Chapter 5

Associations between cellular aging markers and metabolic syndrome: findings from the CARDIA study

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To be submitted.
Abstract

**Background** – Metabolic syndrome (MetS) and its components are suggested to promote accelerated biological aging, which in turn might lead to cardiovascular and aging-related complications. This large-scale study investigated longitudinal relationships between MetS components and two cellular aging markers: leukocyte mitochondrial DNA copy number (mtDNAcn) and leukocyte telomere length (TL).

**Methods** – We included 989 participants from the Coronary Artery Risk Development in Young Adults study. Leukocyte mtDNAcn (baseline and after 10 years) and TL (baseline and after 5 and 10 years) were measured using quantitative PCR. MetS components (waist circumference, triglycerides, high-density lipoprotein (HDL) cholesterol, systolic blood pressure, and fasting glucose) were determined at baseline and after 5 and 10 years. Generalized estimated equation and linear regression models were used to examine associations between: 1) MetS and cellular aging at all time points, 2) baseline MetS and 10-year changes in cellular aging, 3) baseline cellular aging and 10-year changes in MetS, and 4) 10-year changes in MetS and 10-year changes in cellular aging, while adjusting for sociodemographics and lifestyle.

**Results** – MtDNAcn and TL were both negatively associated with increasing age (mtDNAcn B=-4.76; p<.001; TL B=-51.53; p<.001), and positively intercorrelated (r=0.152; p<.001). High triglycerides were consistently associated with low mtDNAcn, and low HDL cholesterol with short TL. Baseline mtDNAcn and TL did not predict subsequent change in MetS dysregulations, but large baseline waist circumference (B=-7.23; p=.05), glucose (B=-13.29; p=.001), number of metabolic dysregulations (B=-7.72; p=.02), and MetS (B=-28.86; p=.006) predicted larger 10-year decrease in mtDNAcn, but not TL shortening. Further, 10-year increase in waist circumference was associated with 10-year telomere attrition (B=-27.61; p=.04).

**Conclusions** – Our longitudinal data confirmed that MetS dysregulations were negatively associated with both cellular aging indicators. Larger metabolic dysregulations at baseline and metabolic deteriorations over the 10-year follow-up were associated with larger 10-year decrease in mtDNAcn and TL, suggesting that metabolic dysregulations longitudinally contributes to accelerated cellular aging.

**Key words** – Mitochondrial DNA; Telomeres; Metabolic syndrome; Waist circumference; Triglycerides; HDL Cholesterol; Blood pressure; Blood glucose; CARDIA
1. Introduction

Aging-related diseases, such as cardiovascular disease (CVD) and diabetes, account for a large proportion of the incidences of all-disease morbidity and mortality in developed nations. Metabolic syndrome (MetS) is a precursor to CVD and diabetes, receiving extensive attention as an early deterioration state prior to disease development in younger adults. MetS is defined as having at least three of the following risk factors: abdominal obesity, dyslipidemia (low high-density lipoprotein (HDL) cholesterol and high triglycerides), hypertension and hyperglycemia. MetS components are suggested to play a role in accelerated biological aging, and this might reinforce the downward spiral towards aging-related diseases, such as CVD and diabetes mellitus.

In the last decades, telomere length (TL) has been suggested as an important marker for cellular aging. Telomeres are DNA-protein complexes that cap chromosomal ends and promote chromosomal stability. During each somatic cell division, DNA loses telomeric repeats with an estimated shortening rate of 22 to 41 base pairs per year, eventually causing replicative cell senescence or apoptosis. Normal telomere maintenance requires the cellular enzyme telomerase that adds telomeric DNA, thus preserving healthy cell function. Although approximately 64-70% of TL is explained by genetic factors, telomere attrition is thought to be accelerated by cumulative exposure to oxidative stress, pro-inflammatory mediators and endocrine and autonomic dysfunction, both in vitro and in vivo.

Leukocyte TL has been associated with various aging-related diseases and mortality. Another suggested marker of cellular aging is the decrease in mtDNA copy number (mtDNAcn), reported in tissues of both animals and humans. Mitochondria are cellular energy-generating organelles that play an important role in metabolic homeostasis, proliferation, differentiation and apoptosis. Cells contain numerous mitochondria in their cytoplasm, each containing multiple copies of mtDNA. The number of mtDNA copies per cell can vary independently from the number of mitochondria in one cell, and is found to be essential for healthy cellular function. With advancing age, mitochondria produce more reactive oxygen species (ROS) and accumulate mtDNA damage and mutations. The ‘free radical theory of aging’ postulates that this process is bidirectional: increased ROS cause oxidative damage to mtDNA, which negatively impacts mitochondrial function, leading to cellular dysfunction and senescence, and eventually to the onset of aging-related phenotypes.

