Universiteit
Leiden
The Netherlands

Insight into the pathophysiology of cardiometabolic diseases using multiple omics approaches

Wang, W.

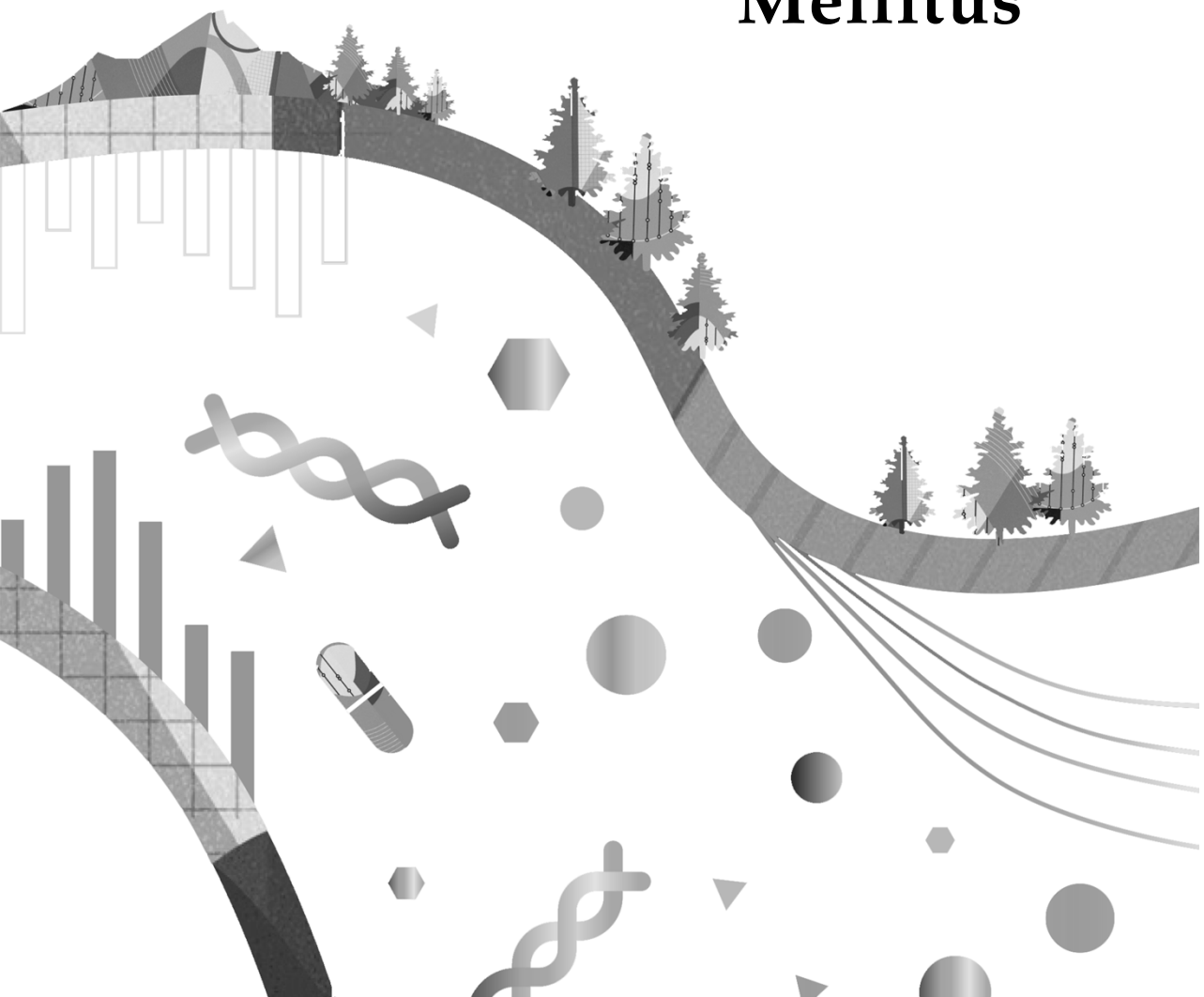
Citation
Wang, W. (2024, November 27). *Insight into the pathophysiology of cardiometabolic diseases using multiple omics approaches*. Retrieved from https://hdl.handle.net/1887/4169999

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4169999

Note: To cite this publication please use the final published version (if applicable).

Part II

Pathophysiology of Type 2 Diabetes Mellitus



Chapter 3

Assessment of the bi-directional relationship between blood mitochondrial DNA copy number and type 2 diabetes mellitus: a multivariable-adjusted regression and Mendelian Randomization study

Wenyi Wang, Jiao Luo, Ko Willems van Dijk, Sara Hägg, Felix Grassmann, Leen M `t Hart, Diana van Heemst, Raymond Noordam

Diabetologia (2022) 10.1007

The supplemental information for this paper is available online at https://drive.google.com/drive/folders/1UoSstJZtu2ylarbtkhn6Kmr3rz68hhSu?usp=sharing

Abstract

Aims/hypothesis: Mitochondrial dysfunction, which can be approximated by blood mtDNA copy number (mtDNA-CN), has been implicated in the pathogenesis of type 2 diabetes mellitus. Thus far, however, insights from prospective cohort studies and Mendelian Randomization (MR) analyses on this relationship are limited. We assessed the association between blood mtDNA-CN and incident type 2 diabetes using multivariable-adjusted regression analyses, and the associations between blood mtDNA-CN and type 2 diabetes and body mass index (BMI) using bi-directional MR.

Methods: Multivariable-adjusted Cox proportional hazard models were used to estimate the association between blood mtDNA-CN and incident type 2 diabetes in 285,967 unrelated European individuals from UK Biobank (UKB) free of type 2 diabetes at baseline. Additionally, a cross-sectional analysis was performed to investigate the association between blood mtDNA-CN and BMI. Furthermore, we assessed the potentially causal relationship between blood mtDNA-CN and type 2 diabetes (N=898,130 from DIAGRAM, N=215,654 from FinnGen) and BMI (N=681,275 from GIANT) using bi-directional two-sample MR.

Results: During a median follow-up of 11.87 years, 15,111 participants developed type 2 diabetes. Participants with higher level of blood mtDNA-CN have lower risk to develop type 2 diabetes (hazard ratio: 0.90; 95% Confidence Interval (CI): 0.89-0.92). After additional adjustment for BMI and other confounders, these results attenuated moderately and remained present. The multivariable-adjusted cross-sectional analyses show higher blood mtDNA-CN was associated with lower BMI (-0.12 (95% CI: -0.14 ~ -0.10) kg/m2. In the bi-directional MR analyses, we found no evidence for causal associations between blood mtDNA-CN and type 2 diabetes, and blood mtDNA-CN and BMI in either direction.

