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RESEARCH ARTICLE

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Exploring mitochondrial DNA copy number in circulating cell-free DNA and extracellular vesicles across cardiovascular health status: A prospective case–control pilot study

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Abstract

Cardiovascular disease (CVD) is a leading global cause of mortality, difficult to predict in advance. Evidence indicates that the copy number of mitochondrial DNA (mtDNAcn) in blood is altered in individuals with CVD. MtDNA released into circulation may act as a mediator of inflammation, a recognized factor in the development of CVD, in the long distance. This pilot study aims to test if levels of mtDNAcn in buffy coat DNA (BC-mtDNA), in circulating cellfree DNA (cf-mtDNA), or in DNA extracted from plasma extracellular vesicles (EV-mtDNA) are altered in CVD patients and if they can predict heart attack in advance. A group of 144 people with different CVD statuses (50 that had CVD, 94 healthy) was selected from the LifeLines Biobank according to the incidence of new cardiovascular event monitored in 6 years (50 among controls had heart attack after the basal assessment). MtDNAcn was quantified in total cf-DNA and EV-DNA from plasma as well as in buffy coat. EVs have been characterized by their size, polydispersity index, count rate, and zeta potential, by Dynamic Light Scattering. BC-mtDNAcn and cf-mtDNAcn were not different between CVD patients and healthy subjects. EVs carried higher mtDNAcn in subject with a previous history of CVD than controls, also adjusting the analysis for the EVs derived count rate. Despite mtDNAcn was not able to predict CVD in advance, the detection of

Abbreviations: BC-DNA, buffy coat DNA; BC-mtDNAcn, buffy coat mtDNA copy number; BMI, body mass index; Ccf-mtDNA, circulating cell-free mitochondrial DNA; Cf-mtDNA, cell-free mitochondrial DNA; CHD, coronary heart disease; CVD, cardiovascular disease; DAMP, damage-associated molecular pattern; DCR, derived count rate; DLS, dynamic light scattering; EVs, extracellular vesicles; EV-DNA, extracellular vesicles DNA; EV-mtDNA, extracellular vesicles mitochondrial DNA; EV-mtDNAcn, extracellular vesicles mitochondrial DNA copy number; EV-nDNA, extracellular vesicles nuclear DNA; EV-nDNAcn, extracellular vesicles nuclear DNA copy number; gDNA, genomic DNA; HDL, high-density lipoproteins cholesterol; LLDS, LifeLines diet score; LDL, low-density lipoproteins cholesterol; MtDNA, mitochondrial DNA; MtDNAcn, mitochondrial DNA copy number; nDNA, nuclear DNA; nDNAcn, nuclear DNA copy number; NUMTs, nuclear insertions of mitochondrial origin; P/L, platelets/leukocyte ratio; PDI, polydispersity index; SCORE2, Systemic Coronary Risk Estimation 2; tcf-DNA, total cell-free DNA; tcf-mtDNA, total cell-free mitochondrial DNA; tcf-mtDNAcn, total cell-free mitochondrial DNA copy number; tcf-nDNA, total cell-free nuclear DNA; tcf-nDNAcn, total cell-free nuclear DNA copy number; tCHOL, total cholesterol; TG, triglycerides; TTPs, trusted third parties; ZP, zeta potential.

increased EV-mtDNAcn in CVD patients in this pilot study suggests the need for further investigations to determine its pathophysiological role in inflammation.

KEYWORDS

cell-free DNA, CVD, extracellular vesicles, heart attack, mitochondrial DNA

1 | INTRODUCTION

Mitochondrial DNA (mtDNA) is a small circular multicopy genome located in the inner matrix of mitochondria. It consists of 16 569 base pairs, and it contains 37 genes: 13 encoding for oxidative phosphorylation mRNAs, 22 for tRNAs, and 2 for rRNAs.[1] MtDNA exists in cells in a different copy number (mtDNAcn) depending on the tissue type, health status of cells or environmental exposures.[2-13] Also, increasing evidence demonstrated the presence of mtDNA in body fluids as circulating cell-free mtDNA (ccf-mtDNA).[14] Ccf-mtDNA can circulate in body fluids as naked or contained in lipid-based vesicles.[14] The mechanisms underlying the release of mitochondrial DNA from cells into the extracellular compartment have not been fully elucidated, prompting the formulation of various hypotheses to address this phenomenon. In passive mechanisms, mtDNA is believed to be released into the extracellular space following cellular apoptosis or necrosis, packaged within apoptotic bodies, intact mitochondria, or as ccf-mtDNA.[14,15] Ccf-mtDNA can also be released by cells through an actively regulated process, residing inside extracellular vesicles (EVs), such as exosomes, mitochondria-derived vesicles, or neutrophils/eosinophils extracellular traps.[15]

There is a growing body of evidence associating both mtDNAcn and ccf-mtDNA with human health status. In particular, mtDNAcn has been associated with metabolic and cardiovascular disease (CVD).[2,12,16,17] Measurement of mtDNAcn in human buffy coat/circulating leukocytes has revealed an inverse association with prevalent and incident CVD outcomes.[18] Also, mtDNAcn in whole blood has been correlated to all cause of mortality and cardiovascular disease in peripheral arterial disease patients with intermittent claudication.[19] A potential role of ccf-mtDNA in the etiology of some inflammatory diseases has also been hypothesized.[20] MtDNA has been suggested to be inflammogenic and immunostimulatory, acting as a damage-associated molecular pattern (DAMP).[20,21] The immune system may recognize mtDNA as "foreign" due to its bacterial-like sequences, a trait linked to mitochondria's endosymbiotic origins.[20] Several pathways have been suggested through which mtDNA triggers immune responses, including TLR9 and ZBP1 receptor activation, cGAS-STING pathway engagement, NLRP3 and AIM2 inflammasome activation.[20,22] Despite the unclear mechanisms of cell-free mitochondrial DNA (cf-mtDNA) entry into cells and its binding to intracellular receptors, changes in ccf-mtDNA levels have been measured in inflammation-related conditions, including diabetes, coronary heart disease, Parkinson's disease, and Alzheimer's disease.[23-28] A direct involvement of ccf-mtDNA in CVD pathogenesis has been proposed, where mtDNA-LL37 complexes may accumulate in atherosclerotic plaques, leading to the activation of inflammatory cytokines and recruitment of immune cells in vitro.[20,29]

EVs (and their cargoes) have also been implicated in the pathophysiology of CVD and have recently been proposed as potential biomarkers for these conditions.[30] For instance, exosomes derived from vascular smooth muscle cells have been shown to transfer miR-155 from smooth muscle to endothelial cells, resulting in endothelial cell damage and accelerated atherosclerosis.[31] Also, EVs released by endothelial cells may contribute to plaque formation by inducing a proliferative and migratory phenotype in vascular smooth muscle cells following arterial injury.[32,33] Among cargoes carried by EVs, DNA is also included. Extracellular vesicles DNA (EV-DNA), possibly both nuclear and mitochondrial, can be located on the surface or inside the vesicle.[34] The level of mtDNA carried by EVs, particularly exosomes, has been studied in different pathologies,[35-38] with an observed increase in patients with chronic heart failure.[39]

Despite significant evidence supporting mtDNAcn as a biomarker for CVD, the translation of this evidence into clinical practice remains limited. Uncertainties persist regarding if mtDNAcn from the buffy coat or ccf-DNA or its fractions is associated to CVD, as well as whether the impact of blood composition on this biomarker affects its predictive capabilities. In the light of this evidence, this case–control pilot study aims at testing if levels of mtDNA in buffy coat (BC-mtDNAcn), ccf-mtDNA or extracellular vesicles mitochondrial DNA (EV-mtDNA) are altered in CVD patients, and if they can predict heart attack in advance, compared to other established CVD predictors (i.e., Systemic Coronary Risk Estimation 2 (SCORE2),[40] and triglycerides/high-density lipoprotein cholesterol ratio (TG/HDL)[41,42]). Additionally, this research aims to characterize EVs in different cardiovascular health statuses, seeking to discern whether the properties of these particles can

serve as predictive indicators of CVD outcomes or if they are associated with their mtDNA content.

