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## Mitochondrial DNA copy number variation and pancreatic cancer risk in the prospective EPIC cohort

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### Conflict of interest:

The authors declare no potential conflicts of interest.

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## Abstract

**Background**—Mitochondrial DNA (mtDNA) copy number in peripheral blood has been found to be associated with risk of developing several cancers. However, data on pancreatic ductal adenocarcinoma (PDAC) are very limited.

**Methods**—To further our knowledge on this topic we measured relative mtDNA copy number by a quantitative real-time PCR assay in peripheral leukocyte samples of 476 PDAC cases and 357 controls nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

**Results**—We observed lower mtDNA copy number with advancing age ( $p=6.54 \times 10^{-5}$ ) and with a high BMI level ( $p=0.004$ ) and no association with sex, smoking behavior and alcohol consumption. We found an association between increased mtDNA copy number and decreased risk of developing PDAC with an OR=0.35 (95% C.I 0.16-0.79),  $p=0.01$  when comparing the 5<sup>th</sup> quintile with the 1<sup>st</sup> using an unconditional logistic regression and OR=0.19 (95% C.I 0.07-0.52),  $p=0.001$  with a conditional analysis. Analyses stratified by BMI showed an association between high mtDNA copy number and decreased risk in the stratum of normal weight, consistent with the main analyses.

**Conclusions**—Our results, suggest a protective effect of a higher number of mitochondria, measured in peripheral blood leukocytes, on PDAC risk.

**Impact**—Our findings highlight the importance of understanding the mitochondrial biology in pancreatic cancer.

### Keywords

Pancreatic cancer; mitochondrial copy number; mtDNA copy number; cancer risk; prospective cohort; leukocyte

## Introduction

Pancreatic cancer is a relatively rare disease with an incidence of 17.8/100,000 (crude rate) in Europe, and with a very high mortality rate, considering a 5-year survival lower than 5% (1). Established risk factors are few compared to other cancer types and include smoking, diabetes mellitus, obesity and chronic pancreatitis (2). In the last decade PDAC mortality has continued to increase and it has been estimated that by 2030 it will be the second most frequent cancer for mortality in Europe (3). The poor prognosis is caused by several factors, including the aggressiveness of the disease, lack of effective treatments, lack of knowledge about biological markers for early detection and for risk prediction (4).

A better understanding of risk factors and availability of better risk markers might lead to improved chances of stratifying the population according to their risk and, in the long term, to a faster diagnosis (5–7). In addition, the discovery of novel risk markers could further our knowledge on the biology of this disease.

One such possible risk marker might be mitochondrial DNA. Mitochondria are organelles involved in the regulation of critical cellular functions such as apoptosis, calcium homeostasis and energy production via the oxidative phosphorylation reaction and are responsible for the production of reactive oxygen species (ROS) (8,9). Mitochondria possess own copies of DNA (mtDNA), which are maternally inherited, and in each eukaryotic cell there can be hundreds or thousands of copies of their genomes. mtDNA copy number represents the number of mitochondria contained in each cell, and this number is in a constant range in order to sustain the energetic needs of the cell (10). mtDNA copy number varies by cell type, but, in general, there is a correlation between the amount of mtDNA in different cell types (11). Therefore, mtDNA copy number measured in circulating leukocytes could represent a good and non-invasive indicator of the average amount of mtDNA copy number in other tissues (11). In cells under normal physiological conditions, the amount of

mtDNA is relatively stable. Several reports have highlighted that mitochondrial copy number increases to compensate for mtDNA damage and mitochondrial dysfunction (12) and in addition it could also represent a marker of endogenous and exogenous stressors including oxidative stress (13,14). The number of epidemiologic studies investigating the association of mtDNA copy number measured in leukocytes with cancer risk has been increasing in recent years, summing up to about thirty studies across various tumour types, including breast (11,15–17), colorectal (18–20), prostate (21–23), lymphoma (24,25) and pancreatic cancer (26). The results of these reports are very heterogeneous, with some studies showing an association between high number of mtDNA and increased risk while others showing the opposite. For pancreatic cancer only one prospective study, performed in male smokers in Finland, reported an association between high mtDNA copy number and increased risk to develop pancreatic ductal adenocarcinoma (PDAC) (26).

Given the importance of finding new risk factors for pancreatic cancer we performed a case-control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study (27) investigating the possible association between mtDNA copy number variation in peripheral blood and risk of developing PDAC.