Conclusions/interpretation: The results from the present study indicate that the observed association between low blood mtDNA-CN and higher risk of type 2 diabetes is likely not causal.

Introduction

Type 2 diabetes mellitus, which is characterized by a combination of insulin resistance and insufficient insulin secretion, is a major contributor to the burden of morbidity and mortality worldwide [1]. Multiple risk factors have been associated with an increased risk of developing type 2 diabetes, including obesity, adverse lifestyle and genetic factors [2].

Mitochondria generate most of the cell's need for chemical energy in the form of adenosine triphosphate (ATP), and mitochondrial dysfunction has been implicated in various aspects of the development and complications of type 2 diabetes including insulin resistance, obesity and beta-cell dysfunction [3]. Mitochondrial DNA (mtDNA) is a circular and double-stranded DNA molecule comprising 37 genes, of which thirteen genes are involved in the electron transport chains and generation of ATP to provide energy for cells, while the remaining genes encode proteins involved in the assembly of amino acids into functional proteins [4]. Although mitochondria have been implicated in the onset of type 2 diabetes, only a few of the rare mtDNA genetic variants are associated with diabetes mellitus [5].

MtDNA-copy number (mtDNA-CN) is considered as a proxy for mitochondrial function [6]. Low peripheral blood mtDNA-CN has been associated with various age-related diseases, including type 2 diabetes, cardiovascular disease and cancer [7-9]. A lower mtDNA-CN has also been observed in skeletal muscle and adipose tissue of individuals with obesity or type 2 diabetes [10, 11]. In line, a lower mtDNA-CN in beta cells has also been associated with decreased beta cell function [12].

Thus far, epidemiological studies investigating the relationship between mtDNA-CN and type 2 diabetes are limited. To the best of our knowledge, only four cohort studies have investigated the association between blood mtDNA-CN and type 2 diabetes, among which three were conducted using a prospective study design [13-16]. Two out of the four studies showed that low blood mtDNA-CN was associated with an increased risk of developing type 2 diabetes. However, these studies were performed in a relatively small population with a low number of (incident) type 2 diabetes cases, which warrants replication of the findings in larger study samples. In addition, none of these studies have examined the question whether the relationship between blood mtDNA and type 2 diabetes is causal. Therefore, the aim of our study was to investigate the association between blood mtDNA-CN and

incident type 2 diabetes using data from the UK Biobank (UKB). Additionally, the bi-directional relationship between blood mtDNA-CN and type 2 diabetes and body mass index (BMI) was assessed by bi-directional two-sample Mendelian Randomization (MR) analyses [17].

Method

Prospective analyses

Study population

The data used in the present study was derived from the UKB. The UKB is a large-scale prospective cohort study containing in-depth genetic and health information from over 500,000 participants aged 40-70 years at recruitment from the United Kingdom (UK). Participants were recruited between 2006 and 2010 at 22 assessment centers across the UK. Baseline examinations in all participants included physical measurements (e.g., blood pressure), lifestyle and environmental information (e.g., diet, exercise, smoking, alcohol), biological sampling (e.g., blood and urine), medical history, etc. The UKB study was approved by the North-West Multi-center Research Ethics Committee. Access to information to invite participants was approved by the Patient Information Advisory Group from England and Wales, and National Information Governance Board for Health and Social Care. All participants in the UKB study provided written informed consent and detailed research ethics approval can be found on official website (https://www.ukbiobank.ac.uk/learn-more-about-uk-biobank/about-us/ethics). The present study was conducted using accepted proposal 22474.

The genotype data released from UKB contains genotype of 488,377 participants from diverse ancestries. A total of 195,203 participants were removed based on the following exclusion criteria: i) samples did not meet the quality control criteria (N=81,552); ii) participants are related (N=38,642); iii) participants are from non-European ancestry (N=65,498); iv) informed consents were withdrawn (N=71); v) standard deviation (SD) of autosomal probes (last step of mtDNA-CN calculation) > 0.37 (N=9,440). More details on exclusion criteria can be found elsewhere [18]. After filtering, a total of 293,174 unrelated European participants were included in the present study.

Computation of mtDNA copy number in blood

Blood mtDNA-CN, the exposure of our interest, was calculated based on the intensities of genotyping probes on the mitochondrial chromosome on the Affymetrix array in the UKB participants. The pipeline for calculating mtDNA-CN from intensities of probes mapping to the mitochondrial

genome has been described in detail previously [18, 19]. Briefly, the relative amount of mtDNA hybridized to the array at each probe was the log2 transformed ratio (L2R) of the observed genotyping probe intensity divided by the reference intensity. Initial values of mtDNA-CN were computed as the median L2R values across all 265 variants passing quality control on the mitochondrial DNA. The single mtDNA-CN estimated for each individual was derived by computation of weighted mtDNA-CN by using initial values and, normalization of mtDNA-CN to a mean of 0 and SD of 1 over 96 genotyping plates. Finally, quality control was performed by eliminating individuals with SD of autosomal probe intensities higher than 0.37. More details on calculations and used analysis pipeline of mtDNA-CN can be found online (https://github.com/GrassmannLab/MT_UKB).

Outcome definition

Prevalent and incident diagnosis of type 2 diabetes was identified in UKB as the date of first appearance of non-insulin-dependent diabetes mellitus (data-field 130708 in the UKB Database). This variable has been composed through a standard algorithm, performed by the UKB data management team, combining the data derived from hospital admissions (through linkage with the medical records from the National Health Service), general practitioners, death records, and self-report. Detailed information of the algorithm and process could be found online: https://biobank.ndph.ox.ac.uk/showcase/ukb/docs/first_occurrences_outcomes.pdf. Based on the data of first appearance of type 2 diabetes and the data of enrollment information, we defined whether a case was prevalent (before enrolment) or incident (after enrolment).

Covariates

Covariates used in the study included genotyping batch, genetic principal component 1, genetic principal component 2, white blood cell counts, platelet counts, demographic measures (age, sex), anthropometric measures (BMI in kg/m^2, waist circumference in centimeter, height in centimeter), self-reported lifestyles (use of cholesterol-lowering medication [Yes/No], smoking status [Never/Past/Current], physical activities [hours of being physically active per week]) and family (maternal + paternal) history of type 2 diabetes [Yes/No]. Information on the covariates was collected at the moment of study recruitment.

