypaque
density gradient separation technique, and then the PBMCs were
suspected in PBS at a final concentration of ~105 cells/ml for flow
cytometry. The JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-
imidacarbocyanine iodide) (Beyotime, Shanghai, China) was used for
\( \Delta \Psi \text{m} \) assessment in PBMCs. JC-1 forms J-aggregates within
healthy mitochondria that exhibits red fluorescence (emission,
590 nm) in polarized \( \Delta \Psi \text{m} \). In cells with altered mitochondrial
function, JC-1 only forms monomers exhibiting green fluores-
cence (emission, 527 nm) in the cytoplasm in depolarized \( \Delta \Psi \text{m} \).
The changes in \( \Delta \Psi \text{m} \) were recorded by flow cytometry for the
determination of cells with green fluorescence. All staining was
performed following the manufacturer’s instructions and was an-
alyzed using fluorescence-activated cell sorting (FACS) software.

**Statistical Analyses**

Data are expressed as the mean±standard error (SEM) or fre-
cuency, as appropriate. The distribution of the data was tested
using the Kolmogorov–Smirnov test. Normally distributed data
were analyzed using an independent t-test. Nonnormally
distributed data were analyzed using the Mann-Whitney test.
Spearman’s tests were applied to determine the associations
between continuous variables. Categorical data between 2
groups were compared using the chi-square test (\( \chi^2 \) test) with
Fisher’s exact test. Logistic regression was used to describe


**Table 1**

| Characteristic          | SP (n=23) | NSP (n=82) | p-value |
|-------------------------|-----------|------------|---------|
| Age, years              | 52±10     | 50±10      | 0.81    |
| BMI, kg/m²              | 26±5      | 23±5       | 0.02    |
| Ccr, ml/min/1.73m²      | 1.2±0.3   | 1.5±0.4    | 0.01    |
| Total cholesterol, mg/dl| 180±30    | 160±20     | 0.03    |

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of cytoplasmic mtDNA in PBMCs of SP patients was significantly increased (Figure 1A). Correlation analysis revealed a positive correlation between PBMCs cytoplasmic mtDNA and circulating mtDNA (Figure 1B). Next, it will be interesting to investigate whether the cytosol-accumulated mtDNA initiates the subsequent inflammatory response.

**Table 1.** Characteristics and laboratory data of MHD patients with and without sarcopenia.

| Variable                   | NSP (n=82)       | SP (n=23)       | t/z²/Z         | p        |
|----------------------------|------------------|-----------------|---------------|---------|
| Age (years)                | 55.40±1.58       | 59.78±3.14      | -1.282        | 0.203   |
| Gender (M/F)               | 45/37            | 14/9            | 0.262         | 0.609   |
| Dialysis duration (years)  | 4.56±0.32        | 6.32±0.82       | -1.976        | 0.048   |
| BMI (kg/m²)                | 23.09±0.54       | 20.26±0.72      | -2.553        | 0.011   |
| HS (kg)                    | 24.71±0.53       | 17.73±1.20      | -4.733        | <0.01   |
| SMI (kg/m²)                | 7.93±0.20        | 5.07±0.26       | -6.240        | <0.01   |
| Cr (μmol/L)                | 900.32±53.17     | 876.37±61.06    | -0.221        | 0.825   |
| SBP (mmHg)                 | 147.88±4.23      | 148.69±9.59     | -0.391        | 0.696   |
| DBP (mmHg)                 | 90.57±2.72       | 83.70±5.55      | 1.161         | 0.248   |
| TC (mmol/L)                | 3.78±0.17        | 4.07±0.39       | -0.662        | 0.508   |
| TG (mmol/L)                | 2.59±0.25        | 1.93±0.36       | -1.364        | 0.173   |
| LDL-C (mmol/L)             | 2.87±0.16        | 2.95±0.29       | -0.046        | 0.963   |
| FBS (mmol/L)               | 7.76±0.44        | 7.91±0.74       | -0.666        | 0.505   |
| Ab (g/L)                   | 28.26±1.14       | 27.18±2.34      | -0.504        | 0.615   |
| Hb (g/L)                   | 88.08±2.73       | 89.87±3.35      | -0.328        | 0.744   |
| VItD (ng/mL)               | 30.00±1.01       | 25.13±1.93      | 2.259         | 0.026   |
| Ca (mmol/L)                | 2.03±0.04        | 1.96±0.08       | -0.608        | 0.543   |
| P (mmol/L)                 | 1.88±0.11        | 1.42±0.17       | -2.038        | 0.042   |
| hs-CRP (mg/L)              | 6.73±0.58        | 7.56±1.28       | -0.535        | 0.593   |
| ccf-mtDNA (10^5 copies/μL)| 3.72±0.27        | 6.19±0.77       | -3.452        | <0.01   |

BMI – body mass index; HS – handgrip strength; SMI – skeletal muscle index; Cr – creatinine; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC – total cholesterol; TG – triglyceride; LDL-C – low-density lipoprotein cholesterol; FBS – fasting blood sugar; Ab – albumin; Hb – hemoglobin; VItD – vitamin D; Ca – calcium; P – phosphorus; hs-CRP – high-sensitivity C-reactive protein; ccf-mtDNA – circulating cell-free mitochondrial DNA.

