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Mitochondrial DNA copy number (mtDNA-CN) in human health and disease: a systematic re-appraisal and critical evaluation of published meta-analyses

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Giacon, M; Terrazzino, S.

Corresponding author:
Salvatore Terrazzino

salvatore.terrazzino@uniupo.it

Author Affiliation:
Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy.

ADMINISTRATIVE INFORMATION

Support - No support.

Review Stage at time of this submission - Preliminary searches.

Conflicts of interest - None declared.

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Amendments - This protocol was registered with the International Platform of Registered Systematic Review and Meta-Analysis Protocols (INPLASY) on 22 July 2026 and was last updated on 22 July 2026.

INTRODUCTION

Review question / Objective The primary objective of this study is to conduct a systematic re-appraisal and evidence synthesis of published meta-analyses investigating the association between mitochondrial DNA copy number (mtDNA-CN) and various clinical outcomes (e.g., oncology, cardiology, neuropsychiatry, and environmental toxicology).
To move beyond a descriptive overview, this study implements a dual-framework evaluation to provide a formal grading of the current literature:
1. Methodological Quality Assessment: To evaluate the structural rigor and internal validity of the included systematic reviews and meta-analyses using the AMSTAR-2 (A MeaSurement Tool to Assess Systematic Reviews) instrument, identifying critical flaws in the review processes.
2. Epidemiological Credibility Grading: To assess the robustness of the reported clinical associations by applying the Venice criteria, which stratifies evidence into a hierarchy (Strong, Moderate, or Weak credibility) based on the amount of evidence, replication of results, and protection from bias.
Ultimately, this re-appraisal will provide a comprehensive mapping of the current evidence landscape, stratifying the reported associations between mtDNA-CN and clinical phenotypes based on their methodological rigor and scientific certainty. By doing so, the study aims to distinguish robust genetic-clinical associations from those requiring further validation due to inherent limitations in the primary meta-analyses.

Rationale Mitochondrial DNA copy number (mtDNA-CN) has gained significant attention as a non-invasive surrogate biomarker of mitochondrial function and cellular aging. Over the last decade, numerous meta-analyses have been published across diverse medical fields, often claiming significant associations between mtDNA-CN levels and disease risk or progression. However, the field is characterized by high methodological heterogeneity, varying tissue types (e.g., whole blood, leukocytes, saliva), and inconsistent reporting of statistical metrics such as publication

bias and prediction intervals. Currently, there is a lack of a systematic "overview of reviews" that applies a standardized grading system to evaluate the credibility of these findings. This re-appraisal is necessary to distinguish robust genetic-clinical associations from those potentially inflated by small-study effects or poor methodological quality.

Condition being studied Variation in mitochondrial DNA copy number (mtDNA-CN) in relation to human health and disease. The study covers multiple clinical macro-categories, including oncology (e.g. risk and prognosis of solid and hematological tumors), cardiology (e.g. cardiovascular events, heart failure, and mortality), neuropsychiatry (e.g. autism spectrum disorder, ADHD, and neurodegenerative diseases), and environmental toxicology (e.g. exposure to particulate matter and pollutants).

METHODS

Search strategy A systematic search will be conducted in PubMed, Web of Knowledge (ISI), Scopus, Cochrane Library, and OpenGrey. The search terms will include: ("mitochondrial DNA copy number" OR "mtDNA-CN" OR "mtDNA content" OR "mitochondrial DNA amount") AND ("meta-analysis" OR "systematic review"). No language or date restrictions will be applied. The reference lists of all identified papers will be manually screened for additional relevant studies. The screening and selection of studies will be performed independently by two reviewers (M.G. and S.T.), with any discrepancies resolved through discussion and consensus.

Participant or population Human participants (all age groups) included in published systematic reviews and meta-analyses.

Intervention The "exposure" is defined as the quantification of mitochondrial DNA copy number (mtDNA-CN), which serves as a surrogate biomarker of mitochondrial density and function. Measurement methods typically include quantitative PCR (qPCR), droplet digital PCR (ddPCR), or bioinformatic derivation from high-throughput sequencing datasets, such as Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES). This exposure encompasses measurements performed on various human biological samples, most commonly peripheral blood (including whole blood, buffy coat, or leukocytes), but also saliva, muscle tissue, or other clinical specimens as reported in the included meta-analyses. Technically, mtDNA-CN is expressed as the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) content, reflecting the average number of mitochondrial genomes per cell. The specific nuclear reference genes used for normalization (e.g., ALB, B2M, RPP30, HGB) will be recorded to evaluate potential sources of technical variability.

Comparator The comparison is based on the contrast established in the primary meta-analyses, incorporating both dichotomous and continuous variables. This encompasses categorical comparisons, such as high versus low mtDNA-CN quantiles (e.g., tertiles or quartiles), as well as continuous assessments of the difference in mean mtDNA-CN levels between clinically diagnosed cases and healthy controls. The comparator thus reflects the specific statistical approach of each included study, whether treating mtDNA-CN as a binary exposure or as a continuous measure of mitochondrial density.