Power calculation

The statistical power in the MR analysis on type 2 diabetes was calculated using an online tool for binary outcome (https://shiny.cnsgenomics.com/mRnd/). Using the data of Diagram consortium, we had sufficient power to detect a causal association with an odds ratio of at least 1.07 per SD increase in blood mtDNA-CN using either of summary statistics from UKB and Longchamp et. al paper with significance level of 0.05 (**Electronic supplementary material (ESM) Fig. 1**)

Statistical analyses

Study characteristics for the population at the baseline were expressed as mean with SD stratified by quartiles of blood mtDNA-CN. Missing values of parameters were imputed using the package MICE which uses Bayesian polytomous regression for prediction of categorical values, predictive mean matching models for continuous missing values, and logistic regression model for prediction of binary missing values [20].

Participants without type 2 diabetes at baseline were followed until the occurrence of type 2 diabetes, mortality or loss of follow-up, whichever occurred first. Kaplan-Meier survival curves of blood mtDNA-CN in quartiles was provided. For the primary analysis, the association between blood mtDNA-CN as continuous variable on type 2 diabetes was assessed by using Cox proportional hazard model. As secondary analyses, the analyses were repeated with blood mtDNA-CN stratified into quartiles. The 1^{st} and 4^{th} quartiles represent the lowest 25% and the highest 25% of blood mtDNA-CN, respectively. Three multivariable-adjusted Cox proportional hazard models were used to analyze the association between blood mtDNA-CN and type 2 diabetes: model 1 was adjusted for genotyping batch, the first 2 genetic principal components to correct for possible population stratification, white blood cell counts, platelet counts, age and sex; model 2 was additionally adjusted for BMI (in kg/m^2); model 3 was additionally adjusted for use of cholesterol lowering medication (Yes/No), smoking status (Never/Past/Current), physical activities (hours of being physical active per week), waist circumference (Centimeter) and family history of type 2 diabetes (Yes/No).

The potential interactions between blood mtDNA-CN and sex and age were tested by adding an (multiplicative) interaction term to the fully adjusted model. In addition, we also performed prespecified subgroup analyses stratified by sex and age (<=50 years, 50~60 years, >=60 years).

In addition, multivariable-adjusted cross-sectional analyses were performed to investigate the association between blood mtDNA-CN and BMI by linear regression adjusted for genotyping batch, first 2 genetic principal components, white blood cell counts, platelet counts, age, sex, use of cholesterol lowering medication, smoking status, physical activities and family history of type 2 diabetes. Additionally, similar models were applied to investigate the association between blood mtDNA-CN and whole-body lean mass, whole-body fat mass, liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT). All parameters except blood mtDNA-CN were log-transformed to approximate a normal distribution. The linear model of blood mtDNA-CN on whole-body lean mass was adjusted for genotyping batch, the first 2 genetic principal components, white blood cell counts, platelet counts, age, sex, height and fat mass. A similar model was used to investigate the association between blood mtDNA-CN and whole-body fat mass by replacing the fat mass with lean mass. The linear models of blood mtDNA-CN on liver enzymes were adjusted for genotyping batch, the first 2 genetic principal components, white blood cell counts, platelet counts, age, sex and glomerular filtration rate. The estimates derived from all linear models were exponentiated to assess the original effect of each parameter per 1 SD increase in blood mtDNA-CN.

Bi-directional Mendelian Randomization analyses

Exposure data source

Genome-wide association study (GWAS) for blood mtDNA-CN in the unrelated European population of the UKB was performed to provide a list of independent lead single nucleotide polymorphisms (SNPs) for the following use as instrumental variables for the exposure as well as to use the same data as outcome set in the bi-directional MR. UKB genotyping and imputation data released in March 2018 was used to perform GWAS (https://www.ukbiobank.ac.uk/media/cffi4mx5/ukb-genotyping-and-imputation-data-release-faq-v3-2-1.pdf). The analyses were done on the autosomal chromosomes adjusting for age, sex, white blood cell counts,

platelet counts and the first 10 genetic principal components. We excluded SNPs with a minor allele frequency <0.01 as well as SNPs with an imputation quality <0.3. SNPs with p-values smaller than 5e-8 were extracted and stored for the MR analyses. Instrumental variables were identified after clumping process (window size = 10,000 kb, $R^2 < 0.001$) which estimates linkage disequilibrium between SNPs by using the European population from the 1000 genomes project. GWAS in the present study were performed using linear mixed models implemented in the program BOLT_LMM (version 2.3.2) [21]. Visualization of the results was performed using the R-based packages ggplot2 [22] and EasyStrata [23]. Variance in blood mtDNA-CN explained by genetic variants was calculated by using equations that have been described previously [24].

In addition, 129 significant SNPs (p<5e-8) associated with blood mtDNA-CN from the paper of *Longchamps. et. al* [25] were used as exposure as validation of our initial analyses only based on UKB data, which were provided in **ESM Table 1**. This GWAS study was performed in 465,809 European individuals from Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) and the UKB, adjusting for age, sex, principal components, DNA collection site, family structure, and cell composition.

Outcome data source

Summary statistics of type 2 diabetes were extracted from two large databases, notably the Diabetes Genetics Replication and Meta-analysis (Diagram) consortium (available at: https://www.diagram-consortium.org/downloads.html) and the FinnGen study (available at: https://finngen.gitbook.io/documentation/). The summary statistics of BMI was retrieved from The Genetic Investigation of Anthropometric Traits (GIANT) consortium, which is available in the IEU GWAS database (available at: https://gwas.mrcieu.ac.uk/datasets/ieu-b-40/). More information of these data sources can be found in **ESM Table 2**.

MR analyses

Two-sample MR analyses were performed using the R-based statistical package TwoSampleMR (available at: http://github.com/MRCIEU/TwoSampleMR) [26]. This package is able to

harmonize the effect sizes of SNPs for exposure and outcome, and connects to database with thousands of complete GWAS summary data.

The study design for MR analyses is shown in **Figure 1**. In the first set of two-sample MR analyses, we considered blood mtDNA-CN (UKB) (GWAS was performed in the present study) as exposure, and type 2 diabetes (DIAGRAM and FinnGen) and BMI (GIANT) as outcome. Analyses were repeated using data from the *Longchamps et al* paper as exposure [25]. The second set of MR analyses handled reverse causality taking type 2 diabetes (DIAGRAM) and BMI (GIANT) as exposure, and blood mtDNA-CN (UKB) from GWAS as outcome.