MtDNAcn decline seems to be a valid marker of cellular aging, although the literature remains inconsistent. Some studies have even suggested that a higher mtDNAcn may represent a marker of poor mitochondrial health or mitochondrial allostatic load, as the copy number might be increased to compensate for DNA damage or mitochondrial dysfunction. Nevertheless, TL and
mtDNAcn are reported to be positively correlated \(^43\text{-}46\), suggesting that telomeres and mitochondria are functionally linked and associated with the aging process \(^47\). Moreover, lower mtDNAcn has been associated to various health outcomes such as MetS, diabetes, cancer and mortality \(^31;48\text{-}50\).

In cross-sectional studies, MetS and its components were associated with short TL \(^51\text{-}57\), and with reduced mtDNAcn \(^48;58\text{-}63\). However, longitudinal literature is scarce. Some studies showed that short baseline TL is associated with worse MetS outcomes at follow-up \(^51;64\), and vice versa, that baseline MetS components predict shorter TL over time \(^4;6\). Only few studies have repeatedly measured both MetS and TL, and reported that telomere attrition paralleled deterioration in obesity measures \(^4;6;65\). No longitudinal study has yet examined MetS components and mtDNAcn.

This large-scale study investigated the longitudinal relationships between MetS components and mtDNAcn and TL in the Coronary Artery Risk Development in Young Adults (CARDIA). We first examined whether MetS components were consistently associated with mtDNAcn and TL throughout a 10-year period. Then, we investigated whether baseline MetS would predict 10-year changes in cellular aging markers, or vice versa, whether baseline cellular aging would predict 10-year changes in MetS. At last, we correlated 10-year changes in MetS components with the 10-year changes in mtDNAcn and TL. We hypothesized that a disadvantageous metabolic state at baseline would be associated with decreased mtDNAcn and shorter TL at follow-up, and that larger metabolic deterioration would be accompanied by accelerated cellular aging.

2. Methods

2.1. Study sample

The sample population is from the Coronary Artery Risk Development in Young Adults (CARDIA) study. Details of study design, recruitment, and procedures have been published elsewhere \(^66\). In short, during 1985 to 1986, CARDIA performed community-based recruitment of 5,115 research study participants in Birmingham, AL, Chicago, IL, and Minneapolis, MN and from the membership of a prepaid health care plan in Oakland, CA. The study sample was balanced by race, sex and education. Follow-up examinations were conducted at years 2, 5, 7, 10, 15, 20, and 25. All participants signed consent forms at each examination, with all aspects reviewed and approved by the institutional review board of each participating institution. The current study reports data from a sub-study, for which participants were selected if two conditions were satisfied: (1) stored DNA from whole blood was available from years 15, 20 and 25 inclusive, and (2) coronary artery calcification data from all three years inclusive were also available. Within this sub-study, TL was measured at Y15, Y20 and Y25, whereas mtDNAcn was measured at Y15 and Y25. For the purpose of the current study, CARDIA Years 15, 20 and 25 will be described as Year 0, Year 5 and
Year 10, respectively. Overall, we selected participants with complete data on the cellular aging measured at baseline (N=989). MetS had missing values on the different components at baseline (N=1-46), 5-year (N=0-60) and 10-year follow-up (N=4-40).

2.2. Blood draw and sample preparation

Blood samples were drawn by venipuncture, according to a standard protocol, in the morning after an overnight fast (>8 hours) using EDTA-containing tubes. Persons were asked not to smoke or perform heavy physical activity for two hours before their examination visit. Blood was taken from participants, and centrifuged, with aliquots stored at −70°C until shipped on dry ice to the Genetics laboratory of Dr. Fornage at the University of Texas Health Sciences Center (Houston, TX, USA). Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation gradient from whole blood using Ficoll-Paque PLUS (Amersham, density 1.077g/mL) according to the manufacturer’s instructions and cryopreserved and stored in liquid nitrogen until assays were performed. DNA for TL was prepared using Gentra Puregene Cell kit (QIAGEN, Valencia, CA, USA).