## Materials and Methods

### Study population

A full description of the EPIC cohort study has been given elsewhere (27). Briefly, EPIC consists of about 520,000 volunteers, recruited between 1992 and 2005 in 10 European countries. After providing informed consent, diet, lifestyle, and personal medical history questionnaires were collected and blood was drawn from participants at recruitment. Diagnosis of cancer determined after recruitment into the cohort was identified through local and national cancer registries (in Italy except the Naples center, Spain, Sweden, Netherlands, United Kingdom, Norway, Denmark) or by a combination of contacts with participants and local tumor registries, national health insurances or physicians and clinics within an active follow-up (in France, Germany, Greece and the Naples center in Italy). In this study 476 incident pancreatic cancer cases and 357 controls from eight countries (France, Germany, Greece, Italy, Spain, Sweden, Netherlands, and the United Kingdom) were used. Of these 833 individuals, 483 and 350, respectively, were women and men in the same proportions in cases and controls. Cases were diagnosed with exocrine pancreatic cancer, mainly pancreatic ductal adenocarcinoma (ICD-10, C25.0-25.3, 25.7-25.9). Due to the different etiology, endocrine pancreatic tumours (CD-10 C25.4,) were not included in this study. For a subgroup of cases (n=301) and an equal number of controls we performed a matching by center, sex, age at recruitment ( $\pm 6$  months), date at entry in the cohort, and time between blood sampling and time of last consumption of food or drinks (<3, 3-6, and 6 hours) using an incidence density sampling protocol. The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics review board of the International Agency for Research on Cancer (IARC) in Lyon.

## Sample preparation and DNA extraction

Blood samples from France, Germany, Greece, Italy, the Netherlands, Spain and the United Kingdom were conserved in liquid nitrogen (-196°C) in a central biorepository at IARC. Swedish samples were stored in Sweden in freezers at -70°C. DNA was extracted from leukocytes in batches of 96 with an Autopure instrument with Puregene chemistry. To minimise any possible bias due to differential handling each sample was extracted using the same method. DNA concentration and quality were measured using Qubit 4 Fluorometer (Thermo Fisher, Waltham, Massachusetts, US).

## qPCR measurement of mtDNA copy-number

mtDNA copy number was measured in 833 study subjects (476 cases and 357 controls). To measure mtDNA we used a quantitative polymerase chain reaction (qPCR) that quantifies the copy number of the mitochondrial gene NADH dehydrogenase, subunit 1 (*ND1*) using as a reference the nuclear single copy gene albumin (*ALB*). Each reaction was performed in triplicate, in an optical 384-well reaction plate, in a 10 µL reaction volume using 2 µL of 5X HOT FIREPol Probe qPCR Mix Plus with ROX (Solis Bio-Dyne, Tartu, Estonia), 1.5 µM of Syto 9 (Invitrogen, Carlsbad, CA), 5 ng of genomic dried DNA and 8 µL of water. Two primers for *ND1* copy number, and two primers for quantifying *ALB* copy number were used. Each *ALB* primer was modified adding a GC-clamp to the 5' end in order to raise the melting temperature (primer sequences are shown in supplementary materials) (28).

The real-time PCR experiments were carried out using a Viia-7 sequence detection system (Applied Biosystems) using two subsequent (#1 *ND1*; #2 *ALB*) PCR cycling conditions performed in the same plates, to acquire the respective cycle thresholds (Ct) values for copy numbers of *ND1* and *ALB* (control) gene.

The conditions for amplification of *ND1* repeats were 95 °C/ 5 min, 2 cycles of 94 °C/15 sec and 60 °C/1 min, followed by 30 cycles of 85 °C/15 sec with the signal acquisition at 65 °C/1 min. Thermal conditions for *ALB* gene were 35 cycles of 95 °C/15 sec, 85 °C/30 sec, with the signal acquisition at 84 °C/30 sec. The specificity of all amplifications was determined by melting curve analysis done at default settings (95 °C/15 sec, 60 °C/1 min with the continuous signal acquisition at 0.05 °C/sec ramping, 95 °C/15 sec). A serial dilution (1:2) from 20 ng to 0.3 ng of genomic DNA pooled from 50 healthy individuals was included to generate the standard curves for *ND1* and *ALB* genes. The standard curve was used to quantify the *ND1* repeats and *ALB* gene, based on the respective Ct values. For each data point the obtained triplicate values were averaged. Individual values that deviated from the average of the triplicates by more than 5% of the standard deviation were discarded.