The primary MR analysis was conducted using the inverse variance weighted (IVW) method. The magnitudes of the causal effect were estimated by weighted regression of the average SNP-outcome effect on the average SNP-exposure effect with the intercept constrained to zero [27]. Given its assumption that all genetic instruments included are valid, this method could yield biased estimates in the presence of unbalanced horizontal pleiotropy, especially in case the analysis contained many genetic instruments. Therefore, we also performed two additional sensitivity analyses by using other MR models, the weighted median estimator approach and the MR Egger. The weighted median estimator approach implemented the weighted median of the Wald ratios for all instrumental variables, which is able to tolerate up to (not including) 50% of the weight coming from invalid instrumental variables [28]. The MR Egger method is sensitive to horizontal pleiotropy and the estimated value of intercept can be interpreted as an estimate of average pleiotropic effect across the genetic variants [29]. Results for MR analyses were reported in either odds ratio for binary outcomes or estimates for continuous outcomes with accompanied 95% confidence interval.

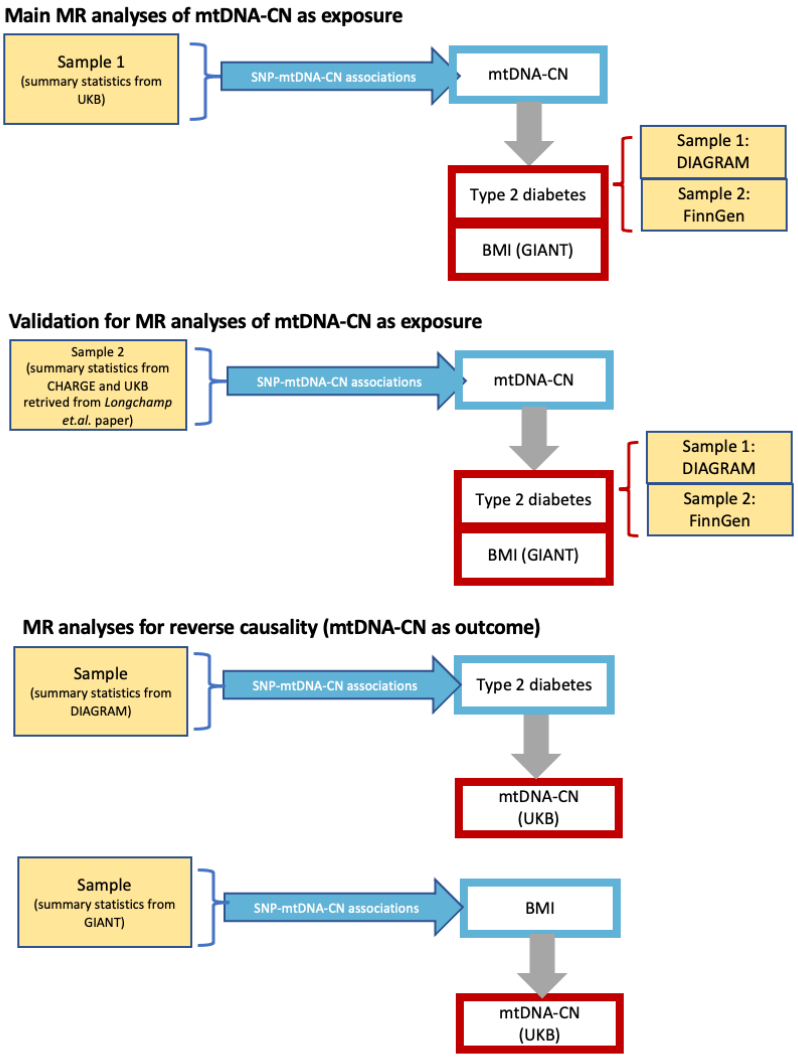


Figure 1 Study design diagram for bi-directional MR analyses. Gray arrows present MR effect estimates, traits in blue rectangles present exposures, traits in red rectangles present outcomes and GWAS data used for traits presented in yellow rectangles. mtDNA-CN: mitochondrial DNA copy number; BMI: body mass index; DIAGRAM: Diabetes Genetics Replication and Meta-analysis consortium; GIANT: The Genetic Investigation of Anthropometric Traits consortium.

Results

Study population

The multivariable-adjusted analyses were conducted in a final sample of 285,967 individuals after excluding 7,207 individuals with type 2 diabetes at baseline. During the follow-up (median follow-up time: 11.9 years; interquartile range: 11.2-12.6 years), 15,111 individuals developed type 2 diabetes. The characteristics of participants stratified by quartile of blood mtDNA-CN after imputation are shown in **Table 1** and the original baseline characteristics before imputation are shown in **ESM Table 3**. The mean age for the whole population is 56.8 (SD = 8.00) years and 54.1% participants are women.

Multivariable-adjusted analyses on incident type 2 diabetes

The Kaplan-Meier curve (**Figure 2A**) illustrated that participants with a higher level of blood mtDNA-CN had a proportionally lower risk of incident type 2 diabetes.

The results from the multivariable-adjusted prospective analyses are presented in **Figure 2B** and **ESM Table 4**. The multivariable-adjusted Cox proportional models show the higher level of blood mtDNA-CN is associated with lower risk of developing type 2 diabetes with hazard ratio (HR) of 0.90 (95% Confidence Interval (CI): 0.89-0.92) for model 1, 0.95 (95% CI: 0.93-0.97) for model 2 and 0.95 (95% CI: 0.94-0.97) for model 3 per 1 SD increase in blood mtDNA-CN. Similarly, taking the participants in the 4th quartile as reference group, model 1 shows participants in quartile 1 for blood mtDNA-CN had the highest risk to develop type 2 diabetes (HR: 1.30; 95% CI: 1.24-1.36); in addition, both quartile 2 and quartile 3 had higher risk of incident type 2 diabetes compared with the reference group (respectively, HR: 1.17; 95% CI: 1.12-1.23, and HR: 1.08; 95% CI: 1.03-1.13). Results did somewhat attenuate after adjustment for BMI (Model 2), and remained similar after further adjustment for other considered confounders (Model 3).

We did not observe any interaction between blood mtDNA_CN with sex or age with p-values for interaction of 0.06 and 0.61, respectively, as was also observed in subgroup analyses (**ESM Table 5**, **ESM Table 6**).

Higher blood mtDNA-CN was associated with lower BMI (-0.12 (95% CI: -0.14 ~ -0.10) kg/m2) in multivariable-adjusted cross-sectional analyses. The results from linear models of blood mtDNA-CN with whole-body lean mass, whole-body fat mass and liver enzymes are shown in **ESM Table 7**. Blood mtDNA-CN was not associated with whole-body lean mass, ALP, AST and ALT. A 1% decrease in whole-body fat mass and GGT were observed per SD increase in blood mtDNA-CN.