2.3. Cellular aging markers

2.3.1. Telomere length (TL)

DNA samples were shipped in 96-well plates to UCSF Blackburn lab. All DNA samples were stored in their original plates in a -80°C freezer upon arrival. Of the original DNA samples, 5ul was diluted with 10ul of sterile Millipore H2O to reach final concentration of 33.3ng/ul. The diluted DNA samples were plated in 96-well plates and stored in a -80°C freezer. DNA samples were thawed on ice on the day of the assay and transferred to 384-well plates. The telomere length measurement assay was adapted from the published original method. TL values were measured from DNA by a quantitative PCR (qPCR) assay that determines the ratio of telomere repeat copy number to single-copy gene copy number (T/S) ratio in experimental samples as compared with a reference DNA sample. Higher T/S ratio signifies longer mean TL. Primers for the telomere PCR (T runs) are tel1b [5’-CGGTTT(GTTTGG)5GTT-3’], used at a final concentration of 100 nM, and tel2b [5’-GGCTTG(CCTTAC)5CCT-3’], used at a final concentration of 900 nM. The primers for the single-copy gene (human beta-globin) PCR (S runs) are hbg1 [5’ GCTTCTGACACAACTGTGTTCACTAGC-3’], used at a final concentration of 300 nM, and hbg2 [5’-CACCAACTTACCATCCACGTTACC-3’], used at a final concentration of 700 nM.

2.3.2. Mitochondrial DNA copy number (mtDNAcn)

Leukocyte mitochondrial DNA copy number (mtDNAcn) was measured by qPCR. A 69 bp fragment within the ND1 gene in mtDNA (nucleotides 3485-3553) and a 87 bp fragment within the RNase P gene in the nuclear DNA were simultaneously amplified by a multiplex TaqMan-based qPCR reaction adapted from. This was used to
determine the relative number of mtDNA copies per diploid nuclear genome (i.e., per cell). The VIC-labelled probe and primer set for RNase P were obtained from Life Technologies (cat#4403328). The primer and probe sequences for ND1 (IDT) are:

ND1-forward 5’-CCCTAAAACCGCCACATCT-3’
ND1-reverse 5’GAGCGATGGTGAGAGCTAAGGT-3’
ND1-FAM probe 5’ FAM-CCATCACCCTCTACATCACCGCCC-TAMRA-3’.

The reaction contained 12.5 ng of genomic DNA, 100nM of ND1 probe, 300 nM of ND1-forward primer and ND1-reverse primer each, 1X RNase P copy number Reference Assay, 1X LightCycler® 480 Probe Master (Roche, cat# 04902343001) in a 10 ul reaction. All samples were run in triplicate wells in 384-well plates in a Roche Lightcyler 480. PCR conditions are 95°C 10 min 45 cyles of 95 °C 10 sec, 60°C 30 sec, 72°C 1 sec with data acquisition at 72°C. Crossing point (CP) for each well is derived by the LightCycler 480 program using the second derivation method. Relative copy number is calculated by the following formula: relative mtDNAcn/diploid genome=POWER[2, (CP_{ND1}-CP_{RNaseP})]*2. Inter-assay CV using 85 CARDIA samples was 3.4% and intra-assay CV using four control DNA samples, averaged from all 21 CARDIA sample plates, was 1.7%.

2.4. Metabolic syndrome components

All five MetS components were measured at baseline, 5-year and 10-year follow-up. Waist circumference was measured as the average of two measures at a level midway between the lowest rib and the iliac crest. Seated blood pressure was measured after a five-minute rest, taking an average of second and third readings of the first- and fifth-phase Korotkoff sounds. Fasting blood glucose was measured by Roche Modular P-hexokinase method. Plasma lipids were measured at the University of Washington Northwest Lipid Research Clinic Laboratory (Seattle). Triglycerides were measured by UV method and determined enzymatically on the Abbott Spectrum (using Hitachi 917–R1Buffer/4–chlorophenol/enzymes), and HDL cholesterol was measured by Trinder-type method and determined enzymatically after dextran sulfate-magnesium precipitation on the Abbott Spectrum. The continuous measures were adjusted for medication use based on the estimated effects of the medication. According to the standards of medical care in diabetes, the goal of antidiabetic medication should be to lower the fasting glucose level to <7.0 mmol/L. 69 In agreement with these standards, for persons using antidiabetic medication (baseline N=17; 5-year N=44; 10-year N=72) when glucose level was <7.0 mmol/L, a value of 7.0 mmol/L was assigned. For persons using antihypertensive medication (baseline N=64; 5-year N=161; 10-year N=266), 10 mmHg was added to the SBP according to the average decline in blood pressure in antihypertensive trials 70.
Based on the recommendations of the National Cholesterol Education Program (NCEP) Expert Panel, MetS diagnosis was defined as having at least three of these dysregulations: 1) abdominal obesity: waist circumference ≥102 cm in men and ≥88 cm in women; 2) hypertriglyceridemia: triglycerides ≥1.7 mmol/L or medication for hypertriglyceridemia; 3) low HDL cholesterol <1.03 mmol/L in men and <1.30 mmol/L in women or medication for reduced HDL cholesterol; 4) hypertension: blood pressure: systolic ≥130 and/or diastolic ≥85 mmHg or antihypertensive medication; 5) hyperglycemia: fasting plasma glucose ≥5.6 mmol/L or antidiabetic medication. Furthermore, we calculated a summarizing variable from the number of MetS dysregulations (range 0-5), reflecting MetS severity.