Standard curves were graphically represented as a semi-log regression line plot of Ct values and log of standard DNA concentration. The real-time PCR efficiency (E) of each reaction was calculated using standard curve points in the exponential phase according to the equation:  $E = 10^{[-1/\text{slope}]}$ . Samples whose Ct average was not within the standard curve range were discarded (26 samples) as “out of range” and not included in the analyses.

The mtDNA copy number was expressed as the ratio between *ND1/ALB*, using the Pfaffl method (29), which is best suited to the type of data obtained from a qPCR with efficiencies



not perfectly identical between the amplification reactions of *NDI* and *ALB*, using as a calibrator the Ct of the standard curve to the equivalent of 5ng of DNA.

### Statistical analysis

Association between mtDNA copy number and potential confounders at baseline, i.e. age, body mass index (BMI, kg/m<sup>2</sup>), smoking behavior and alcohol consumption, was tested using a generalized linear model (GLM) in the control group.

The mtDNA copy number was categorized into quintiles based on the distribution of mtDNA copy number in the controls and modelled as a categorical variable. The phenotype (pancreatic cancer case or control) was expressed as a dichotomic variable, age was expressed as a continuous variable, the center of origin was expressed as a categorical variable, the plate of PCR-reactions was expressed as a categorical variable, BMI was expressed as a categorical variable with four values (underweight (BMI<19); normal weight (19 BMI<25); overweight (25 BMI<30); obese (BMI ≥ 30)), smoking behavior was expressed as a dichotomic variable (never, ever), and the same for alcohol consumption (non-drinker, drinker).

Odds ratios (ORs) and 95% confidence intervals (CIs) of the association between mtDNA copy number and pancreatic cancer risk were estimated using unconditional logistic regression adjusted by sex, age, center, BMI and plate.

Conditional logistic regression was also used on a subgroup of the subjects (n=301 case-control pairs), using center, sex, age at recruitment (±6 months), date at entry in the cohort, interval between blood sampling and time of last consumption of food and drink (<3, 3-6, and ≥ 6 hours) as matching variables. The analysis was further adjusted for BMI and plate. We also performed a conditional and unconditional analysis adjusting for diabetes and smoking status. However we had this information only in a subgroup of the subjects enrolled in the study.

Analyses were also performed by strata of lag time between blood collection and diagnosis of pancreatic cancer (<7 years, ≥ 7 years). We also performed analyses stratified by smoking, considering only males (77 pancreatic cancer cases and 80 controls), and the two sexes combined (142 pancreatic cancer cases and 134 controls). These two analyses were performed to compare the results with those of Lynch and collaborators (26). Finally, we performed analyses stratified by classes of BMI and age, because these two covariates are associated with mtDNA copy number, as reported in Supplementary Table 1.

A p<0.05 was considered statistically significant. All statistical analyses were two-sided.

### Results

A detailed description of the study population is shown in Table 1.

We tested in controls the possible association between mtDNA copy number in leukocytes and age, BMI, smoking behavior and alcohol consumption. We observed lower mtDNA copy number with advancing age ( $p=6.54 \times 10^{-5}$ ) and with a high BMI level ( $p=0.004$ ). For

sex, smoking behavior, diabetes status and alcohol consumption we observed no statistically significant associations (Supplementary Table 1).

The results from the unconditional logistic regression show an association between high mtDNA copy number and decreased risk of developing PDAC when analyzing mtDNA copy number categorized in quintiles (Table 2). We compared the fifth quintile (highest) with the first (lowest) and obtained OR=0.35 (95% CI 0.16-0.79,  $p=0.01$ ). We also analyzed the quintiles of mtDNA copy number as continuous variable (i.e. the unit of measurement was the increase of one quintile) and obtained OR=0.79 (95% CI 0.66-0.96,  $p=0.019$ ) (Table 2).