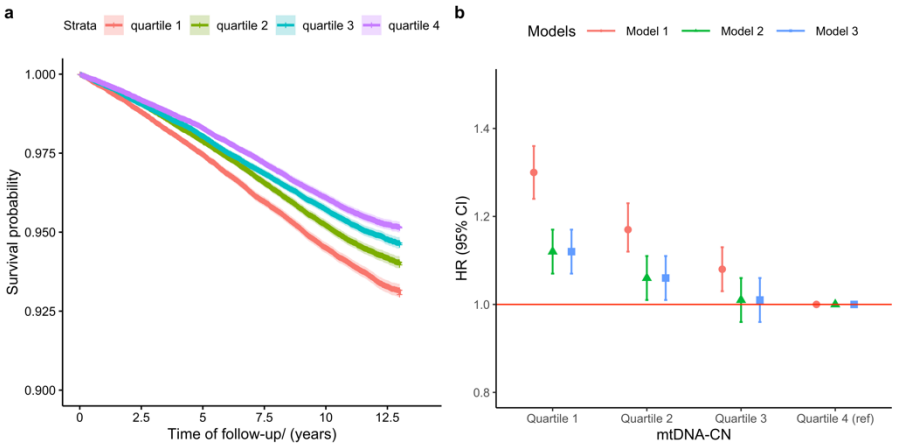


Figure 2 (**A**) Kaplan-Meier curve presenting time to develop incident type 2 diabetes by quartile of mtDNA copy number. X-axis represents follow-up time in years. Y-axis represents the overall survival probability of developing type 2 diabetes. (**B**) Hazard ratio for incident type 2 diabetes by quartiles of mtDNA copy number. Model 1 was adjusted for genotyping batch, principal component 1, principal component 2, white blood cell counts, platelet counts, age and sex; model 2 was additionally adjusted for BMI based on model 1; model 3 was additionally adjusted for BMI, medication use for cholesterol, smoking status, physical activities, waist circumference and family history of type 2 diabetes based on model 1.

Bi-directional Mendelian Randomization analyses

In our newly-conducted GWAS of blood mtDNA-CN in UKB, 3075 significant SNPs (P < 5e-8) were identified, of which 55 were independent lead SNPs (R^2<0.001), which were used in the MR analyses as exposure (**ESM Table 8**). These independent lead SNPs explained 1.3% of variance in blood mtDNA-CN levels. The -log10(p-value) plot and QQ plot for GWAS of blood mtDNA-CN are presented in **ESM Fig. 2**.

The results from the MR analyses taking 55 SNPs of blood mtDNA-CN from the UKB as exposure are shown in **Figure 3** and **ESM Table 9**. We observed that the odds ratio per SD increase of blood mtDNA-CN on type 2 diabetes using the IVW method was 1.07 (95% CI: 0.89-1.29) for DIAGRAM as outcome data source and 1.11 (95% CI: 0.90-1.38) for FinnGen as outcome data source. Similar results were obtained when we used the previously identified SNPs from the larger *Longchamps et al* study [25] as exposure (**ESM Fig. 3** and **ESM Table 9**) which explained 2.2% of variance in blood mtDNA-CN levels. The results from the MR analyses of blood mtDNA-CN on BMI using either UKB data or data from *Longchamps et al*. as exposure gave similar results indicating no evidence for a causal association between blood mtDNA-CN and BMI (**Figure 4** and **ESM Table 10**). The results from weighted median and MR Egger were consistent with the IVW method in the above analyses.

The reverse MR was applied to investigate the potential causal association between genetically-influenced BMI and type 2 diabetes on blood mtDNA-CN, respectively. **Figure 5** illustrated the estimates of type 2 diabetes on blood mtDNA-CN and BMI on blood mtDNA-CN. For both exposures, we did not find evidence for a causal association, and consistent results were derived in the weighted median and MR Egger analyses (**ESM Table 11**).

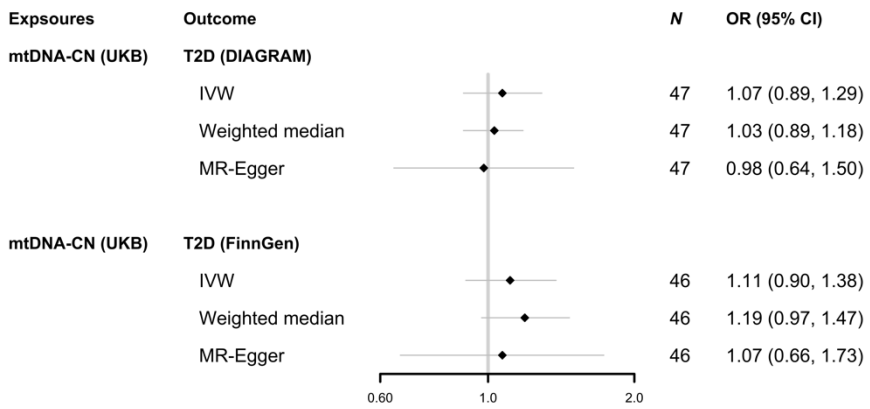


Figure 3 Forest plot of MR results for mtDNA-CN (UKB) and type 2 diabetes (DIAGRAM and FinnGen). N: number of SNPs analyzed in the MR analyses; OR: odds ratio per 1 SD increase in mtDNA-CN; 95% CI: 95% confidence interval; IVW: inverse variance weighted.

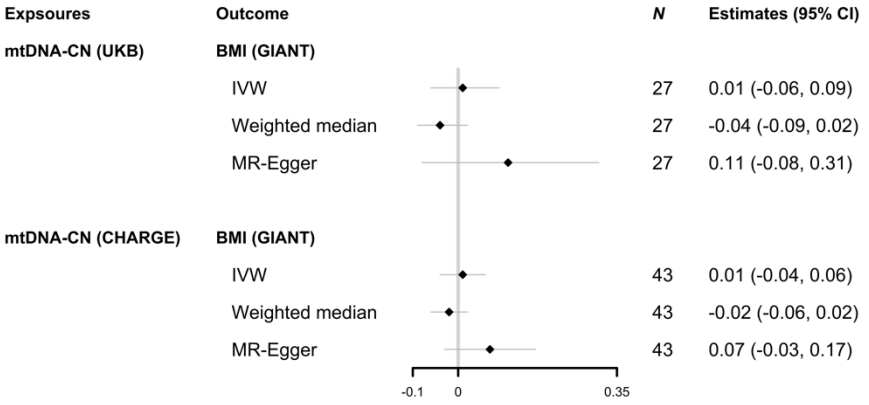


Figure 4 Forest plot of MR results for mtDNA-CN (UKB and CHARGE) and BMI (GIANT). N: number of SNPs analyzed in the MR analyses; Estimates are reported per 1 SD increase in mtDNA-CN; 95% CI: 95% confidence interval; IVW: inverse variance weighted.