2.5. Covariates
All covariates were recorded at the three time points. Sociodemographic factors included age, sex, race (whites vs. blacks) and educational achievement (less than high school or less vs. high school and college vs. post-college education), and were based on standardized questionnaires. Lifestyle factors included smoking (never, former and current smoker), alcohol consumption (no drinker, mild-moderate drinker 1–14 (women) / 1–21 drinks per week (men), heavy drinker >14 (women) / >21 (men) drinks per week), and habitual physical activity as measured by the CARDIA Physical Activity History, a simplified version of the Minnesota Leisure Time Physical Activity Questionnaire.

2.6. Statistical analyses
Sample characteristics were described as percentages or means and standard deviations. For non-normally distributed factors the median and interquartile range were calculated. Associations between MetS and both cellular aging markers at the three time points were analysed using generalized estimating equations (GEE) with an exchangeable correlation structure, taking into account the within-person correlations at repeated measurements, and handling missing observations. Separate GEE models were run with mtDNAcn (two time points) and with TL (three time points) as the outcomes. First, only time-varying age was entered into the model to examine the effects of age on the two cellular aging markers. Then the MetS components, the number of metabolic dysregulations and MetS diagnosis were entered as a separate predictors, adjusted for sex, race, and time-varying age, education, smoking, alcohol and physical activity.

Next, we calculated change scores of MetS components, mtDNAcn and TL by subtracting baseline values from the 10-year follow-up values for all participants with available data at both time points. We tested the associations between baseline mtDNAcn and TL and 10-year changes in MetS components, while adjusting for sociodemographic and lifestyle factors and baseline cellular aging values using linear regression models. Vice versa, we tested the fully adjusted associations between
baseline MetS and 10-year change in mtDNAcn and TL, while adjusting for baseline values of the cellular aging marker.

Last, we conducted linear regression analyses to examine the associations between 10-year changes in MetS components (per increase in one SD) or the number of MetS dysregulations with 10-year changes in mtDNAcn and TL. Changes in MetS components or changes in the number of MetS dysregulations were entered into separate models as predictors, and 10-year mtDNAcn/TL as the outcome, while adjusting for baseline covariates, baseline mtDNAcn or TL, and baseline MetS. All analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Statistical significance level was set at p<0.05, two-tailed.

3. Results

Table 1 shows the sample characteristics at baseline, 5-year and 10-year follow-up (N=989). Overall, more subjects meet the criteria for MetS over time, increasing from 12.7% at baseline to 20.2% at the 10-year follow-up. Both cellular aging markers significantly decreased over time (p<.001), and were significantly associated with age in GEE analyses: mtDNAcn (B=-4.76; SE=0.40; p<.001) and TL (B=-51.53; SE=1.18; p>.001). MtDNAcn and TL were highly correlated with their follow-up measures (r>.45, all p-values <.001), and positively correlated with each other at baseline (r=0.15; p<.001).

Table 2 shows associations from GEE analyses between MetS components, the number of MetS dysregulations and MetS diagnosis, and the cellular aging markers mtDNAcn (at two time points) and TL (three time points). Higher levels of triglycerides (p=.05) were consistently associated with lower mtDNAcn, and lower levels of HDL cholesterol were associated with shorter TL (p=.04).

Next, we investigated the associations between baseline mtDNAcn and TL predicting 10-year changes in MetS components. We found that the baseline cellular aging markers did not significantly predict changes in MetS (Table 3). Conversely, the associations between baseline MetS components and 10-year change in mtDNAcn and TL are shown in Table 4. Larger baseline waist circumference (p=.05), glucose (p=.001), number of metabolic dysregulations (p=.02), and MetS diagnosis (p=.006) predicted larger 10-year decrease in mtDNAcn. No significant associations were found between baseline MetS components and 10-year change in TL.

Finally, we examined the associations between 10-year changes in MetS components and 10-year changes in cellular aging markers, while adjusting for all covariates, baseline values of MetS components and baseline values of cellular aging markers (Table 5). Changes in MetS components were not associated to changes in mtDNAcn. However, an increase in waist circumference was associated with significant telomere attrition over the 10-year follow-up (p=.04).
Table 1: Sample characteristics at all time points of subjects with complete data on telomere length and mitochondrial DNA copy number at baseline (N=989)