2 | MATERIALS AND METHODS

2.1 | Participant recruitment and data collection

The study is in collaboration with the LifeLines Biobank. LifeLines[43,44] is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviors of 167 729 persons living in the North of the Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical, and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity. The LifeLines study was approved by the ethics committee of the University Medical Centre Groningen, document number METC UMCG METc 2007/152. LifeLines operates in the highest ethical standards and strictly controls, and it takes into account rules regarding the irreversible pseudonymization of participants, encryption of data, use of trusted third parties (TTPs), and controlled data access. This will guarantee that both personal data and samples respect the EU Directive 2004/23 on standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells and EU Regulation 2016/679 of the European Parliament and of the Council of April 27, 2016, on the protection of natural persons with regard to the processing of personal data and on the free movement of such data. All participants signed an informed consent (http://wiki.lifelines.nl/doku.php?id=informed_consent).

From 2007 to 2013, over 167 000 participants were included at baseline (1A), with the aim to follow up for at least 30 years.[44] Questionnaires completed during the study generated around 8000 variables and cover a broad range of topics (detailed information are available at LifeLines Wiki [http://wiki-lifelines.web.rug.nl] or catalog [https://data-catalogue.lifelines.nl]). Subjects enrolled have been invited to complete two follow-up questionnaires within the following 6 years [from 2011 to 2013] (time 1B and 1C, about 1.5y and 2.5y after baseline assessment, respectively). A second assessment (2A) to collect health-related data, physical measurements, and additional biological samples was performed after 6y from baseline [from 2014 to 2017].

For the participants included in this study (according to the study design described in paragraph 2.3), SCORE2 was calculated. SCORE2 is as an established predictive biomarker for CVD, as detailed in the 2021 European Society of Cardiology Guidelines on cardiovascular disease prevention in clinical practice. SCORE2 algorithm estimates the 10-year risk of fatal and non-fatal CVD events in apparently healthy people aged 40–69 years.[45] SCORE2 estimates the risk of CVD events based on sex, systolic blood pressure (mmHg), total cholesterol (mmol/L) (tCHOL), HDL cholesterol (mmol/L) smoking habits (being or not being a smoker), and geographical origin. In this study, SCORE2 has been calculated according to the formulas contained in the supplementary materials of the paper published in 2021 by SCORE2 working group and ESC Cardiovascular risk collaboration.[40] We applied the formulas specific for low-risk CVD countries (as The Netherlands is indexed).

2.2 | Sample collection, processing, and storage

Blood samples have been collected at LifeLines center using BD Vacutainer® 10.0 mL K2E (EDTA) 18.0 mg Plus blood collection tubes, immediately centrifuged for 15 min at 2500 RCF to isolate plasma and buffy coat, then stored at −80° upon shipment. Data about tCHOL, HDL, low-density lipoproteins cholesterol (LDL), triglycerides (TG), and blood composition (i.e., neutrophilic, basophilic, eosinophilic granulocytes, monocytes, leukocytes, lymphocytes, thrombocytes) were collected by LifeLines operators at the laboratory of the University Medical Centre Groningen (certified according to NEN-EN-ISO 9001:2008 and NEN-EN-ISO 15189:2012 standards). Genomic DNA (gDNA) was extracted from the buffy coat at LifeLines center using the Perkin-Elmer Chemagic 360 to perform magnetic bead DNA extraction. Both gDNA from buffy coat (BC-DNA) and plasma from selected samples were shipped to the University of Camerino for further analysis.

2.3 | Study design

In this pilot study, information about sex, gender, age, body mass index (BMI), blood pressure (measured automatically using the DinaMap PRO100 or DinaMap PRO100V2), dietary records, diet quality (according to the LifeLines Diet Score [LLDS]),[46] smoking, physical activity, cardiovascular health, and any other disease onset was used to select a subcohort of 144 people according to the experimental design shown in Figure 1 and detailed as follows. Recruited participants were selected according to three different CVD statuses: 50 individuals reported a CVD event that previously occurred (within 2 years before baseline assessment) (γ group). 94 individuals were

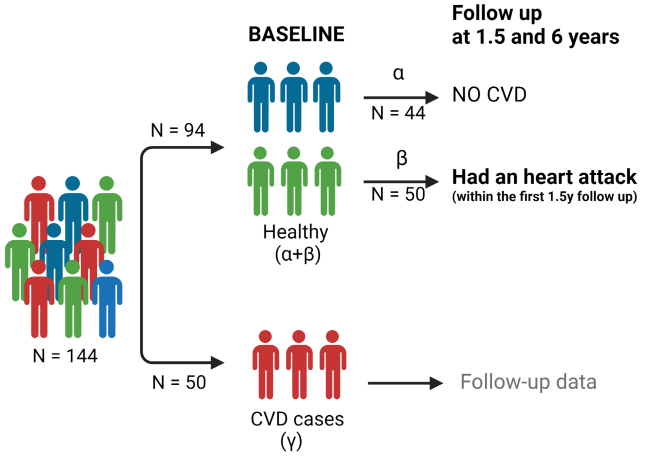

FIGURE 1 Study design: A total of 144 subjects were included in the study according to their cardiovascular health status. 50 individuals reported a CVD event that previously occurred within 2 years before baseline assessment (γ group). In all, 94 individuals were healthy at baseline assessment. Of them, 50 individuals reported a heart attack occurred after baseline assessment (within the following 1.5 years) (β group). The remaining 44 did not report any cardiovascular event for the following 6 years (α group). Individuals were recruited according to specific inclusion and exclusion criteria to control for confounding factors that has been associated to mtDNAcn by previous studies (see paragraph 2.3 for details). Individuals were matched so that the selected groups did not show significant differences in age, ethnicity, country of origin, sex, body composition, dietary habits, and physical activity levels. Figure created with BioRender.com.

healthy at baseline assessment. Of them, 50 individuals reported a heart attack occurred after baseline assessment (within the following 1.5 years) (β group). The remaining 44 did not report any cardiovascular event for the following 6 years (α group), representing healthy controls (Figure 1). Individuals who were healthy at baseline but reported a heart attack during follow-up (β group) were selected among new cases of heart attack reported within the following 1.5y after baseline assessment. Healthy controls (groups α) were selected matching individuals from β group for age, sex, and BMI. Considering potential confounding factors able to affect mtDNAcn according to previous literature, we selected individuals so that groups did not show significant differences in age, ethnicity, country of origin, sex, body composition, dietary habits, and physical activity levels (see results section). To this aim, the selection was performed according to the following inclusion and exclusion criteria. Exclusion criteria were BMI <18 or ≥ 40; age <45 or >65; no fasting; missing info about LLDS; other ethnicities than Caucasian; other birthplaces than Netherlands; individuals who reported at baseline one or more of the following pathologies: cancer, stroke, diabetes, atherosclerosis, aneurism, or coagulopathy. Inclusion criteria were $18 \leq \text{BMI} < 40$, $45 \leq \text{age} \leq 65$, blood fasting samples available, available data about LLDS, Caucasian ethnicity, and Netherlands birthplace. This study design allowed comparison of matched cases and controls considering a significant number of new cases of heart attack over time in a restricted number of samples, controlling for numerous other confounding conditions.

2.4 | Relative quantification of buffy coat mtDNA copy number (BC-mtDNAcn)

Relative quantification of mtDNAcn (mtDNAcn/nuclear DNA copy number [nDNAcn]) has been assessed in gDNA extracted from buffy coat by qPCR using CFX96 (Biorad, Hercules, California, USA). The following genes have been amplified for the detection of mitochondrial and nuclear DNA, respectively, using the listed primers: mtDNA-tRNALeu (Fw: 5′-CACCCAAGAACAGGGTTTGT-3′; Rv: 5′-TGGCCATGGGTATGTTGTTA-3′) and beta-2-microglobulin(B2M)(Fw:5′-TGCTGTCTCCATGTTTGATGTATCT-3′; Rv: 5′-TCTCTGCTCCCCACCTCTAAGT-3′). These primers have been validated by Fazzini and colleagues[47] and verified for their specificity (unique amplification of mtDNA) and for the absence of co-amplified nuclear insertions of mitochondrial origin (NUMTs).