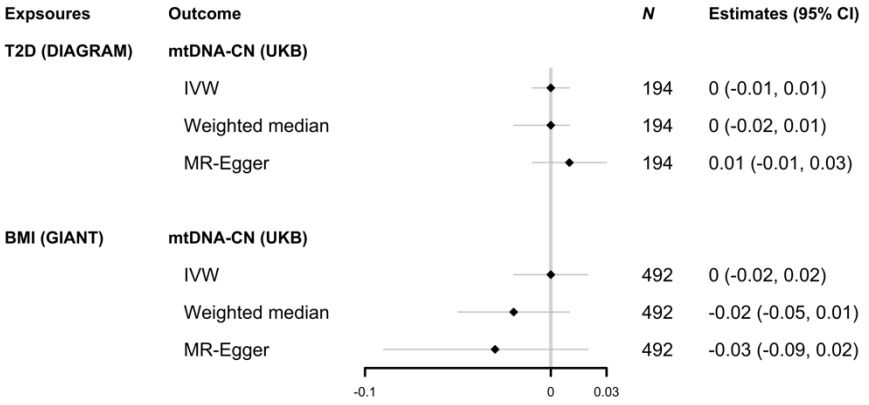


Figure 5 Forest plot of MR results for BMI (GIANT) and type 2 diabetes (DIAGRAM) associating with mtDNA-CN (UKB). N: number of SNPs analyzed in the MR analyses; Estimates were reported as having type 2 diabetes versus not having type 2 diabetes for MR analyses between type 2 diabetes and mtDNA-CN; Estimates were reported as per 1 kg/m2 increase in BMI for MR analyses between BMI and mtDNA-CN; 95% CI: 95% confidence interval; IVW: inverse variance weighted.

Table 2 Baseline characteristics of the population by quartile of mtDNA copy number

	Quartile 1 (N=71492)	Quartile 2 (N=71492)	Quartile 3 (N=71491)	Quartile 4 (N=71492)	Overall (N=285967)
mtDNA-CN (SD)	-1.12 [-5.09, -0.658]	-0.313 [-0.658, -0.00423]	0.303 [-0.00420, 0.656]	1.14 [0.656, 4.68]	-0.00423 [-5.09, 4.68]
Sex (Female)	37587 (52.6%)	38340 (53.6%)	38911 (54.4%)	39801 (55.7%)	154639 (54.1%)
Age (years)	57.4 (8.01)	56.9 (8.00)	56.6 (7.97)	56.1 (7.97)	56.8 (8.00)
White blood cell count	7.36 (1.83)	6.96 (1.72)	6.72 (1.70)	6.40 (2.50)	6.86 (1.99)
Platelet count	247 (57.8)	252 (57.8)	256 (59.0)	259 (62.9)	253 (59.6)
BMI (kg/m^2)	27.6 (4.88)	27.4 (4.66)	27.2 (4.57)	26.9 (4.47)	27.3 (4.65)
Waist circumference (cm)	91.0 (13.6)	90.3 (13.3)	89.7 (13.1)	88.8 (12.9)	90.0 (13.2)
Whole-body fat mass (kg)	25.2 (9.80)	24.8 (9.41)	24.5 (9.26)	24.0 (9.05)	24.6 (9.39)
Whole-body lean mass (kg)	53.7 (11.5)	53.5 (11.5)	53.2 (11.4)	53.0 (11.4)	53.3 (11.5)
Physical activity (hours/week)	26.4 (33.9)	27.0 (34.4)	26.9 (33.9)	27.1 (34.0)	26.8 (34.1)
Cholesterol-lowering medicine	11941 (16.7%)	11468 (16.0%)	10974 (15.4%)	10430 (14.6%)	44813 (15.7%)

	Quartile 1 (N=71492)	Quartile 2 (N=71492)	Quartile 3 (N=71491)	Quartile 4 (N=71492)	Overall (N=285967)
Smoking status					
Never	38362 (53.7%)	39170 (54.8%)	39715 (55.6%)	40525 (56.7%)	157772 (55.2%)
Previous	25027 (35.0%)	24996 (35.0%)	24806 (34.7%)	24904 (34.8%)	99733 (34.9%)
Current	8103 (11.3%)	7326 (10.2%)	6970 (9.7%)	6063 (8.5%)	28462 (10.0%)
Family history of T2D	11262 (15.8%)	10959 (15.3%)	11125 (15.6%)	11156 (15.6%)	44502 (15.6%)
Follow-up time (years)	11.2 (2.30)	11.3 (2.12)	11.4 (2.06)	11.5 (1.99)	11.4 (2.13)
Height (cm)	169 (9.21)	169 (9.24)	169 (9.26)	169 (9.25)	169 (9.24)
ALT (U/L)	20.3 [3.10, 495]	20.2 [3.18, 490]	20.1 [3.25, 425]	19.7 [3.10, 491]	20.1 [3.10, 495]
ALP (U/L)	81.4 [8.00, 1360]	80.4 [14.8, 1420]	79.7 [14.2, 1360]	78.9 [14.1, 1420]	80.1 [8.00, 1420]
AST (U/L)	24.5 [5.10, 947]	24.4 [3.30, 711]	24.4 [5.10, 572]	24.3 [4.40, 584]	24.4 [3.30, 947]
GGT (U/L)	26.9 [5.50, 1170]	26.1 [5.10, 1120]	25.8 [5.00, 1160]	25.1 [5.20, 1160]	26.0 [5.00, 1170]

Continuous variables were presented as mean with standard deviation (SD) or median with minimum and maximum values, and categorial variables were presented as number with percentages. BMI: body mass index; T2D: type 2 diabetes; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyl transfer

Discussion and Conclusion

We found a lower level of blood mtDNA-CN to be associated with higher risk of incident type 2 diabetes. A weak association between blood mtDNA-CN and lower BMI was also found. However, no associations were observed between lower genetically-influenced blood mtDNA-CN and higher type 2 diabetes risk nor higher BMI, suggesting that the observed associations are likely not causal. Also in the reverse direction, no evidence was found for a causal association of type 2 diabetes and of BMI with blood mtDNA-CN. Further regression analyses showed that blood mtDNA-CN was not associated with whole-body lean mass, AST, ALT and ALP, but was associated with whole-body fat mass and GGT, however, with very small observed effect sizes.