Study designs to be included Systematic reviews with quantitative synthesis (meta-analysis) based on observational studies (cohort, case-control, cross-sectional).

Eligibility criteria Eligibility will be defined according to the PECO (Population, Exposure, Comparator, Outcome) and Study Design framework:
Inclusion Criteria:
Population (P): Human participants of any age or clinical condition included in published systematic reviews.
Exposure (E): Measurement of mitochondrial DNA copy number (mtDNA-CN) via qPCR, ddPCR, or bioinformatic derivation from sequencing datasets (WGS/WES).
Comparator (C): Contrast groups as defined in the primary meta-analyses, such as high versus low mtDNA-CN levels or clinical cases versus healthy controls.
Outcome/Phenotype (O): Any clinical phenotype or outcome, encompassing both disease status (e.g., the presence or absence of a specific clinical condition in case-control meta-analyses) and longitudinal events, including but not limited to disease risk, prognosis, mortality, or markers of disease progression across oncology, cardiology, neuropsychiatry, and environmental toxicology.
Study Design: Systematic reviews with quantitative synthesis (meta-analysis) based on observational studies (cohort, case-control, or cross-sectional). Studies must report pooled effect sizes (OR, HR, RR, MD, or SMD) with corresponding 95% confidence intervals and p-values.
Exclusion Criteria:

Narrative or Qualitative Reviews: Any review or systematic review that does not provide quantitative meta-analytical data.
Preclinical Studies: Animal-based or in vitro studies.
Mendelian Randomization (MR) studies: MR studies will be excluded as they utilize genetic variants as instrumental variables to infer causality rather than evaluating direct observational associations. This methodology is fundamentally different and unsuitable for standardized credibility assessment via the Venice criteria in this specific re-appraisal framework.

Information sources PubMed, Web of Knowledge (ISI), Scopus, Cochrane Library, OpenGrey, and reference lists of included articles.

Main outcome(s) The primary outcomes are the pooled effect sizes (e.g., OR, HR, RR, MD, or SMD) and their corresponding 95% Confidence Intervals (CIs) extracted from the "overall analysis" of each included meta-analysis. These metrics will be used to characterize the direction and magnitude of the association between mtDNA-CN and the specific clinical phenotypes under study. While the Venice criteria provide a framework for grading the epidemiological credibility of nominally significant associations, the pooled effect sizes and their 95% CIs represent the core clinical findings for all included meta-analyses, allowing for an assessment of the clinical relevance and precision regardless of their statistical significance.

Additional outcome(s) Secondary outcomes include the methodological quality ratings derived from the AMSTAR-2 tool, as well as the statistical metrics required for the assessment of epidemiological credibility. Specifically, we will extract measures of inter-study heterogeneity (I2 and Phet) and indicators of publication bias (Egger's regression test p-value) for every meta-analysis, regardless of its statistical significance. These parameters will serve as the technical basis for the grading of epidemiological credibility via the Venice criteria (where applicable for significant associations) and will be reported for all other meta-analyses as descriptive data in supplementary tables.

Data management Two independent reviewers (M.G. and S.T.) will extract all relevant statistics using a standardized data extraction form, with any discrepancies or disagreements resolved through discussion and consensus. For each meta-analysis, we will extract the pooled effect size (OR, HR, RR, MD, or SMD), 95% CI, p-value, I2 (heterogeneity), and Egger's test p-value. In addition to statistical outcomes, descriptive metadata will be extracted for each study, including the biological matrix (e.g., whole blood, buffy coat, or leukocytes), the specific nuclear reference gene used for normalization, and the last search date to assess the up-to-dateness of the evidence. Furthermore, we will record whether the primary meta-analyses provided estimates adjusted for key biological and technical confounders, such as age, sex, and white blood cell (WBC) counts.
In instances where the Egger's test p-value is not reported in the text but a Forest Plot is available, we will implement a systematic recovery protocol:
1. Extraction: Individual study data (point estimates and 95% CIs) will be extracted directly from the Forest Plot images.
2. Validation: To ensure the accuracy of the extraction process, the overall pooled effect size and 95% CI will be recalculated using the extracted study-level data.
3. Recalculation: The missing Egger's regression test for publication bias will be calculated by reconstructing the meta-analysis from the extracted study-level estimates. The resulting Egger's p-value will be used for the Venice criteria assessment only if the reconstructed pooled estimate matches the original reported value.
4. Missing Data Policy: If the meta-analysis cannot be reconstructed due to missing or insufficient study-level data (e.g., unreadable Forest Plots or lack of numerical estimates), or if the recalculated estimate does not match the original reported value, the Egger's test will be marked as "NR" (Not Reported). In such cases, the "Protection from Bias" domain of the Venice criteria will be graded conservatively as "C".
A systematic cross-check of primary studies included in overlapping meta-analyses will be performed to identify cases of redundancy. This ensures that the evidence synthesis and Venice grading are based on independent or prioritized datasets, while maintaining a full qualitative record of all published systematic reviews.