**Table 2.** Univariate and multivariate logistic regression analysis for the occurrence of sarcopenia.

| Variable     | Univariate regression analysis | Multivariate regression analysis |
|--------------|--------------------------------|---------------------------------|
|              | OR                             | p-values                        | OR                             | p-values                        |
| Dialysis duration | 1.164                         | 0.028                           | 1.218                         | 0.016                           |
| BMI          | 0.877                          | 0.015                           | 0.872                         | 0.032                           |
| VItD         | 0.943                          | 0.004                           | 0.954                         | 0.152                           |
| Phosphorus   | 0.545                          | 0.044                           | 0.739                         | 0.369                           |
| ccf-mtDNA    | 1.286                          | <0.01                           | 1.236                         | 0.013                           |

**PBMCS Exhibit Increased Gene Expression Associated with Proinflammatory Activities in SP Patients**

Previous research has shown that mtDNA recognition is established by pattern-recognition receptors (PRRs), such as TLR9 [31], NLRP3 [32] and cGAS-STING [33], which are essential...
for initiating the adaptive immune response. Based on this concept, we analyzed the mRNA levels of molecules downstream of mtDNA in PBMCs and found that the mRNA levels of TLR9 and IL-6 in SP group were significantly higher than that in the NSP group, whereas IL-1β levels were significantly lower in the SP group. No significant differences in cGAS, NLRP3 or TNF-α were observed between the 2 groups (Figure 2). As inflammation has been postulated to play a significant role in the development of sarcopenia, we speculated that the mtDNA-induced inflammation is likely one of the important causes for the MHD-related sarcopenia.

Mitochondria Appear to be Impaired More Severely in Patients with Sarcopenia

The depletion of ΔΨm, reactive oxygen species generation, increased membrane permeability, and calcium overload are characteristics of mitochondrial injury. To investigate the degree of mitochondrial damage, we assessed the loss of ΔΨm using JC-1. Since we were unable to acquire informed consent to obtain tissues from patients, we chose PBMCs, a mitochondria-rich source, as indicators of mitochondrial dysfunction in lieu of tissue biopsy collection. Notably, FACS analysis showed that ΔΨm was significantly decreased in the SP group compared to the NSP group (Figure 3A, 3B). This finding suggests
that mitochondria might be impaired more severely in the tissues and cells of patients with sarcopenia, even in easily obtainable circulating PBMCs.

**Significant Upregulation of the Mitophagy-Related Genes in Patients with Sarcopenia**

It is known that mitophagy represents a “double-edged sword” in the mtDNA release mechanism. To gain insight into the role of mitophagy in this process, we measured the expression of mitophagy-related genes in PBMCs using RT-qPCR. As shown in **Figure 4**, the mRNA levels of Parkin and LAMP2 in the SP group were significantly higher than those in the NSP group, while mRNA levels of PINK1, LC3 and Beclin1 were not significantly changed. These results suggest that mitophagy may be enhanced in patients with sarcopenia, enabling mtDNA leakage into the extracellular spaces.

**Discussion**

Inflammation is widely recognized as a “hallmark of sarcopenia”. Recently, mtDNA has been in the spotlight as an endogenous danger molecule that can potentially elicit inflammation [30]. However, its actual impact on sarcopenia, especially in MHD patients have yet to be investigated. The first striking observation is that the high copy number of circulating mtDNA was significantly associated with a higher risk of sarcopenia in MHD patients, and that the increase in plasma mtDNA might contribute to the onset and maintenance of proinflammatory status. Furthermore, we analyze the state of mitochondria and the activation of mitophagy and propose a potential mechanism for regulating the emergence of cf-mtDNA.

Many chronic diseases such as cardiovascular disease, cancer, chronic kidney disease, diabetes, and neurodegenerative diseases, among others, are initiated or worsened by systemic inflammation [34,35]. Nevertheless, evidence from recent works suggests that plasma levels of proinflammatory cytokines