Three previous follow-up studies have investigated the association between blood mtDNA-CN and incident type 2 diabetes [13, 15, 16]. Our findings are in line with the results of *Memon et.al.* who found a lower level of blood mtDNA-CN to be associated with increased risk of incident type 2 diabetes in a follow-up study of 2387 middle-aged Swedish women [13]. However, research conducted in the Atherosclerosis Risk in Communities (ARIC) study found that mtDNA-CN in circulating leukocytes was not associated with risk of developing type 2 diabetes among 7713 white participants [15]. Same results as ARIC study were reported by *Erwin Reiling* using European participants from Botnia and Rotterdam studies [16]. Our finding of an association of higher blood mtDNA-CN with lower BMI is similar to the findings of *Liu Xue et.al* [30] and *Skuratovskaia et. al.* [31]. The divergent results on type 2 diabetes may be due to the different characteristics of the populations, different covariates used in the Cox models, the data source of type 2 diabetes and the measurement methods for mtDNA-CN. However, the extremely large UK biobank cohort make it possible to observe the small effect sizes of the associations and the discrepancy may be explained by the differences in power.

We found no evidence for the hypothesis that low blood mtDNA-CN drives the risk of type 2 diabetes and obesity from the results of the MR analyses. This hypothesis is based on the assumption that blood mtDNA-CN reflects overall mitochondrial function including the mitochondrial function of tissues that play a role in type 2 diabetes like muscle and liver. We further elaborated on this assumption by performing linear regression analyses between blood mtDNA-CN and whole-body lean and fat mass, and liver

enzymes. We found one SD increase in blood mtDNA-CN led to no change in lean mass, AST, ALT and ALP, and small decreases in GGT. This indicates that blood mtDNA-CN may not be a marker for mitochondrial function in muscle and a weak marker for mitochondrial function in liver. Although one study in monkeys reported that mitochondrial function in blood was associated with mitochondrial function in muscle [32], human trials have failed to show the correlation between mitochondrial respiration in peripheral blood and muscle in both women and men. This is in agreement with our finding that blood mtDNA-CN may not be a good marker for muscle mitochondrial function [33, 34]. The associations that have been observed between mtDNA-CN and liver disease are not consistent. Increased liver mtDNA-CN was found in patients with non-alcoholic fatty liver disease [35, 36], while decreased blood and liver mtDNA-CN was found in patients with biliary atresia [37, 38]. Although blood mtDNA-CN was associated with liver enzymes GGT in our study, given the small effect sizes, the blood mtDNA-CN is a weak marker for mitochondrial functional in the liver.

Our data showed a clear observational association between blood mtDNA-CN and incident type 2 diabetes. Since the MR analyses did not find evidence for causality, it was expected that adjusting the observational association for BMI and other considered confounders would significantly attenuate the association. However, this was not the case and only adjusting for BMI moderately attenuated the association. The most likely explanation is that the remaining association is due to residual confounding. In addition to unmeasured residual confounding, some measured variables may not be very precise. For example, the physical activities were collected based on the recall of participants, which may not be accurate.

Our study has some strengths and limitations. One of the strengths of our study is that we included 285,967 unrelated European individuals without type 2 diabetes, which is a large sample size and large number (N=15,111) of incident type 2 diabetes cases. To our knowledge, this is the largest investigation on blood mtDNA-CN and type 2 diabetes. One of the limitations was that blood mtDNA-CN may not be a reflection of mitochondrial function in muscle and liver, which are relevant in type 2 diabetes pathogenesis, and therefore blood mtDNA-CN might not be a suitable marker for the involvement of these tissues in type 2 diabetes. Another limitation is that we only included European populations and the results cannot be extrapolated to other populations with different ancestries. In addition, there was some overlap in the data sources for exposure and

outcome in the two-sample MR analyses. However, Minelli *et.al* provide evidence that two-sample MR methods perform well in large one-sample MR studies even when the assumption of independence does not hold. Only results derived with the MR-Egger regression analysis should be interpreted with caution [39].

In conclusion, our results showed that blood mtDNA-CN was prospectively associated with incident type 2 diabetes, but MR analyses did not provide evidence for causality.

Reference

[1]Zheng Y, Ley SH, Hu FB (2018) Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nature Reviews Endocrinology 14(2): 88-98. 10.1038/nrendo.2017.151
[2]Wu Y, Ding Y, Tanaka Y, Zhang W (2014) Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. Int J Med Sci 11(11): 1185-1200. 10.7150/ijms.10001
[3]Montgomery MK, Turner N (2015) Mitochondrial dysfunction and insulin resistance: an update. Endocrine Connections 4(1): R1-R15. 10.1530/EC-14-0092
[4]Dimauro S, Davidzon G (2005) Mitochondrial DNA and disease. Ann Med 37(3): 222-232. 10.1080/07853890510007368
[5]Kraja AT, Liu C, Fetterman JL, et al. (2019) Associations of Mitochondrial and Nuclear Mitochondrial Variants and Genes with Seven Metabolic Traits. Am J Hum Genet 104(1): 112-138. 10.1016/j.ajhg.2018.12.001
[6]Castellani CA, Longchamps RJ, Sun J, Guallar E, Arking DE (2020) Thinking outside the nucleus: Mitochondrial DNA copy number in health and disease. Mitochondrion 53: 214-223. https://doi.org/10.1016/j.mito.2020.06.004
[7]Ashar FN, Zhang Y, Longchamps RJ, et al. (2017) Association of Mitochondrial DNA Copy Number With Cardiovascular Disease. JAMA Cardiology 2(11): 1247-1255. 10.1001/jamacardio.2017.3683
[8]Reznik E, Miller ML, Şenbabaoğlu Y, et al. (2016) Mitochondrial DNA copy number variation across human cancers. Elife 5. 10.7554/eLife.10769
[9]Lee HK, Song JH, Shin CS, et al. (1998) Decreased mitochondrial DNA content in peripheral blood precedes the development of non-insulin-dependent diabetes mellitus. Diabetes Res Clin Pract 42(3): 161-167. 10.1016/s0168-8227(98)00110-7
[10]Kaaman M, Sparks LM, van Harmelen V, et al. (2007) Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. Diabetologia 50(12): 2526-2533. 10.1007/s00125-007-0818-6
[11]Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE (2005) Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes 54(1): 8-14. 10.2337/diabetes.54.1.8
[12]Nile DL, Brown AE, Kumaheri MA, et al. (2014) Age-related mitochondrial DNA depletion and the impact on pancreatic Beta cell function. PLoS One 9(12): e115433. 10.1371/journal.pone.0115433
[13]Memon AA, Sundquist J, Hedelius A, Palmér K, Wang X, Sundquist K (2021) Association of mitochondrial DNA copy number with prevalent and incident type 2 diabetes in women: A population-based follow-up study. Sci Rep 11(1): 4608. 10.1038/s41598-021-84132-w
[14]Fazzini F, Lamina C, Raftopoulou A, et al. Association of mitochondrial DNA copy number with metabolic syndrome and type 2 diabetes in 14 176 individuals. Journal of Internal Medicine n/a(n/a). https://doi.org/10.1111/joim.13242
[15]DeBarmore B, Longchamps RJ, Zhang Y, et al. (2020) Mitochondrial DNA copy number and diabetes: the Atherosclerosis Risk in Communities (ARIC) study. BMJ Open Diabetes Research & Care 8(1): e001204. 10.1136/bmjdrc-2020-001204
[16]Reiling E, Ling C, Uitterlinden AG, et al. (2010) The association of mitochondrial content with prevalent and incident type 2 diabetes. J Clin Endocrinol Metab 95(4): 1909-1915. 10.1210/jc.2009-1775
[17]Emdin CA, Khera AV, Kathiresan S (2017) Mendelian randomization. Jama 318(19): 1925-1926
[18]Luo J, Noordam R, Jukema JW, et al. (2021) Low mitochondrial copy number drives atherogenic cardiovascular disease: evidence from prospective cohort analyses