We also performed a conditional logistic regression analysis using mtDNA copy number categorized in quintiles on a subgroup of matched subjects and the results support the previous observations (OR=0.74, 95% CI 0.59-0.93,  $p=0.008$  for the quintiles of mtDNA copy number analyzed as continuous variable, OR=0.19, 95% CI 0.07-0.52,  $p=0.001$  comparing the 5<sup>th</sup> quintile (highest) with the first (lowest) (Table 2). The results of the analysis adjusted for diabetes confirmed a protective effect of mtDNA, although reaching statistical significance only in one of the quintiles. These analyses were done on a much smaller number of individuals: a total of 447 subjects for the unconditional analysis and 400 for the conditional analysis (Supplementary Table 2). The results of the analysis performed on the subgroup of individuals for which we had smoking history are in general agreement with the unadjusted analyses (Supplementary Tables 2 and 3).

We performed analyses stratified by lag time between blood collection and diagnosis of pancreatic cancer (<7 years, ≥7 years) and we did not observe any statistically significant association in either of the strata (Supplementary Table 4). In addition, we also conducted a sensitivity analysis considering smokers (males and females alone and the two sexes combined); in the analyses of men and both sexes, we observed non-significant increases of risk in the highest quintiles (Supplementary table 5). Analyses stratified by BMI showed an association between high mtDNA copy number and decreased risk in the stratum of normal weight (Supplementary table 6).

## Discussion

The association of mtDNA copy number with cancer risk has been studied in many cancer types with heterogeneous results (11,15–26.). In the present study we observed an association between a high level of mtDNA copy number and reduced risk of developing the disease. The association was consistent using both unconditional and conditional analysis (in a subgroup of individuals) and considering mtDNA quintiles as a continuous or categorical variable.

Lynch and colleagues in a nested case-control study performed in 203 PDAC cases who were Finnish male smokers and 656 male smoker controls within the prospective ATBC cohort found, instead, an association between high mtDNA copy number and increased PDAC risk(26). The differences in the results of the two studies may be explained by the differences in study design. Both are prospective studies, however, in the study by Lynch there are only male smokers while ours is considerably larger and composed by both genders



and unselected for smoking status. We investigated the association between mtDNA copy number and pancreatic cancer risk in male smokers present in our study (77 pancreatic cancer cases and 80 controls), but we observed non-statistically significant results, although we obtained ORs>1 for the highest quintiles, which agrees with the results of Lynch et al. In addition, we also performed an analysis considering all the current smokers and obtained broadly similar results as in the male smokers.

Our study suggests that peripheral blood mtDNA copy number is inversely associated with BMI, and ageing, in agreement with the literature (30–32). We observed a non-statistically significant association between smoking behavior and lower mtDNA, as recently reported by Wu and colleagues in an all-female population (33).

A possible biological explanation of our findings on PDAC risk can be found in the central role that mitochondria have in regulating global variability in gene expression at cellular level (34). In particular, in a very recent study, Marquez-Jurado and colleagues have shown that cells with an increased number of mitochondria have a faster response to external stress, increasing apoptotic protein synthesis and triggering the apoptosis process more quickly (35). The authors also observed that high mitochondrial content is associated with increased apoptotic inducers such as TNF alpha, suggesting a key role for mitochondria in discriminating cell fate (35). Additionally Armstrong and colleagues showed a direct effect of altered mitochondrial bioenergetics in pancreatic acinar cells and in the regulation of apoptosis to necrosis switch (36). Triggering a faster apoptotic response could be instrumental in avoiding tissue necrosis, that can cause inflammation that can degenerate into pancreatitis or acute pancreatitis and in turn lead to PDAC development (37,38). In addition several authors observed that there is a correlation between low blood mtDNA copy number with high levels of circulating inflammatory markers such as IL-6, CRP and neutrophil-to-lymphocyte ratio (39–41) that have been investigated as PDAC risk and prognostic markers (42–44). Given that mtDNA copy number measured in leukocytes is considered to be a good proxy for mtDNA content in other tissues (10,11), one can speculate that low levels of mtDNA copy number, measured in blood, may be associated with an increased level of inflammation, that could increase the risk of developing PDAC (45).

This study has clear strengths, such as its prospective nature that reduces the risk of reverse causation bias, and the fact that we have taken into consideration potential confounding factors, such as body mass index, age, smoking, and alcohol consumption. A limitation is its rather small sample size even though it is the largest study on PDAC on mtDNA copy number attempted so far. In addition our study is based on a single measurement of mtDNA which does not reflect the possible fluctuation of mtDNA over long term. However in a previous study we have shown that mtDNA copy numbers are stable over the years (46).