2.5 | Total cell-free DNA (tcf-DNA) extraction

Plasma samples were thawed at 37° for 5 min and mixed to avoid precipitation of insoluble particles that might reduce the yield of EVs isolation. According to Trumpff et al.,[14] plasma was further centrifuged at 5000 g for 10 min to remove potential residual platelets and large vesicular apoptotic bodies. Clean plasma was used for subsequent analyses. Total cell-free DNA (tcf-DNA) has been isolated from 480 μL of clear plasma using the Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Cat. 55100, Norgen, Thorold, ON, Canada) according to manufacturer's instruction.

2.6 | EVs isolation, characterization, and DNA extraction

Small EVs have been isolated from 850 μL of clear plasma using the Plasma/Serum Exosome Purification Mini Kit (Cat. 57400, Norgen, Thorold, ON, Canada). EVs quantification and characterization have been conducted using Dynamic Light Scattering (DLS) (Zetasizer Nano-S90, Malvern Panalytical, Malvern, UK) as

previously described.[48-55] For DLS analysis, 1000 μL of ultrapure water has been added to 5 μL of EVs extract and 1000 μL of the solution has been transferred in a polystyrene cuvette and equilibrated at 22°C. Z-Average size, Polydispersity Index (PDI), Derived Count Rate (DCR), and Zeta potential (ZP) have been measured. The Z-average size (nm) is defined as the intensity-weighted mean hydrodynamic size of the ensemble collection of particles measured by DLS. PDI is a dimensionless measure of the heterogeneity of a sample based on size.[56] The DCR (kpcs) indicates the number of photons collected by the light detector of the instrument in a second: higher DCR usually indicates higher concentrations, larger particles or higher concentration and larger particles. The ZP (mV), an indicator of colloidal stability, is influenced by the surface charge of extracellular vesicles.[57] The net surface charge of extracellular vesicles, indicated by the ZP, determines the stability of the particles or their tendency to aggregate.[57] After characterization, 100 μL of the solution containing EVs have been used to extract EV-DNA by the Qiamp DNA mini kit (Cat. 51304, Qiagen, Hilden, Germany) according to manufacturer's instructions.

2.7 | Quantification of mtDNA in tcf-DNA and EV-DNA by digital PCR

Absolute quantification of total cell-free mitochondrial DNA (tcf-mtDNA), total cell-free nuclear DNA (tcf-nDNA), EV-mtDNA, and extracellular vesicles nuclear DNA (EV-nDNA) has been performed by QIAcuity digital PCR (Qiagen, Hilden, Germany) according to manufacturer's indication. The imaging profiling has performed setting the instrument on an exposure duration of 350 ms and a gain of 4. MtDNA-tRNALeu and B2M were amplified to detect mtDNA or nuclear DNA (nDNA), respectively. Poisson statistics have been applied to calculate the average amount of target DNA per well (QIAcuity Software Suite 2.1.8.23, Qiagen, Hilden, Germany). A number of copies of target DNA contained in 1 mL of plasma were calculated accordingly.

2.8 | Statistical analysis

Statistical analysis was performed using SPSS (IBM, version 25, USA) and R studio (2023.06.0 + 421 version). Data were tested for normality using the Shapiro–Wilk test and log-transformed prior to analysis where necessary to normalize distribution. Parametric tests were such as unpaired student *t*-test or one-way ANOVA. Tukey's multiple comparison test was used as a post-hoc test to test significant difference between mean's groups. A receiver operating characteristic (ROC) analysis was performed, and the area under curve (AUC) was calculated to test predictiveness of the selected biomarkers. Significance was accepted with $p \leq .05$.

3 | RESULTS

3.1 | Descriptive statistics of the cohort and CVD risk biomarkers

Table 1 presents the descriptive statistics of the selected cohort. No differences between the three groups are measured for age (Kruskal–Wallis; $p = .907$), BMI (Kruskal–Wallis; $p = .680$), or sex distribution (Pearson's chi-square; $p = .970$), in accordance with the case–control design of the study. The three groups were exposed to current similar lifestyle habits among those that may impact cardiovascular risk (smoking, diet, physical activity). In particular, current smokers were not differently distributed among groups (Pearson's chi-square; $p = .551$). Dietary habits (measured considering the LLDS[46] as an index of the overall diet quality) were not significantly different among groups (ANOVA; $p = .457$). Moderate-to-vigorous physical activity was not different between groups neither for hours per week (Kruskal–Wallis; $p = .801$) nor for the activity score (Kruskal–Wallis; $p = .932$).

3.1.1 | Blood composition in the CVD groups

Given that changes in blood composition have been previously observed in patients with CVD or at risk,[58] and it has been hypothesized that thrombocyte levels may influence ccf-mtDNA levels,[14] data on the blood composition of participants have been analyzed. Table 2 shows the differences in blood composition in the three groups. A *p* for trend difference was observed for leukocytes (ANOVA, $p = .082$), where lower levels were measured in α than in β group ($p = .026$). A significantly different distribution between groups was measured for mononuclear cells (ANOVA, $p = .020$), with higher levels in β than α group ($p = .005$). Levels of eosinophilic granulocytes were different in the three groups (ANOVA, $p = .038$), with higher values in γ than α group ($p = .016$). No significant differences between groups were observed for other parameters describing blood cell composition (Table 2).

Difference between the groups in platelets/leukocytes ratio (P/L), a factor believed to influence the quantification of mtDNAcn in blood cells,[59] exhibited a *p* for trend (ANOVA,

TABLE 1 General descriptive statistics of the entire cohort ($N = 144$) and of the groups: α ($N = 44$), β ($N = 50$), α + β ($N = 94$) and γ ($N = 50$).

	α ($N = 44$)				β ($N = 50$)				α + β ($N = 94$)				γ ($N = 50$)				Whole Population ($N = 144$)			
	Min	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***
Age	45	65	55	6.0	45	65	55	6.1	45	65	55	6.1	45	65	55	6.9	45.0	65.0	55.1	6.3
BMI	22.3	38.6	28.0	4.0	22.3	37.8	27.4	3.6	22.2	38.6	27.7	3.8	22.3	35.0	27.7	2.9	22.3	38.6	27.7	3.5
	n		%		*n*		%		*n*		%		*n*		%		*n*		%	
Females	15		34		16		32		31		33		17		34		48		33	
Males	29		66		34		68		63		67		33		66		96		67	

Note: N = number of participants in the group.

Abbreviations: Max, maximum; Min, minimum; *N*, number; *SD*, standard deviation.

TABLE 2 Blood composition in the three groups (α, β, γ).

	α ($N = 44$)					β ($N = 50$)					γ ($N = 50$)					
	N	**Min**	**Max**	**Mean**	***SD***	***N***	**Min**	**Max**	**Mean**	***SD***	***N***	**Min**	**Max**	**Mean**	***SD***	***p***
Basophilic granulocytes (10^9/L)	43	0	0.08	0.030	0.016	49	0	0.07	0.033	0.016	49	0.01	0.15	0.032	0.022	.460
Eosinophilic granulocytes (10^9/L)	43	0.03	0.48	**0.173**	0.1	49	0.03	0.65	**0.235**	0.146	49	0.02	0.65	**0.233**	0.128	**.038**
Erythrocytes (10^{12}/L)	44	4.15	5.79	4.844	0.361	50	3.91	5.74	4.829	0.374	50	4.3	6	4.869	0.35	.856
Leukocytes (10^9/L)	44	2.4	10.2	**5.932**	1.50	50	2.7	13.7	**6.706**	2.187	50	4.1	9	**6.408**	1.111	**.082**
Lymphocytes (10^9/L)	43	0.79	4.07	2.034	0.601	49	1.02	3.01	2.172	0.558	49	1.21	3.95	2.165	0.568	.394
Mononuclear cells (10^9/L)	43	0.22	0.75	**0.470**	0.136	49	0.27	1.17	**0.574**	0.197	49	0.26	0.98	**0.518**	0.143	**.020**
Neutrophilic granulocytes (10^9/L)	43	1.9	7.38	3.284	1.068	49	1.05	7.34	3.547	1.479	49	2.06	5.88	3.416	0.802	.710
Thrombocytes (10^9/L)	44	163	363	242.6	53.184	50	118	358	242.28	61.217	50	160	378	234.62	45.991	.707

Abbreviations: Max, maximum; Min, minimum; *N*, number; *p*, overall *p*-value.; *SD*, standard deviation.