in the UK Biobank combined with Mendelian Randomization. medRxiv: 2021.2007.2001.21259854. 10.1101/2021.07.01.21259854
[19]Hägg S, Jylhävä J, Wang Y, Czene K, Grassmann F (2021) Deciphering the genetic and epidemiological landscape of mitochondrial DNA abundance. Human Genetics 140(6): 849-861. 10.1007/s00439-020-02249-w
[20]van Buuren S, Groothuis-Oudshoorn K (2011) mice: Multivariate Imputation by Chained Equations in R. Journal of Statistical Software 45(3): 1 - 67. 10.18637/jss.v045.i03
[21]Loh PR, Tucker G, Bulik-Sullivan BK, et al. (2015) Efficient Bayesian mixed-model analysis increases association power in large cohorts. Nat Genet 47(3): 284-290. 10.1038/ng.3190
[22]Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York
[23]Winkler TW, Kutalik Z, Gorski M, Lottaz C, Kronenberg F, Heid IM (2015) EasyStrata: evaluation and visualization of stratified genome-wide association meta-analysis data. Bioinformatics (Oxford, England) 31(2): 259-261. 10.1093/bioinformatics/btu621
[24]Teslovich TM, Musunuru K, Smith AV, et al. (2010) Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466(7307): 707-713. 10.1038/nature09270
[25]Longchamps R, Yang S, Castellani C, et al. (2021) Genetic analysis of mitochondrial DNA copy number and associated traits identifies loci implicated in nucleotide metabolism, platelet activation, and megakaryocyte proliferation, and reveals a causal association of mitochondrial function with mortality. bioRxiv: 2021.2001.2025.428086. 10.1101/2021.01.25.428086
[26]Hemani G, Tilling K, Davey Smith G (2017) Orienting the causal relationship between imprecisely measured traits using GWAS summary data. PLoS Genet 13(11): e1007081. 10.1371/journal.pgen.1007081
[27]Bowden J, Del Greco M F, Minelli C, Davey Smith G, Sheehan N, Thompson J (2017) A framework for the investigation of pleiotropy in two-sample summary data Mendelian randomization. Statistics in Medicine 36(11): 1783-1802. https://doi.org/10.1002/sim.7221
[28]Burgess S, Bowden J, Fall T, Ingelsson E, Thompson SG (2017) Sensitivity Analyses for Robust Causal Inference from Mendelian Randomization Analyses with Multiple Genetic Variants. Epidemiology 28(1): 30-42. 10.1097/EDE.0000000000000559
[29]Burgess S, Thompson SG (2017) Interpreting findings from Mendelian randomization using the MR-Egger method. European journal of epidemiology 32(5): 377-389. 10.1007/s10654-017-0255-x
[30]Liu X, Longchamps RJ, Wiggins KL, et al. (2021) Association of mitochondrial DNA copy number with cardiometabolic diseases. Cell Genomics 1(1): 100006. https://doi.org/10.1016/j.xgen.2021.100006
[31]Skuratovskaia DA, Sofronova JK, Zatolokin PA, et al. (2018) Additional evidence of the link between mtDNA copy number and the body mass index. Mitochondrial DNA Part A 29(8): 1240-1244. 10.1080/24701394.2018.1436170
[32]Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Molina AJA (2016) Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: Implications for a minimally invasive biomarker of mitochondrial health. Redox Biology 10: 65-77. https://doi.org/10.1016/j.redox.2016.09.009
[33]Rose S, Carvalho E, Diaz EC, et al. (2019) A comparative study of mitochondrial respiration in circulating blood cells and skeletal muscle fibers in women. Am J Physiol Endocrinol Metab 317(3): E503-e512. 10.1152/ajpendo.00084.2019
[34]Hedges CP, Woodhead JST, Wang HW, et al. (2019) Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation

to high-intensity interval training in healthy young men. J Appl Physiol (1985) 126(2): 454-461. 10.1152/japplphysiol.00777.2018
[35]Kamfar S, Alavian SM, Houshmand M, et al. (2016) Liver Mitochondrial DNA Copy Number and Deletion Levels May Contribute to Nonalcoholic Fatty Liver Disease Susceptibility. Hepat Mon 16(12): e40774-e40774. 10.5812/hepatmon.40774
[36]Ma C, Liu Y, He S, et al. (2020) Association Between Leukocyte Mitochondrial DNA Copy Number and Non-alcoholic Fatty Liver Disease in a Chinese Population Is Mediated by 8-Oxo-2'-Deoxyguanosine. Front Med (Lausanne) 7: 536. 10.3389/fmed.2020.00536
[37]Udomsinprasert W, Poovorawan Y, Chongsrisawat V, Vejchapipat P, Jittikoon J, Honsawek S (2019) Leukocyte mitochondrial DNA copy number as a potential biomarker indicating poor outcome in biliary atresia and its association with oxidative DNA damage and telomere length. Mitochondrion 47: 1-9. https://doi.org/10.1016/j.mito.2019.04.006
[38]Tiao M-M, Lin T-K, Kuo F-Y, et al. (2007) Early Stage of Biliary Atresia Is Associated With Significant Changes in 8-Hydroxydeoxyguanosine and Mitochondrial Copy Number. Journal of Pediatric Gastroenterology and Nutrition 45(3)
[39]Minelli C, Del Greco M F, van der Plaat DA, Bowden J, Sheehan NA, Thompson J (2021) The use of two-sample methods for Mendelian randomization analyses on single large datasets. International Journal of Epidemiology. 10.1093/ije/dyab084