Quality assessment / Risk of bias analysis The methodological quality of each included systematic review will be independently assessed by two reviewers (M.G. and S.T.) using the AMSTAR-2 (A MeaSurement Tool to Assess Systematic Reviews 2) instrument. Any appraisal discrepancies will be resolved through consensus.
The assessment will focus on the 16 items of the tool, with particular emphasis on the seven critical domains: (i) protocol registration, (ii) adequacy of the literature search, (iii) justification for excluding individual studies, (iv) risk of bias assessment of primary studies, (v) appropriateness of meta-

analytical methods, (vi) consideration of risk of bias when interpreting results, and (vii) assessment of publication bias.
Based on the presence of deficiencies in these critical domains, each review will be categorized into one of four levels of confidence: "High," "Moderate," "Low," or "Critically Low." This classification will provide a standardized measure of the overall confidence in the results of the meta-analyses under re-appraisal.

Strategy of data synthesis The epidemiological credibility of each "overall meta-analysis" reporting a nominally significant association ($p < 0.05$) will be graded according to the Venice criteria, a framework designed to assess the quality of evidence in genetic associations across three fundamental domains:
1. Amount of Evidence: Evaluated based on the total number of cases (N) in the meta-analysis (Grade A: $N > 1000$; Grade B: $100 < N \leq 1000$; Grade C: $N \leq 100$).
2. Replication of Results: Assessed through the degree of inter-study heterogeneity (I2) (Grade A: $I2 < 25\%$; Grade B: $25\% \leq I2 < 50\%$; Grade C: $I2 \geq 50\%$).
3. Protection from Bias: Determined by the Egger's regression test p-value, using either the original or the recalculated value as described in Item 20 (Grade A: $P > 0.10$; Grade B: $0.05 < P \leq 0.10$; Grade C: $P \leq 0.05$). In instances where the Egger's test is missing and cannot be validated or reconstructed, this domain will be assigned a conservative Grade "C".
To avoid the double-counting of participants in cases where multiple meta-analyses investigate the same clinical association (i.e., the same mtDNA-CN exposure and the same clinical phenotype/outcome), a hierarchical selection process will be applied for the evidence grading. This selection specifically addresses redundancy, involving overlapping meta-analyses that focus on the same clinical phenotypes/outcomes and represent the same clinical question (e.g., disease risk vs. disease status); in such instances, only the most recent or comprehensive version will be selected for the evidence synthesis.
While all identified meta-analyses will be included in the systematic review and assessed for methodological quality (AMSTAR-2), only the most comprehensive meta-analysis (defined as the one including the largest number of primary studies and cases) will be prioritized for the primary Venice grading. This hierarchical selection also applies when the same clinical phenotype is investigated using different effect size metrics (e.g., Standardized Mean Difference for levels vs. Odds Ratio for risk). If these metrics address the same clinical question (e.g., disease status) and rely on overlapping primary studies, only the most comprehensive meta-analytical unit will be prioritized. Conversely, if different metrics reflect distinct clinical endpoints for the same phenotype —such as disease risk (OR/RR) versus longitudinal prognosis (HR)—they will be analyzed as separate and independent associations.
If meta-analyses are equally comprehensive, the most recently published one (based on the last search date) will be selected, with methodological quality (AMSTAR-2 score) used as a further tie-breaker if necessary.
The final Evidence Classification follows a hierarchical grading system:
Strong Credibility is assigned to associations that achieve a Grade A in all three domains (AAA).
Moderate Credibility is defined by findings that exhibit at least one Grade B and no Grade C deficiencies.
Weak Credibility characterizes associations that receive at least one Grade C in any domain, indicating potential instability or susceptibility to bias.
Non-significant findings: Meta-analyses with a p-value ≥ 0.05 will be categorized as "No evidence of association" and will not undergo formal Venice grading, as the framework is intended to evaluate the robustness of claimed.

Subgroup analysis Subgroup findings (e.g., based on tissue type, ethnicity, or clinical subtypes) will be organized into supplementary tables and synthesized narratively to investigate potential sources of heterogeneity. For each subgroup, we will extract the same primary metrics as the overall analysis (pooled effect sizes, 95% CIs, and p-values), along with statistical heterogeneity measures (I2 and Phet), where reported in the original study. In instances where these metrics are not available, they will be marked as "NR" (Not Reported). These secondary analyses are intended for exploratory purposes and will not undergo formal Venice grading, ensuring the primary focus remains on the robustness and epidemiological credibility of the main meta-analytical evidence.

Sensitivity analysis Not applicable for this re-appraisal.

Language restriction None.

Country(ies) involved Italy.

Keywords mtDNA-CN; mitochondrial DNA copy number; Umbrella review; systematic review; meta-analysis; Venice criteria; AMSTAR-2; clinical phenotypes; clinical outcomes; critical appraisal.

Dissemination plans The final results will be submitted to a peer-reviewed international journal in the field of clinical chemistry, molecular medicine, or pharmacology. Findings may also be presented at national or international scientific congresses.

Contributions of each author

Author 1 - Martina Giacon.
Email: martina.giacon@uniupo.it
Author 2 - Salvatore Terrazzino.
Email: salvatore.terrazzino@uniupo.it