Significant results are in bold.

$p = .059$). In particular, P/L was higher in α (P/L = 43.1 ± 12.6) than γ (P/L = 37.4 ± 8.4) ($p = .033$) or β (P/L = 38.7 ± 13.0) ($p = .042$). β P/L was not different to γ P/L ($p = .913$).

3.1.2 | Classical CVD risk biomarkers

TG/HDL ratio (considered a CVD risk biomarker[41,42]) was different between groups (Kruskal–Wallis; $p = .026$). γ group showed a TG/HDL ratio significantly higher than α ($p = .008$). No significant differences were measured between α and β ($p = .076$) or β and γ ($p = .355$). The three groups did not show significant differences for the SCORE2 profile (Kruskal–Wallis, $p = .060$), which is considered an established predictor of CVD.[40] Results are shown in Table 3.

3.2 | mtDNAcn from buffy coat (BC-mtDNAcn) in different CVD statuses

Relative quantification of buffy coat mtDNAcn (mtDNAcn/nDNAcn) was not associated to age ($p = .703$), which was suggested to affect this parameter in previous studies.[59] This observation could potentially be attributed to the relatively narrow age range within the cohort (Table 1). BC-mtDNAcn was significantly correlated with blood composition. In particular, mtDNAcn was positively correlated with leukocyte levels (Pearson's correlation = .264, $p = .002$), especially mononuclear cells (Pearson's correlation = .232, $p = .006$). A nominal correlation was detected also with neutrophils (Pearson's correlation = .188; $p = .027$) and basophilic granulocytes (Pearson's correlation = .178; $p = .037$) but not with eosinophils ($p = .487$). No correlation with platelets, the other major cellular component of buffy coat, was measured ($p = .219$). Since P/L is considered a confounding factor for mtDNAcn assessments in blood,[59] we tested the association between BC-mtDNAcn and this parameter. Results showed a significant correlation between BC-mtDNAcn and P/L ratio (Pearson's correlation = −.177; $p = .036$).

No differences were observed for BC-mtDNAcn between individuals who were healthy at baseline (α + β) and CVD cases (γ) ($p = .558$). Given the previously mentioned correlation with blood cells, we normalized BC-mtDNAcn for platelet/leukocyte ratio. Still, no significant differences were measured ($p = .995$) (Figure 2A). No significant differences were measured by distinguishing between healthy controls (α), individuals who reported a heart attack in 1.5y (β), and previous CVD cases (γ) ($p = .442$) (Figure 2B).

3.3 | mtDNA from total cell-free DNA (tcf-mtDNA)

Mean levels of total cell-free mitochondrial DNA copy number (tcf-mtDNAcn) were 48068.72 ± 41744.96 copies/mL, while levels of total cell-free nuclear DNA copy number (tcf-nDNAcn) were 1407.52 ± 859.51 copies/mL of plasma. As expected, tcf-mtDNAcn was higher than tcf-nDNAcn detected in the same starting volume of plasma (1 mL). No differences in tcf-mtDNAcn ($p = .510$) (Figure 3A) or tcf-nDNAcn (Figure 3B) ($p = .387$) or tcf-mtDNAcn/tcf-nDNAcn ratio ($p = .922$) have been observed between subjects that were healthy at baseline (α + β) and CVD cases (γ). No differences in tcf-mtDNAcn ($p = .796$) (Figure 3C) or tcf-nDNAcn ($p = .134$) (Figure 3D) or tcf-mtDNAcn/nDNAcn ($p = .296$) amount have been measured between groups.

No correlations between tcf-DNAcn and blood composition (data not shown) were measured, except for tcf-mtDNAcn/nDNAcn ratio, which was significantly correlated with leukocyte abundance in blood (Pearson's correlation = .235; $p = .005$). No correlations between the BC-mtDNAcn and tcf-mtDNAcn ($p = .444$) or tcf-mtDNAcn/nDNAcn ($p = .450$) were measured.

3.4 | Plasma EVs characterization and EV-DNA cargoes

3.4.1 | Plasma EVs characterization

DLS results show a homogeneous population of isolated extracellular vesicles (PDI: 0.29 ± 0.19). DCR, Z-average, and ZP for the overall group are shown in Table 4. No

TABLE 3 Classical biomarkers of CVD (TG/HDL and SCORE 2) in the three groups: α ($N = 44$), β ($N = 50$), and γ ($N = 50$).

	α ($N = 44$)				β ($N = 50$)				γ ($N = 50$)				
	Min	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	***p***
TG/HDL	0.21	7.49	**1.132**	1.250	0.152	5.100	**1.306**	1.024	0.367	3.664	**1.346**	0.802	**.026**
SCORE2	0.010	0.160	0.042	0.028	0.012	0.104	0.046	0.022	0.008	0.086	0.035	0.017	.060
SCORE2 (%)	0.949	16.014	4.190	2.770	1.181	10.426	4.566	2.192	0.826	8.606	3.458	1.686	.060

Abbreviations: Max, maximum; Min, minimum; *N*, number; *p* = *p*-value; *SD*, standard deviation.

Significant results are in bold

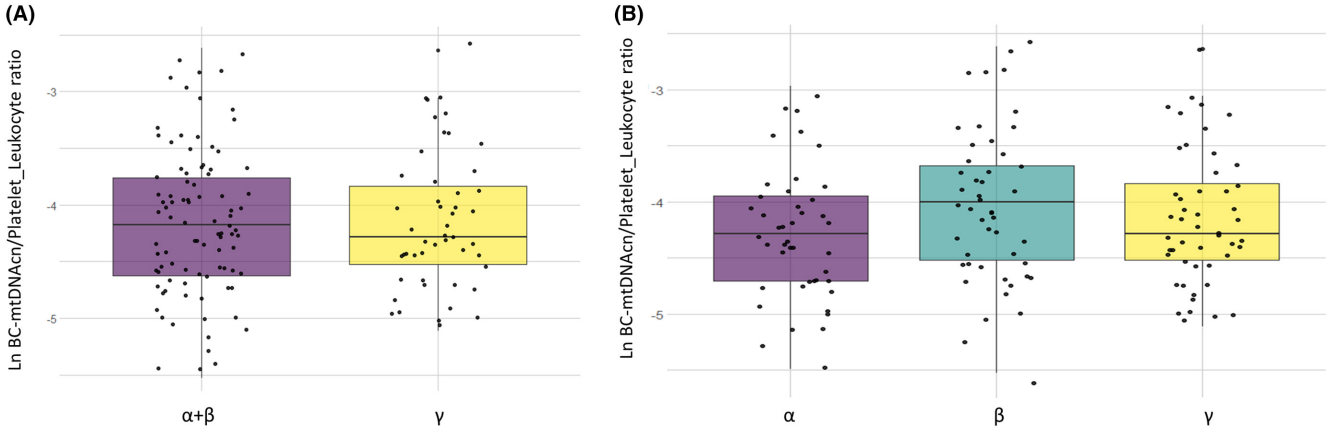

FIGURE 2 BC-mtDNAcn normalized for platelets/leukocytes ratio in (A) healthy individuals at baseline (α + β) and CVD cases (γ) or (B) in the three groups α, β, and γ.