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Year 0</th>
<th>Year 5</th>
<th>Year 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), m (sd)</td>
<td>40.5 (3.6)</td>
<td>45.4 (3.6)</td>
<td>50.4 (3.6)</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>65.5</td>
<td>65.5</td>
<td>65.5</td>
</tr>
<tr>
<td>Race (% black)</td>
<td>40.8</td>
<td>40.8</td>
<td>40.8</td>
</tr>
<tr>
<td>Education completed, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>18.8</td>
<td>21.3</td>
<td>20.0</td>
</tr>
<tr>
<td>High school and college</td>
<td>60.4</td>
<td>56.4</td>
<td>55.1</td>
</tr>
<tr>
<td>At least some post-college education</td>
<td>20.7</td>
<td>22.4</td>
<td>24.9</td>
</tr>
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<table>
<thead>
<tr>
<th>Lifestyle factors</th>
<th>Year 0</th>
<th>Year 5</th>
<th>Year 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>62.2</td>
<td>63.0</td>
<td>63.1</td>
</tr>
<tr>
<td>Former</td>
<td>18.7</td>
<td>21.3</td>
<td>22.9</td>
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<tr>
<td>Current</td>
<td>19.0</td>
<td>15.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Drinking, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-drinker</td>
<td>27.5</td>
<td>24.7</td>
<td>23.0</td>
</tr>
<tr>
<td>Mild-moderate drinker</td>
<td>63.2</td>
<td>65.1</td>
<td>66.1</td>
</tr>
<tr>
<td>Heavy drinker</td>
<td>9.3</td>
<td>10.2</td>
<td>10.9</td>
</tr>
<tr>
<td>Physical activity (total intensity score), m (sd)</td>
<td>336 (273)</td>
<td>335 (275)</td>
<td>335 (274)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cellular aging markers</th>
<th>Year 0</th>
<th>Year 5</th>
<th>Year 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomere length (base pairs), m (sd)</td>
<td>5594 (481)</td>
<td>5679 (451)</td>
<td>4971 (276)</td>
</tr>
<tr>
<td>Mitochondrial DNA copy number, m (sd)</td>
<td>513 (141)</td>
<td>-</td>
<td>459 (124)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolic syndrome components</th>
<th>Year 0</th>
<th>Year 5</th>
<th>Year 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (cm), m (sd)</td>
<td>88.4 (13.8)</td>
<td>91.1 (14.5)</td>
<td>93.3 (14.8)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L), Median (IQR)</td>
<td>0.94 (0.67)</td>
<td>1.01 (0.79)</td>
<td>1.05 (0.70)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L), m (sd)</td>
<td>1.32 (0.36)</td>
<td>1.41 (0.43)</td>
<td>1.53 (0.46)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg), m (sd)</td>
<td>111.9 (13.7)</td>
<td>115.4 (14.6)</td>
<td>117.9 (15.4)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L), Median (IQR)</td>
<td>2.34 (0.31)</td>
<td>2.48 (0.33)</td>
<td>2.41 (0.36)</td>
</tr>
<tr>
<td>Number of metabolic dysregulations, m (sd)</td>
<td>1.10 (1.12)</td>
<td>1.27 (1.25)</td>
<td>1.35 (1.28)</td>
</tr>
<tr>
<td>Metabolic syndrome diagnosis, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic syndrome diagnosis ≥ 3 dysregulations</td>
<td>12.7</td>
<td>18.2</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Footnote: m = Mean; sd = Standard deviation; IQR = Interquartile range; a Metabolic syndrome dysregulations defined as: 1) waist circumference >102 cm (men) and >88 cm (women); 2) triglycerides >1.7 mmol/L; 3) high-density lipoprotein (HDL) cholesterol <1.03 mmol/L (men) and <1.30 mmol/L (women); 4) blood pressure: systolic >130 and/or diastolic >85 mm Hg or antihypertensives; 5) fasting plasma glucose >6.1 mmol/L or antidiabetic medication; b Metabolic syndrome ≥ 3 dysregulations
Table 2: Associations with GEE analyses between metabolic syndrome components (3 measurements) and mitochondrial DNA copy number (2 measurements) and telomere length (3 measurements) (N=989)

<table>
<thead>
<tr>
<th>MetS components</th>
<th>Mitochondrial DNA copy number</th>
<th>Telomere length</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Years 0+5+10 (per standard deviation)</td>
<td>Years 0+10</td>
<td>Years 0+5+10</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
<td>p</td>
</tr>
<tr>
<td>Waist circumference (per 13.8 cm)</td>
<td>-1.54</td>
<td>3.57</td>
<td>.67</td>
</tr>
<tr>
<td>Triglycerides (per 1.21 mmol/L)</td>
<td>-4.35</td>
<td>2.20</td>
<td>.05</td>
</tr>
<tr>
<td>HDL cholesterol (per 0.36 mmol/L)</td>
<td>4.25</td>
<td>3.65</td>
<td>.25</td>
</tr>
<tr>
<td>Systolic BP (per 13.7 mmHg)</td>
<td>-0.16</td>
<td>3.20</td>
<td>.96</td>
</tr>
<tr>
<td>Fasting glucose (per 0.72 mmol/L)</td>
<td>-2.28</td>
<td>4.55</td>
<td>.62</td>
</tr>
<tr>
<td>Number of metabolic dysregulations a</td>
<td>-1.88</td>
<td>2.64</td>
<td>.48</td>
</tr>
<tr>
<td>Metabolic syndrome diagnosis b</td>
<td>1.54</td>
<td>8.37</td>
<td>.85</td>
</tr>
</tbody>
</table>