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**Figure 3.** (A, B) Mitochondria appear to be impaired more severely in patients with sarcopenia. Flow cytometry analysis showing the loss of ΔΨm in PBMCs from SP (n=20) and NSP (n=20) groups. * p<0.05 compared to NSP.
increase with the disease progresses independently of chronic antigenic stimulation, such as infections [36]. It is therefore possible that stimuli different from “classical” antigens derived from bacteria and viruses can also contribute to the inflammatory phenomenon. In this regard, it has been reported that intramitochondrial components, including mitochondrial DNA, cardiolipin, and formyl peptides, can be released extracellularly, enter the blood flow, and act as DAMP agents to activate membrane or cytoplasmic PRRs, such as TLR9 [31], NLRP3 [32] and cGAS-STING mediated pathways [33] and cause inflammation [12]. Thus, mitochondria not only participate in danger signaling inside the cell, but are also a major source of molecule able to activate an innate immune response. Recently, it has been shown that plasma levels of mtDNA are increased during pathological conditions, such as trauma, sepsis, aging, cancer, kidney diseases and immune-mediated diseases, which are characterized by a chronic inflammatory status [9-13,15]. However, the possible contribution of mtDNA to the inflammatory milieu that characterizes muscle wasting disorders remains to be explored.

Muscle atrophy (sarcopenia) is a muscle wasting syndrome associated with several pathological conditions in humans such as aging, diabetes, cancer and kidney diseases, and the presence of sarcopenia worsens outcome [37]. Among the possible pathogenic mechanisms of sarcopenia, mitochondrial dysfunction has been actively investigated. Indeed, mitochondrial impairment and systemic inflammation are hallmarks of sarcopenia. The existence of crosstalk between inflammation and mitochondrial DAMPs in skeletal muscle at a cellular level. This investigation made use of Opa1-deficient muscle cell and reported that mtDNA signal co-localized with TLR9 and drove muscle inflammation in this context [31]. In the current study, we observed higher levels of ccf-mtDNA in the SP group. Univariate analysis and multivariate regression analyses both revealed that ccf-mtDNA levels were independently associated with the occurrence of sarcopenia in MHD patients. Thus, it could be implied that ccf-mtDNA can be employed as a predictive marker for MHD-related sarcopenia. It has previously been shown that the “out of place” mtDNA can be easily engulfed by immune cells and acts as a DAMP [30]. Accordingly, a similar trend of increased mtDNA was also observed in the mitochondria-free cytoplasm of PBMCs from SP patients, together with higher expression of TLR9 and IL-6 in this group. The above results support our hypothesis that high levels of circulating mtDNA, as those observed in sarcopenia subjects, polarize adaptive immune responses by activating monocytes, macrophages and possibly other antigen-presenting cells. However, it is noteworthy that such an increase was not present for cGAS, NLRP3, IL-1β or TNF-α in PBMCs, suggesting that alternative mechanisms might exist, which warrant further investigation.

Mitophagy is the selective degradation of damaged mitochondria by autophagy, contributing to maintenance of a healthy population of mitochondria. Moderate mitophagy is primarily responsible for the elimination of mitochondria with damaged mtDNA [38-40]. Nevertheless, excessive mitophagy triggers mitochondrial damage, resulting in mitochondrial membrane rupturing and mtDNA leakage into extracellular fluid [41,42]. Hence, mitophagy acts as a “double-edged sword” in the process of mtDNA release. PBMCs are abundantly rich in mitochondria.

![Figure 4](image_url) Significant upregulation of the mitophagy-related genes in patients with sarcopenia. Expression of Parkin, PINK1, LC3, Beclin1 and LAMP2 in PBMCs was detected by RT-qPCR. Data are normalized to GAPDH and are expressed relative to the NSP group. * p<0.05 compared to NSP.
ΔΨm is considered an important parameter of mitochondria function and the loss of ΔΨm is regarded as a hallmark of mitochondrial impairment. Recently, tests of PBMCs have been proposed to offer valid information about “general” mitochondrial health [24-26]. We found that ΔΨm was dramatically decreased in PBMCs from SP patients. Furthermore, RT-qPCR analysis showed a significant increase in Parkin and LAMP2 mRNA levels in SP group compared to NSP group. The combination of the data presented in this paper paint a picture of severely compromised mitochondria in patients with sarcopenia, and that the excessive induction of mitophagy may destroy the mitochondria and lead to the release of mtDNA into the blood circulation.

There were several limitations in our study that need to be acknowledged. First, due to the retrospective cross-sectional study design, a cause-and-effect relationship needs to be established in future studies. Second, although we observed the upregulation of mitophagy-related genes in SP patients, mitophagy may be induced as an adaptive rather than toxic response, which deserves further investigation. Third, we used PBMCs as an alternative cellular source, as no biopsies were performed. Therefore, more experiments are required to unequivocally determine the source of ccf-mtDNA.

Conclusions

In summary, we found that the higher copy number of cell-free mtDNA in peripheral blood is significantly associated with a higher risk of sarcopenia in MHD patients. A schematic diagram of the proposed pathway is shown in Figure 5. From a future perspective, the identification of the role of mtDNA could be of importance not only to identify possible new markers of diseases, but also in designing new therapeutic strategies against circulating mtDNA or its receptors to reduce a harmful immune activation.

Declaration of Figures’ Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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