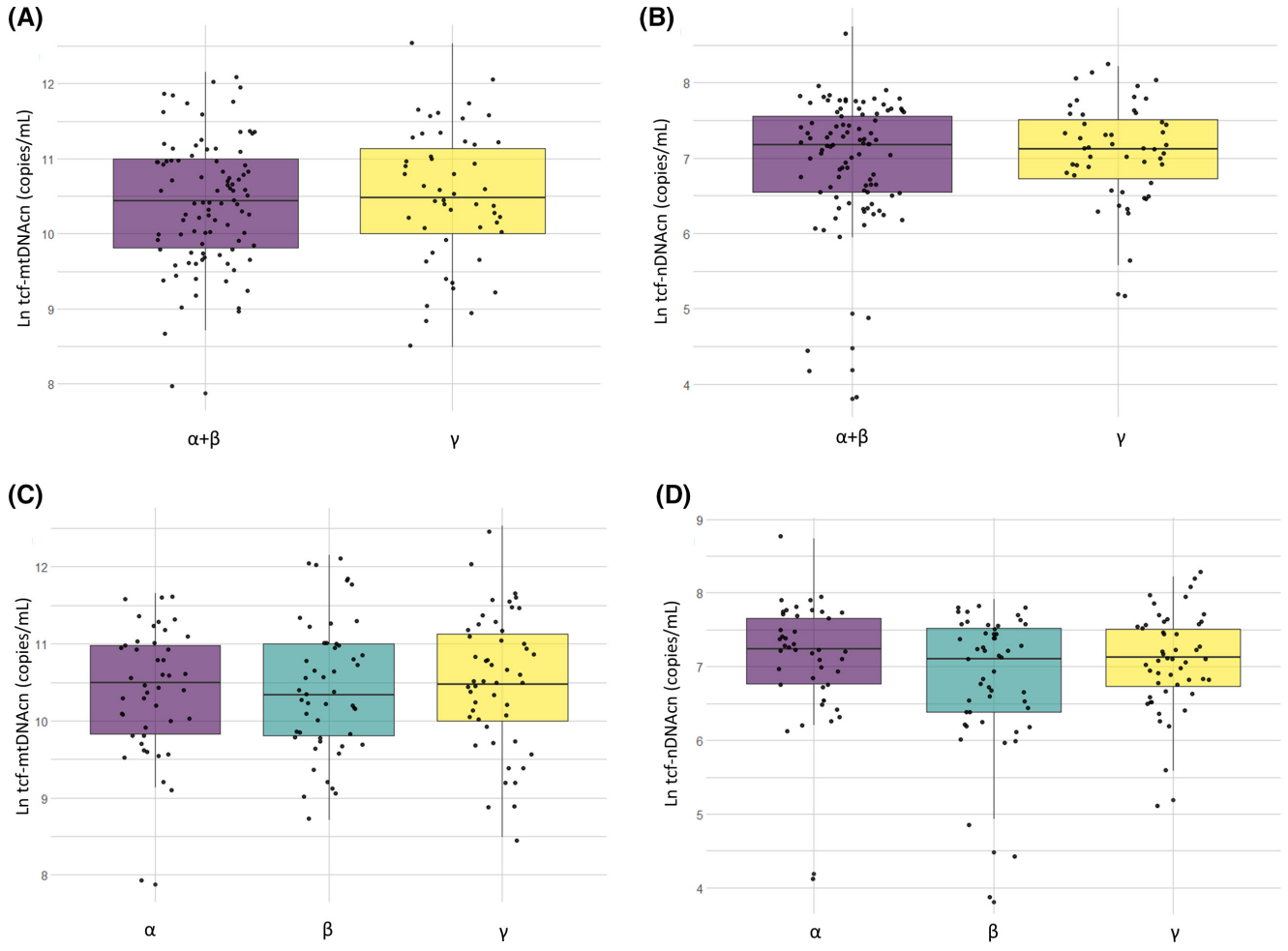

FIGURE 3 Tcf-mtDNAcn and tcf-nDNAcn measured in healthy subjects at baseline (α + β) versus CVD cases (γ) (A, B). Tcf-mtDNAcn and tcf-nDNAcn measured in the three groups α, β, and γ (C, D).

significant differences were measured in terms of DCR and PDI (Table 4). Thus, no differences in DNA cargoes between CVD groups can be attributed to differential processing of the samples during EVs extraction. Also, no significant differences among groups were observed for EVs Z-average and ZP (Table 4), suggesting that no major differences in EVs dimension and charge can be measured in different cardiovascular health statuses.

TABLE 4 Characterization of plasma EVs by DLS in α ($N=44$), β ($N=50$), and γ ($N=50$) groups.

	α ($N=44$)				β ($N=50$)				γ ($N=50$)				
	Min	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	**Min**	**Max**	**Mean**	***SD***	***p***
DCR (kcps)	182	5417	1640	1177	306	7413	1453	1316	343	7473	2001	1726	.22
Z-average (nm)	105	357	236	62	118	368	242	63	120	366	241	60	.87
PDI	0.03	0.69	0.27	0.18	0.39	0.77	0.31	0.19	0.04	0.68	0.30	0.20	.53
Z-potential (mV)	−25.6	−3.0	−11.3	4.8	−22.4	−2.4	−11.3	3.9	−25.6	−3.1	−10.6	4.9	.61

Note: Kruskal–Wallis test was applied.

Abbreviations: Max, maximum; Min, minimum; *N*, number; *p*, *p*-value; SD, standard deviation.

3.4.2 | Quantification of mtDNA and nDNA in plasma EVs

Both mtDNA and nDNA were detected by QIAcuity dPCR in the DNA extracted from EVs. In particular, EV-mtDNAcn plasma levels were 1840.0 ± 2663.5 copies/mL, while EV-nDNAcn was 126.4 ± 105.7 copies/mL of plasma. EV-mtDNAcn/EV-nDNAcn ratio was 51.00 ± 83.99 in the whole sample.

EV-mtDNAcn was significantly positively correlated with the DCR (Pearson's correlation = .294; $p = 3.5*10^{-4}$), proving that mtDNA is a cargo in the isolated EVs. Since the DCR depends on the number of particles and their average size, we also tested the correlation between the EV-mtDNAcn and the DCR adjusting for the average size. The correlation between mtDNAcn and DCR is confirmed (β = 0.328, $p = .003$), with no contribution of the average size ($p = .766$) to the model. This corroborates the hypothesis that EV-mtDNcn is correlated with the abundance of EVs, and even eventual nanoparticle aggregates are not responsible for differences observed between groups. EV-mtDNAcn was correlated with Z-average ($p = .044$) but not with the ZP ($p = .574$). Since ZP depends on the EVs surface charge, these results suggest that it is unlikely that mtDNA is passively carried on the surface of the EVs, while it is rather carried as expected within EVs.

On the contrary, EV-nDNA was not correlated to extracellular vesicles DCR ($p = .315$), Z-average ($p = .626$), or ZP ($p = .284$). This suggests that the low level of nDNA measured in EVs is likely a residual contamination rather than a real EVs cargo. According to this hypothesis, the ratio EV-mtDNA/EV-nDNA was not associated to exosomes DCR ($p = .725$), Z-average ($p = .427$), or ZP ($p = .445$).

EV-mtDNA was not associated to the blood composition in the whole group (Additional file 1: Table S1). EV-nDNAcn was correlated to the levels of basophilic granulocytes (Pearson's correlation = 0.272; $p = .001$) and mononuclear cells (Pearson's correlation = .205; $p = .017$). EV-nDNA was also correlated with BC-mtDNAcn (Pearson's correlation = .223; $p = .009$), tcf-mtDNAcn (Pearson's correlation = .209; $p = .013$), and tcf-nDNAcn (Pearson's correlation = .226; $p = .007$). This evidence (EV-nDNA correlating to total levels of DNA circulating in plasma but not to the abundance of EVs) supports the hypothesis that EV-nDNA detected in these samples is rather a contamination than EV-DNA cargo. On the contrary, EV-mtDNAcn (which was associated to the abundance of EVs in the sample) was correlated neither with BC-mtDNAcn nor with the tcf-mtDNAcn or tcf-nDNAcn levels (Table 5). This supports the hypothesis that EV-mtDNAcn is due to a biological phenomenon independent of the total cell-free DNA levels.

3.4.3 | EVs DNA cargoes in CVD groups

Individuals who were healthy at baseline (α + β) had lower levels of EV-mtDNAcn than in CVD cases (γ) ($p = .006$) (Figure 4A). In particular, the γ group showed significantly higher levels of EV-mtDNAcn than the α group ($p = .019$) and the β group ($p = .016$), with no differences between α and β groups ($p = .995$) (Figure 4C). The association between EV-mtDNAcn and CVD status remained significant (β = 0.195; $p = .022$) also adjusting the analysis for the blood composition (that differed between CVD groups), suggesting that increased EV-mtDNAcn is not a direct consequence of blood cell composition differences.