Footnote: Adjusted for: sex and time-varying age, education, race, smoking, alcohol, physical activity; a Metabolic syndrome dysregulations defined as: 1) waist circumference >102 cm (men) and >88 cm (women); 2) triglycerides >1.7 mmol/L; 3) high-density lipoprotein (HDL) cholesterol <1.03 mmol/L (men) and <1.30 mmol/L (women); 4) blood pressure (BP): systolic >130 and/or diastolic >85 mm Hg or antihypertensives; 5) fasting plasma glucose >6.1 mmol/L or antidiabetic medication; b Metabolic syndrome ≥ 3 dysregulations

Table 3: Linear regression with baseline mitochondrial DNA copy number (mtDNA) and telomere length (TL) predicting 10-year changes in MetS components (N=989) (continuation on next page)

<table>
<thead>
<tr>
<th>Baseline cellular aging (1 SD decrease)</th>
<th>10-year Δ waist circumference</th>
<th>10-year Δ triglycerides</th>
<th>10-year Δ HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
<td>p</td>
</tr>
<tr>
<td>mtDNA (per 141 l)</td>
<td>0.327</td>
<td>0.245</td>
<td>.18</td>
</tr>
<tr>
<td>TL (per 481 l)</td>
<td>-0.304</td>
<td>0.231</td>
<td>.19</td>
</tr>
</tbody>
</table>

Footnote: Adjusted for age, sex, education, race, smoking, alcohol, physical activity; a Metabolic syndrome dysregulations defined as: 1) waist circumference >102 cm (men) and >88 cm (women); 2) triglycerides >1.7 mmol/L; 3) high-density lipoprotein (HDL) cholesterol <1.03 mmol/L (men) and <1.30 mmol/L (women); 4) blood pressure (BP): systolic >130 and/or diastolic >85 mm Hg or antihypertensives; 5) fasting plasma glucose >6.1 mmol/L or antidiabetic medication; B = Unstandardized Beta; SD= Standard deviation; SE = Standard Error.
### Table 4: Linear regressions with baseline metabolic syndrome (components) predicting 10-year changes in mitochondrial DNA copy number (mtDNAcn) and telomere length (TL) (N=989)

<table>
<thead>
<tr>
<th>Baseline predictors (per standard deviation increase)</th>
<th>10-year Δ mtDNAcn</th>
<th>10-year Δ TL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
</tr>
<tr>
<td>Waist circumference (per 13.8 cm)</td>
<td>-7.23</td>
<td>3.71</td>
</tr>
<tr>
<td>Triglycerides (per 1.21 mmol/L)</td>
<td>-4.02</td>
<td>3.50</td>
</tr>
<tr>
<td>HDL cholesterol (per 0.36 mmol/L)</td>
<td>7.18</td>
<td>3.83</td>
</tr>
<tr>
<td>Systolic BP (per 13.7 mmHg)</td>
<td>-2.23</td>
<td>3.68</td>
</tr>
<tr>
<td>Fasting glucose (per 0.72 mmol/L)</td>
<td>-13.29</td>
<td>3.98</td>
</tr>
<tr>
<td>Number of metabolic dysregulations a</td>
<td>-7.72</td>
<td>3.19</td>
</tr>
<tr>
<td>Metabolic syndrome diagnosis b</td>
<td>-28.86</td>
<td>10.41</td>
</tr>
</tbody>
</table>

Footnote: Adjusted for: age, sex, education, race, smoking, alcohol, physical activity, baseline cellular aging marker; a Metabolic syndrome dysregulations defined as: 1) waist circumference>102 cm (men) and >88 cm (women); 2) triglycerides >1.7 mmol/L; 3) high-density lipoprotein (HDL) cholesterol <1.03 mmol/L (men) and <1.30 mmol/L (women); 4) blood pressure (BP): systolic >130 and/or diastolic >85 mm Hg or antihypertensives; 5) fasting plasma glucose >6.1 mmol/L or antidiabetic medication; b Metabolic syndrome ≥3 dysregulations