In conclusion, several evidences, including our results, suggest a protective effect of a higher number of mitochondria, measured in peripheral blood leukocytes, on PDAC risk and highlight the importance of understanding the mitochondrial biology in pancreatic cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Availability of data and materials

For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at <http://epic.iarc.fr/access/index.php>.

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**Table 1****Participant characteristics.**

Distribution across countries and characteristics of the cases and controls participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study.

Country	Controls	Cases	Total
France	11	10	21
Germany	55	90	145
Greek	21	32	53
Italy	33	56	89
Spain	37	49	86
Sweden	119	117	236
Netherlands	39	58	97
United Kingdom	42	64	106
Total	357	476	833
<b>Sex</b>			
Men	155 (43%)	195 (41%)	350 (42%)
Women	202 (57%)	281 (59%)	483 (58%)
<b>Mean age (years)</b>	57	57	57
<b>Median age at recruitment</b>	60	58	59
(25 <sup>th</sup> -75 <sup>th</sup> percentile)	36-75	35-75	30-75
<b>BMI (median-mean)</b>	25 - 25.95	26 - 26.76	26 - 26.41
<b>BMI (%)</b>			
Underweight (BMI<19)	10 (3%)	1 (<1%)	11 (1%)
Healthy weight (19<BMI<25)	141 (42%)	182 (40%)	324 (41%)
Overweight (25<BMI<30)	131 (39%)	192 (42%)	323 (41%)
Obese (BMI>30)	54 (16%)	85 (18%)	139 (17%)
<b>Cigarette smokers</b>			
Never	130 (49%)	174 (45%)	304 (47%)
Ever	137 (51%)	211 (55%)	348 (53%)
- Current	49 (18%)	109 (28%)	158 (24%)
- Former	88 (33%)	102 (27%)	190 (29%)
<b>Alcohol use</b>			
Non-drinker	41 (14%)	49 (18%)	90 (16%)
Drinker	251 (86%)	223 (82%)	474 (84%)
<b>Diabetes status</b>			
Diabetic	13 (5%)	29 (8%)	42 (7%)
Non-diabetic	227 (95%)	317 (92%)	544 (93%)

**Table 2**  
**Association between mtDNA copy number and PDAC risk using an unconditional and a conditional analysis.**

<b>Unconditional analysis</b>	<b>Controls</b>	<b>Cases</b>	<b>Total</b>	<b>OR</b>	<b>95% C.I.</b>	<b>P-value</b>
Continuous variable log-transformed	357	476	833	0.47	0.28; 0.79	<b>0.004</b>
Continuous variable divided in quintiles	357	476	833	0.79	0.66, 0.96	<b>0.019</b>
Analysis by quintiles						
1 <sup>st</sup> quintile	76	126	202	ref	-	-
2 <sup>nd</sup> quintile	70	105	175	0.91	0.57, 1.56	0.809
3 <sup>rd</sup> quintile	68	117	185	0.85	0.51, 1.52	0.639
4 <sup>th</sup> quintile	74	71	145	0.49	0.24, 1.08	0.077
5 <sup>th</sup> quintile	69	57	126	0.35	0.16, 0.79	<b>0.010</b>
<b>Conditional analysis</b>						
Continuous variable log-transformed	301	301	602	0.35	0.19; 0.64	<b>0.001</b>
Continuous variable divided in quintiles	301	301	602	0.74	0.59; 0.93	<b>0.008</b>
Analysis by quintiles						
1 <sup>st</sup> quintile	61	68	129	ref	-	-
2 <sup>nd</sup> quintile	60	56	116	0.81	0.47, 1.40	0.448
3 <sup>rd</sup> quintile	55	69	124	0.94	0.52, 1.68	0.827
4 <sup>th</sup> quintile	61	63	124	0.34	0.13, 0.87	<b>0.025</b>
5 <sup>th</sup> quintile	64	45	109	0.19	0.07, 0.52	<b>0.001</b>

Unconditional logistic regression performed using mitochondrial copy number variable (Pfaffl) categorized in quintiles and analyzed as continuous variable log-transformed, and continuous variable divided in quintiles (whereby the unit of measurement is a single quintile). Analyses were adjusted for sex, age, BMI, plate and recruitment center. Individual matching in conditional analysis was done by center, gender, age at recruitment ( $\pm 6$  months), date at entry in the cohort, time between blood sampling, and time of last consumption of food and drink (<3, 3-6, and >6 hours). This analysis was adjusted for plate and BMI.