EV-nDNAcn was significantly higher in CVD cases than in healthy subjects at baseline ($p = .033$) (Figure 4B). Also, EV-nDNAcn was significantly higher in γ than β group ($p = .009$), but it did not differ from α group ($p = .167$) (Figure 4D). However, a multivariate linear regression model adjusted for blood composition showed no significant association between EV-nDNAcn and CVD status ($p = .477$). Similarly, no significant associations were detected adjusting for DCR, Z-average, or ZP between EV-nDNA with the CVD status ($p = .326$). On the contrary, the association between EV-mtDNAcn and CVD status was significant (β = 0.180; $p = .025$) even adjusting the model for the DCR (that is per se associated to the EV-mtDNAcn; β = 0.294; $p = .003$), the Z-average, and the ZP (which do not contribute to this association). These results suggest

TABLE 5 Pearson correlation coefficient (r) and *p*-value (*p*) of the correlation between BC-mtDNAcn, Tcf-mtDNAcn, Tcf-nDNAcn, Tcf-mtDNAcn/Tcf-nDNAcn, EV-mtDNAcn, EV-nDNAcn, and EV-mtDNAcn/EV-nDNAcn.

	BC-mtDNAcn		Tcf-mtDNAcn		Tcf-nDNAcn		Tcf-mtDNAcn/ Tcf-nDNAcn		EV-mtDNAcn		EV-nDNAcn		EV-mtDNAcn/ EV-nDNAcn	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
BC.-mtDNAcn	1		−0.065	.444	−0.154	.068	0.064	.450	0.163	.054	**0.223**	**.009**	0.064	.450
Tcf-mtDNAcn	−0.065	.444	1		**0.288**	**$4*10^{-4}$**	**0.658**	**$3*10^{-19}$**	−0.032	.699	**0.209**	**.013**	**0.658**	**$3*10^{-5}$**
Tcf-nDNAcn	−0.154	.068	**0.288**	**$4*10^{-4}$**	1		**−0.532**	**$6*10^{-12}$**	−0.057	.495	**0.226**	**.007**	**−0.532**	**$6*10^{-12}$**
Tcf-mtDNAcn/Tcf-nDNAcn	0.065	.450	**0.658**	**$3*10^{-19}$**	**−0.532**	**$6*10^{-12}$**	1		0.016	.845	0.016	.851	**1**	**0**
EV-mtDNAcn	0.163	.054	−0.032	.699	−0.057	.495	0.016	.845	1		0.187	.28	0.16	.845
EV-nDNAcn	**0.223**	**.009**	**0.209**	**.013**	**0.226**	**.007**	0.016	.851	0.187	.28	1		−0.016	.851
EV-mtDNAcn/EV-nDNAcn	0.064	.450	**0.658**	**$3*10^{-5}$**	**−0.532**	**$6*10^{-12}$**	**1**	**0**	0.016	.845	0.016	.851	1	

Significant results are in bold

that both the EVs abundance and their mtDNA cargoes contribute to explain the difference between the three groups.

To understand if the concentration of circulating EVs (DCR) and/or EV-mtDNAcn were able to distinguish between healthy subjects (α+β) and CVD patients (γ), we performed a multivariate logistic regression. The analysis showed that EV-mtDNAcn (β=1.673; $p=.019$), but not the EVs DCR ($p=.410$), significantly contributed to the prediction model. This suggests the hypothesis that EV-mtDNAcn cargo (rather than the number of EVs) is the major driver of the association between EV-mtDNAcn and the cardiovascular health status.

Considering the possibility to distinguish in advance subjects that are going to develop CVD from really healthy subjects (i.e., comparing α with β group), neither EV-mtDNAcn ($p=.994$) nor EVs DCR ($p=.396$) were able to predict the onset of CVD in advance.

3.5 | CVD risk prediction

No significant correlations were measured between CVD risk biomarkers such as TG/HDL or SCORE2 and BC-mtDNAcn, tcf-mtDNAcn, or EV-mtDNAcn (Table 6). However, SCORE2 was correlated to the EVs DCR (total number of exosomes isolated from the same amount of plasma) (β=0.171; $p=.049$), independently from the EV-mtDNAcn (β=0.014; $p=.875$), and the CVD status (β=−0.152; $p=.072$).

To assess the predictive power and compare the selected biomarkers for CVD, we conducted ROC analysis. Results showed that only EV-mtDNAcn (AUC=0.648; $p=.002$) was predictive of CVD presence at baseline (α+β vs. γ). A *p* for trend was measured for TG/HDL (AUC=0.595; $p=.051$). No significant results were obtained for SCORE2 (AUC=0.411; $p=.068$), tcf-mtDNAcn (AUC=0.544; $p=.390$), or BC-mtDNAcn (AUC=0.450; $p=.337$) (Figure 5A). Testing the predictiveness of heart attack in advance (α vs. β), none of the classifiers resulted to be significantly associated to the outcome [EV-mtDNAcn, AUC=0.648; $p=.002$; TH/HDL, AUC=0.595; $p=.051$; SCORE2, AUC=0.570; $p=.068$; tcf-mtDNAcn, AUC=0.544; $p=.390$; BC-mtDNAcn, AUC=0.450; $p=.337$] (Figure 5B).

4 | DISCUSSION

A large body of literature describes the link between CVD and inflammation,[60] which in turn is connected to mitochondrial homeostasis.[61] Remarkably, Chen et al. recently showed that small EVs from young plasma reverse

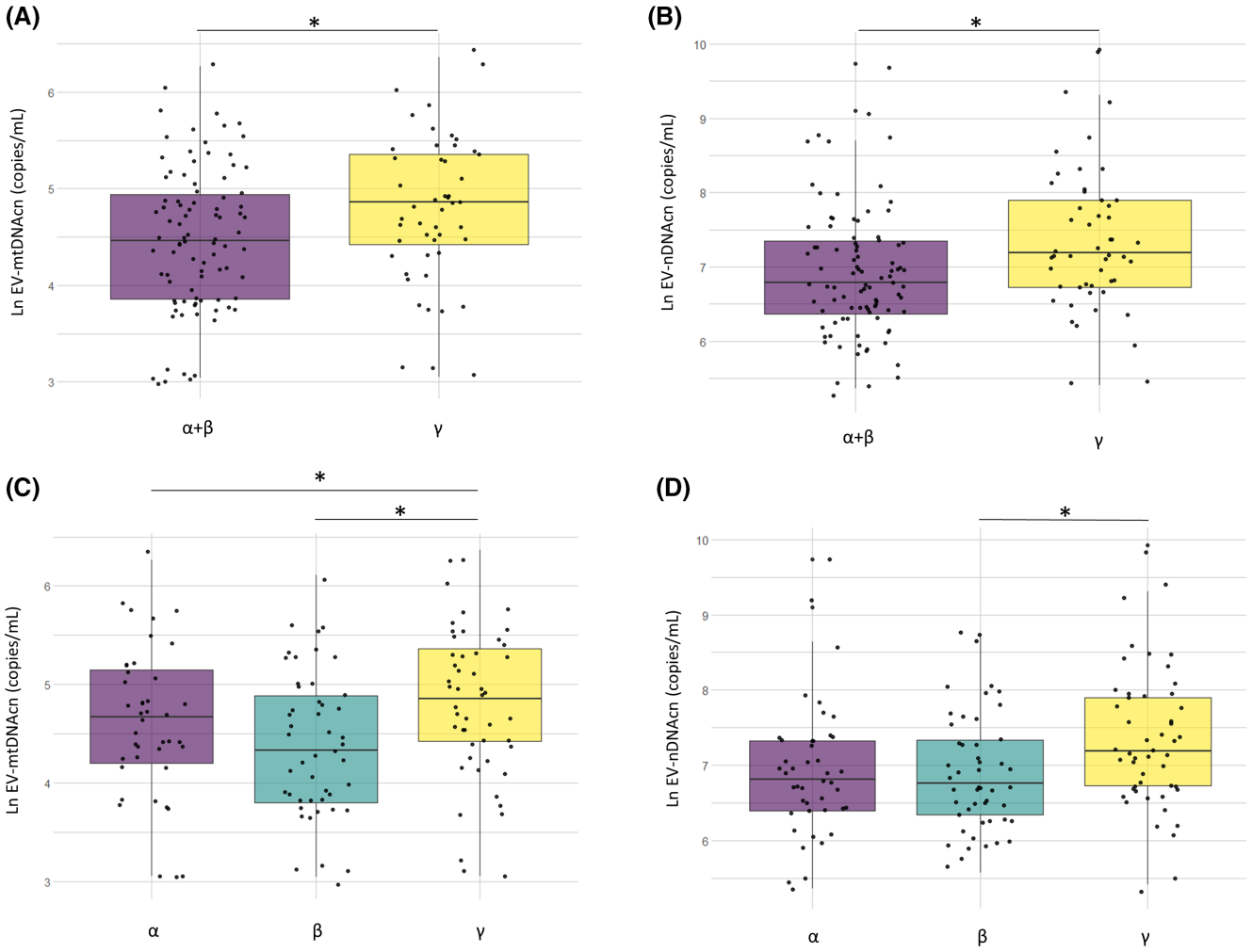

FIGURE 4 EV-mtDNAcn and EV-nDNAcn measured in healthy subjects at baseline (α + β) and in CVD cases (γ) (A, B). EV-mtDNAcn and EV-nDNAcn measured in the three groups α, β, and γ (C, D). *$p < .005$.