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### Table 3: Linear regression with baseline mitochondrial DNA copy number (mtDNA) and telomere length (TL) predicting 10-year changes in MetS components (N=989) (continued from previous page)

<table>
<thead>
<tr>
<th>10-year Δ systolic BP</th>
<th>10-year Δ fasting glucose</th>
<th>10-year Δ number of dysregulations a</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>SE</td>
<td>p</td>
</tr>
<tr>
<td>0.452</td>
<td>0.460</td>
<td>.33</td>
</tr>
<tr>
<td>-0.147</td>
<td>0.437</td>
<td>.74</td>
</tr>
</tbody>
</table>

---

Révész (2016) - The interplay between biological stress and cellular aging: an epidemiological perspective
Table 5: Linear regressions with 10-year changes in metabolic syndrome predicting 10-year changes in mitochondrial DNA copy number (mtDNAcn) and telomere length (TL) (N=989)

<table>
<thead>
<tr>
<th>10-year Δ predictors (Per standard deviation change)</th>
<th>10-year Δ mtDNAcn</th>
<th>p-value</th>
<th>10-year Δ TL</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (+ 13.8 cm)</td>
<td>5.63</td>
<td>6.58</td>
<td>.39</td>
<td>27.61</td>
</tr>
<tr>
<td>Triglycerides (+ 1.21 mmol/L)</td>
<td>-4.67</td>
<td>6.27</td>
<td>.46</td>
<td>-8.50</td>
</tr>
<tr>
<td>HDL cholesterol (+ 0.36 mmol/L)</td>
<td>-3.74</td>
<td>4.24</td>
<td>.38</td>
<td>3.38</td>
</tr>
<tr>
<td>Systolic BP (+ 13.7 mmHg)</td>
<td>-4.64</td>
<td>3.48</td>
<td>.18</td>
<td>-2.48</td>
</tr>
<tr>
<td>Fasting glucose (+ 0.72 mmol/L)</td>
<td>-0.38</td>
<td>2.50</td>
<td>.88</td>
<td>-7.10</td>
</tr>
<tr>
<td>Number of metabolic dysregulations a</td>
<td>-1.27</td>
<td>3.53</td>
<td>.72</td>
<td>-7.15</td>
</tr>
</tbody>
</table>

Footnote: Adjusted for: age, sex, education, race, smoking, alcohol, physical activity, baseline predictor, baseline cellular aging marker; * Metabolic syndrome dysregulations defined as: 1) waist circumference>102 cm (men) and >88 cm (women); 2) triglycerides >1.7 mmol/L; 3) high-density lipoprotein (HDL) cholesterol <1.03 mmol/L (men) and <1.30 mmol/L (women); 4) blood pressure (BP): systolic >130 and/or diastolic >85 mm Hg or antihypertensives; 5) fasting plasma glucose >6.1 mmol/L or antidiabetic medication.

4. Discussion

This large-scale study investigated the longitudinal relationships between MetS components and mtDNAcn and TL throughout a 10-year period. First, mtDNAcn and TL were positively associated with each other and with age. Next, at the three time points, higher levels of triglycerides were consistently associated with low mtDNAcn, and low HDL cholesterol was associated with shorter TL. Furthermore, large baseline waist circumference, low HDL cholesterol, high glucose and a high number of MetS dysregulations were associated with a large 10-year decrease in mtDNAcn, whereas they did not predict the 10-year telomere attrition. Conversely, baseline cellular aging markers did not predict metabolic deterioration. Lastly, 10-year increase in waist circumference was associated with 10-year telomere attrition, but none of the other changing MetS components were running parallel with 10-year changes in cellular aging markers.

Over time, mtDNAcn decreased during the 10-year follow-up with a yearly decrease of approximately 5 mtDNA copies, similar to the rate seen in other studies. The mean TL showed a slight increase from baseline to the 5-year follow-up, and a large decrease towards the 10-year follow-up, with an average attrition of 52bp per year, slightly higher than the averages reported in a systematic review. Although mtDNAcn decline seems to be a valid marker for cellular aging, the literature is inconsistent. The number of mtDNA copies per cell is shown to decrease with age in various cell types, such as blood leukocytes, human pancreatic cells, fibroblasts, skeletal muscle cells, but not all studies have confirmed these age-related mtDNAcn decreases. One study also found that the decline in mtDNAcn starts around the age of 48. Within the current study, we found that metabolic dysregulations and increasing age were associated with the low mtDNAcn in PBMCs, and that mtDNAcn was positively correlated with the more established cell aging marker TL.
Overall, only higher triglycerides were consistently associated with decreased mtDNAcn, and only lower HDL cholesterol with shorter TL. Our results do not fully confirm the associations reported in earlier studies between the other MetS components and mtDNAcn or TL. Neither did we find that baseline MetS components predicted 10-year TL attrition, as seen before, although our finding is in line with some earlier studies that did not observe this association either with MetS components or with body weight. It is unclear why these findings differ. We previously showed that subjects with a disadvantageous metabolic profile at baseline have shorter TL over time, but not a faster attrition rate, possibly due to a strong homeostatic mechanism. However, a novel finding was that greater baseline waist circumference, higher glucose and MetS severity did predict the 10-year decline of mtDNAcn. At last, we confirmed earlier studies that found an increase in waist circumference parallels telomere attrition. Perhaps TL is more a marker of the current metabolic state, whereas mtDNAcn responds with long-term changes. Future studies should be designed to investigate the complex relationship between these two cellular aging markers.