TABLE 6 Pearson coefficient and *p*-value (*p*) of the correlation between BC-mtDNAcn, Tcf-mtDNAcn, EV-mtDNAcn, and TG/HDL ratio and SCORE2.

	BC-mtDNAcn	*p*	Tcf-mtDNAcn	*p*	EV-mtDNAcn	*p*
TG/HDL ratio	−0.032	.704	−0.035	.675	0.089	.289
SCORE2	0.075	.376	−0.064	.447	0.034	.686

age-related functional declines by improving mitochondrial energy metabolism, suggesting a functional link between EVs and mitochondrial functions.[62] Intracellular mtDNAcn, initially proposed as a surrogate biomarker of mitochondrial functions, has been associated to metabolic and cardiovascular health in humans.[12] Also, ccf-mtDNA may be implicated in the pathogenesis of CVD, owing to its potential pro-inflammatory properties.[14,20,22] Thus, the hypothesis that mtDNAcn might be used as a predictive tool for CVD prevention and risk stratification has been postulated.[18] Previous studies have shown an inverse correlation between whole blood[63-65] or BC-mtDNAcn[18,66] (relative to nDNA) and both prevalent and incident CVD.[18] However, concerns about this measurement have been raised,[59] particularly considering that blood composition might influence this parameter. This concern arises from the varied abundance of mtDNAcn in different cell types, with platelets, in particular, contributing to the measurement with mtDNA but not nDNA. Therefore, mtDNAcn measured from whole blood or buffy coat may serve as an index of overall blood composition rather than specifically reflecting mitochondrial functions. In our study, BC-mtDNAcn was not different in subjects that were healthy at baseline (α + β) than in CVD cases (γ). No significant difference has been seen neither in adjusting the analysis for platelet/leukocyte ratio. The BC-mtDNAcn was

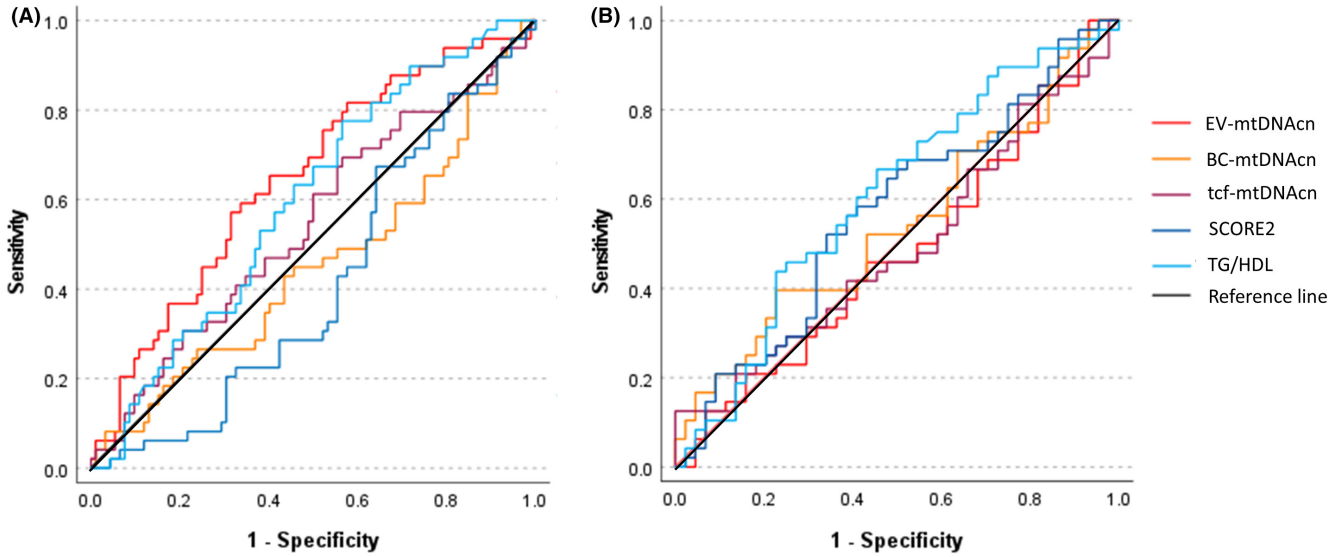

FIGURE 5 ROC curve analysis evaluating the predictiveness of the selected biomarkers for CVD presence at baseline (A) or after 1.5 years from baseline (B).

not associated to SCORE2 or TG/HDL. Despite platelets and leukocytes being major contributors to the release of ccf-mtDNA, no correlations between BC-mtDNAcn and tcf-mtDNAcn were observed. It is worth noting that platelets and leukocytes, while significant contributors, are not the sole contributors to this phenomenon.[14] In any case, BC-mtDNAcn was not informative of the cardiovascular health status in this cohort.

While numerous studies have tested the associations between CVD and mtDNAcn from whole blood, there is a scarcity of data from human cohorts concerning ccf-mtDNAcn in CVD. Berezina et al.[67] showed that heart failure patients ($N = 120$) have higher ccf-nDNAcn but lower ccf-mtDNAcn than controls ($N = 120$). In contrast, Liu et al.[24,68] measured an increase in mtDNA in diabetic patients with coronary heart disease (CHD) ($N = 50$) compared to those without CHD ($N = 44$). Wiersma et al.[69] showed increased levels of ccf-mtDNAcn in paroxysmal atrial fibrillation ($N = 100$) but reduced levels in persistent atrial fibrillation ($N = 116$) and longstanding-persistent atrial fibrillation ($N = 20$) compared to controls ($N = 84$). Ueda et al.[70] measured higher levels of both ccf-mtDNAcn and ccf-nDNAcn in patients with atherosclerotic plaques ($N = 62$) than controls ($N = 21$). Only Ye et al.[39] selectively investigated the plasma exosomes-derived mtDNA (by droplet digital PCR), showing that both the plasma exosome particle numbers and the exosomal mtDNAcn were elevated in chronic heart failure patients ($N = 20$) compared to controls ($N = 20$). In our study, no significant differences were observed between healthy subjects ($\alpha + \beta$) and CVD patients (γ) in terms of mtDNAcn measured in the total fraction of cell-free DNA (tcf-mtDNAcn). Tcf-mtDNAcn was not different neither between α and β groups. However, the specific fraction of mtDNAcn carried in EVs was higher in CVD patients (γ) than healthy subjects ($\alpha + \beta$), while no difference was observed between individuals who reported a heart attack after 1.5 years (β) and those remaining healthy in the following 6 years (α). The association between EV-mtDNAcn and CVD status remained significant even after adjusting the analysis for both the abundance of EVs and blood composition. This observation suggests that variations in EV-mtDNAcn may offer more insights into cardiovascular health compared to tcf-mtDNAcn. This is significant because tcf-mtDNA includes both passively released mtDNA (resulting from necrosis or apoptosis) and actively released mtDNA, while EV-mtDNA specifically originates from an active and regulated process.[14] Little is known about the mechanistic explanation of the packaging of mtDNA in EVs but increasing attention has recently started to be addressed to this phenomenon. EVs containing mtDNA have been hypothesized to derive from mitochondria-derived vesicles,[22] given that they transport mitochondrial proteins.[71] However, a recent study denied the presence of mtDNA in mitochondria-derived vesicles[72] proposing the hypothesis that different mechanisms could be implicated in the translocation of mtDNA to EVs, or that other transporters of mtDNA from mitochondria to EVs may exist.[22] Our results lead us to speculate that cells may initiate an active response to CVD, resulting in the packaging of mtDNA inside EVs released into the bloodstream. Indeed, a positive correlation between the EV-mtDNAcn and EVs abundance was measured in our cohort. The role of mtDNA transfer by EVs in CVD has been previously investigated,[73] especially in vitro.[74,75]