It remains hard to explain why some MetS components are associated with accelerated cellular aging in certain studies, but not in others. The most robust association is the one between the abdominal obesity and dyslipidemia components of metabolic syndrome and cellular aging. Within the so-called ‘adipocyte overflow hypothesis’ enlarged adipocytes reach their fat storage capacities, causing an ‘overflow’ of fatty acids into sites such as the liver and muscle. These fatty acids not only promote further deteriorations of other MetS components (e.g. insulin resistance), but they also increase systemic inflammation and oxidative stress, both catalysts of telomeric attrition. This ‘metabolic oversupply’ in cells is also shown to fragment mitochondria, increasing reactive oxygen species production promoting the accumulation of mtDNA damage, whereas an undersupply is shown to promote mitochondrial fusion and limit mtDNA damage. Therefore, maintenance of metabolic balance appears to be important to preserve mitochondrial function, and excessive mitochondrial damage may contribute to the pro-aging effects of MetS. The association between fasting glucose and mtDNAcn is also very interesting given the strong dose-response relationship between fasting glycemia and all-cause mortality, and the damaging effects of hyperglycemia on mtDNA. Measurements of mitochondrial integrity and function, such as mtDNA damage and respiratory chain function, would be informative to probe the functional significance of mtDNAcn changes in the present study, and to further understand the role of mitochondria in MetS and cell aging.

The present findings should be interpreted with some caution, and the following limitations should be taken into account. In the current study, TL and mtDNAcn were measured with qPCR in cellular homogenates, and although this is a widely used and cost-efficient high-throughput technique, one might argue mean bulk
TL does not capture sub-populations of cells with abnormally short telomeres, and that various mistakes can occur when measuring the mitochondrial genome to nuclear genome ratio, and qPCR may thus fail to capture biologically meaningful changes over time in comparison to other methods. Also, both mtDNAcn and TL are measured in leukocytes, as they are easily accessible and the results are comparable with most other studies. For TL, high correlations have been observed between various tissues and leukocytes, but these correlations have not yet been examined for mtDNA. Methodologically, one potential explanation for the lack of consistent results with mtDNAcn across studies could lie upon differences in biological material examined, including for instance whole blood, purified total leukocytes, mononuclear cells (PBMCs), and granulocytes. This is because cellular composition, particularly platelet content (which contain high abundance of mtDNA but no nuclear DNA) in whole blood or platelet contamination in isolated PBMCs, may cause or mask apparent differences in mtDNAcn. To enable interpretation of findings across studies in this developing field, detailed isolation methods or lack thereof should be reported. Lastly, telomerase, dietary intake and distribution of cell subtypes were not assessed in this study. By measuring telomerase, more information would have been available about the telomeric homeostasis system, as telomerase predominantly acts to lengthen shortest telomeres relative to longer ones. Nevertheless, this is the first study not only looking at two novel cellular aging markers simultaneously, but also describing a 10-year follow-up in ~1000 participants. Some other major strengths of this study were the extensive measurement of MetS components, medication use and relevant sociodemographic and lifestyle factors.

Overall, this large-scale longitudinal investigation showed that both mtDNAcn and TL decreased over a 10-year period, consistent with their use as proxies for cellular aging in human population studies. Although baseline MetS did not predict LT attrition, it did predict greater decline in leukocyte mtDNAcn, an effect apparently driven mostly by abdominal obesity and fasting glucose. However, baseline cellular aging did not predict metabolic deteriorations, suggesting that metabolic stress promotes cellular aging but not the converse. When looking at the parallel deterioration of metabolic dysregulations and cellular aging, increased abdominal obesity was associated with accelerated telomere attrition. Overall, these findings provide the basis for future mechanistic studies aimed at understanding how metabolic stress promotes cellular aging, and the interplay between mitochondria and telomeres in humans. Ultimately, resolving these mechanisms and identifying modifiers of this relationship should help to discover novel targets in the prevention or delay of aging-related morbidities and health impairments.
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