While the molecular pathways activated by mtDNA in EVs are only partially defined, compelling evidence regarding the pro-inflammatory potential of ccf-mtDNA has been gathered.[20,22,76] Fan et al.[77] showed increased levels of inflammatory biomarker in chronic kidney disease patients with high mtDNAcn. Also, mtDNA released within exosomes has been recently shown to promote inflammation in Behçet's syndrome, a chronic systemic inflammatory disorder.[78] Indeed, exosomes, which represent a large part of EVs, play a crucial role in the process of intracellular and inter-organ communication transporting fundamental biological signals which can have paracrine or long-rage effects.[34] The uptake of exosomes by recipient cells involves the endosomal pathway.[79] As a DAMP, mtDNA may activate TLR9, cGAS-STING, and NLRP3.[22] Of note, previous studies have shown that the cGAS-STING-IRF3 or the STING–NF-kB pathway is activated when oxidized mtDNA leaks into the cytosol.[80] Also, the activation of the NLRP3 inflammasome requires the release of oxidized mtDNA.[81] These findings suggest that biochemical modifications of mtDNA (not limited to oxidation but potentially including other modifications, such as methylation or hydroxymethylation) might represent an additional layer of regulation of these mechanisms and modulate the pro-inflammatory potential of the mtDNA over long distances. This interesting hypothesis might represent an additional level of regulation of mtDNA pro-inflammatory effects, warranting further investigations currently ongoing in our laboratories.

A role for exosomes per se has been suggested in the development of CVD,[30,33] with previous literature showing higher levels of plasma exosomes in chronic heart failure patients and acute ischemic stroke patients than controls.[39,82] In our study, EV-mtDNAcn remained associated with CVD even after adjusting for the characteristics of EVs and blood composition, suggesting that the cargo, rather than the number and size of EVs, may play a crucial role in the biological phenomena occurring after a heart attack. Nevertheless, we found a correlation between the abundance of EVs and SCORE2, an international index considered a predictor of CVD in the long range,[40] corroborating the importance of EVs in defining the risk for CVD.

In our study, EV-mtDNAcn was not associated with the EVs surface charge, suggesting that the DNA is contained within the vesicles rather than being externally associated with the vesicle surface. This evidence aligns with our hypothesis, suggesting a cellular response in the context of CVD, leading to the active packaging of mtDNA as a cargo within EVs. The presence of double-stranded DNA on the surface of EVs has been previously reported.[83] Complexes constituted by double-stranded DNA and histones such as H2A, H2B, and H3 have been found on the surface of exosomes.[84] In line with this evidence, a few copies of nuclear DNA were also detected in EVs in our study, where EV-nDNAcn was higher in CVD patients than in subjects who were going to develop a heart attack. No differences have been seen in the EV-nDNAcn levels between CVD patients and controls. However, EV-nDNAcn was not associated to the abundance of EVs (measured as DCR by DLS), while it was correlated with both mtDNAcn and nDNAcn from the overall cell-free fraction, as well as to the number of blood mononuclear cells and basophilic granulocytes. This hinted at the hypothesis that the few copies of nDNA detected in our EVs samples could potentially be passively carried on the surface of the vesicles rather than being a real EVs cargo. Also, nDNA detected in EVs might be a remnant of nDNA from the total cell-free DNA fraction. Indeed, despite some studies that reported nDNA in EVs,[85,86] the presence of genomic DNA in EVs is still a matter of debate. In particular, the mechanism by which nDNA, which is compartmentalized in the nucleus, is transported into EVs remains an open question.[87] A hypothesis posits that micronuclei, structural formations in the nuclear membrane that arise during cell division in the event of errors in chromosome distribution, may collapse, releasing their DNA content into the cytoplasm.[87] In turn, the nDNA released by micronuclei may be loaded into exosomes. Indeed, higher levels of DNA have been found in the exosomes produced by cancer cells or by cells exposed to genotoxic conditions which contains a higher number of micronuclei.[86-88] Despite this intriguing hypothesis, our findings do not confirm that the low levels of nDNA detected in EVs, even with advanced and highly sensitive technologies like digital PCR, represent a reliable signal.

Concerning the possibility to predict CVD, neither classical predictors (TG/HDL,[41] SCORE2[40]) nor levels of mtDNAcn (both in buffy coat or EVs) were able to predict heart attack 1.5y in advance (distinguishing α from β group). In contrast, SCORE2 was significantly able to predict CVD onset after 6y.

This study shows preliminary findings, and some drawbacks have to be acknowledged. The first limitation arises from the unavailability of blood samples from acute cases of heart attack for analysis. These samples would have been valuable as positive controls. Unfortunately, this constraint originates because the LifeLines cohort primarily focuses on studying healthy individuals. Consequently, we weren't able to identify a significant number of samples still matching cases and controls by adhering to the selection and matching criteria. The sample size of 144 individuals is a second limitation, potentially increasing the risk of false negative outcomes. However, this risk is mitigated by the study's robust case–control design, which is founded

on stringent inclusion and exclusion criteria outlined earlier. These criteria consider not only information at basal assessment (i.e., age, smoking, diet, physical activity, other disease presence) but also prospective disease onset (i.e., we excluded individuals who developed any other diseases than heart attack in 1.5y). For this reason, this is a unique cohort, where differences between samples are likely attributed to the presence or onset of heart attack, controlling for numerous confounding factors, which are rarely used as selection criteria in bigger cohorts. Unfortunately, it was not possible to consider familial history for CVD and to compare predictiveness of mtDNAcn with other specific biomarkers of cardiovascular health in this cohort. Future studies addressing this research question in larger cohorts and including these information would enhance the potential for translating the evidence into clinical practice.

In conclusion, risk stratification and prediction of cardiovascular event remains a challenge, emphasizing the need for further research investments. This is particularly crucial given the substantial impact of these pathologies on the health system. Although several studies suggested the usage of mtDNAcn as a predictor of CVD,[2,18,19,89-91] applications of this evidence in clinical practice remain to be validated. Nevertheless, our preliminary findings suggest EVs and their cargoes (including mtDNA) as a promising and novel focus for a better understanding of CVD pathophysiology. Further research is warranted to investigate how mtDNA released in plasma exerts its biological effects, especially in the context of inflammation-driven pathologies.

AUTHOR CONTRIBUTIONS

Chiara Rucci: sample analysis, manuscript drafting; Gaia de Simone: sample analysis; Saniya Salathia: DLS analysis for EVs characterization; Roberta Censi and Cristina Casadidio: manuscript revision, supervision of EVs characterization; Laura Bordoni: conceptualization, data analysis and interpretation, manuscript drafting and revision, supervision, found acquisition.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data may be obtained from a third party and are not publicly available. Researchers can apply to use the LifeLines data used in this study. More information about how to request LifeLines data and the conditions of use can be found on their website (https://www.lifelines.nl/researcher/how-to-apply).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The LifeLines study was approved by the ethics committee of the University Medical Centre Groningen, document number METC UMCG METc 2007/152. LifeLines operates with the highest ethical standards and strictly controls and takes into account rules regarding the irreversible pseudonimization of participants, encryption of data, use of trusted third parties (TTPs), and controlled data access. This will guarantee for both personal data and samples respect the EU Directive 2004/23 on standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells and EU Regulation 2016/679 of the European Parliament and of the Council of April 27, 2016, on the protection of natural persons with regard to the processing of personal data and on the free movement of such data. All participants signed an informed consent (http://wiki.lifelines.nl/doku.php?id=informed_consent).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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