Mitochondrial DNA copy number is associated with mortality and infections in a large cohort of patients with chronic kidney disease

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Damage of mitochondrial DNA (mtDNA) with reduction in copy number has been proposed as a biomarker for mitochondrial dysfunction and oxidative stress. Chronic kidney disease (CKD) is associated with increased mortality and risk of cardiovascular disease, but the underlying mechanisms remain incompletely understood. Here we investigated the prognostic role of mtDNA copy number for cause-specific mortality in 4812 patients from the German Chronic Kidney Disease study, an ongoing prospective observational national cohort study of patients with CKD stage G3 and A1-3 or G1-2 with overt proteinuria (A3) at enrollment. MtDNA was quantified in whole blood using a plasmid-normalized PCR-based assay. At baseline, 1235 patients had prevalent cardiovascular disease. These patients had a significantly lower mtDNA copy number than patients without cardiovascular disease (fully-adjusted model: odds ratio 1.03, 95% confidence interval [CI] 1.01-1.05 per 10 mtDNA copies decrease). After four years of follow-up, we observed a significant inverse association between mtDNA copy number and all-cause mortality, adjusted for kidney function and cardiovascular disease risk factors (hazard ratio 1.37, 95% CI 1.09-1.73 for quartile 1 compared to quartiles 2-4). When grouped by causes of death, estimates pointed in the same direction for all causes but in a fully-adjusted model decreased copy numbers were significantly lower only in infection-related death (hazard ratio 1.82, 95% CI 1.08-3.08). A similar association was observed for hospitalizations due to infections in 644 patients (hazard ratio 1.19, 95% CI 1.00-1.42 in the fully-adjusted model). Thus, our data support a role of mitochondrial dysfunction in increased cardiovascular disease and mortality risks as well as susceptibility to infections in patients with CKD.

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Mitochondria are double-membrane organelles located in the cytosol of eukaryotic cells. They play a critical role in energy production of the cell by synthesizing adenosine triphosphate through the process of oxidative phosphorylation. Mitochondria are dynamic organelles, as their number can fluctuate by continuous cycles of fission and fusion processes that help to maintain a functional balance when cells experience metabolic demand or environmental stress.[1]

Mitochondria play a central role in kidney function, as the kidney is second to only the heart in oxygen consumption and mitochondrial count.[2] Kidneys require an enormous amount of energy to perform their tasks.[3] Mitochondria are especially abundant in proximal tubular cells as they are involved in reabsorption of filtrate[4] and secretion of toxins. Accumulating experimental evidence shows an increase of fragmented mitochondria in kidney tubules in CKD models.[5,6] Moreover, a role of mitochondrial dysfunction in renal pathologies has been described in several studies,[7–10] and persistent mitochondrial dysfunction contributes to renal impairment leading to CKD.[4] However, the mechanism underlying this relationship is still poorly understood. It has been hypothesized that increased production of mitochondrial reactive oxygen species (ROS), a common feature in acute kidney injury[8] (AKI) and CKD,[11] can contribute to an imbalance in

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mitochondrial homeostasis and consequently to mitochondrial dysfunction. In light of these findings, improving mitochondrial function has been proposed as a therapeutic strategy in AKI[9,12] and CKD.[13]

Moreover, evidence indicates that oxidative stress in patients with CKD is not limited to the kidneys and may contribute to an increased risk of cardiovascular disease and premature mortality in CKD patients.[14] MtDNA copy number in circulating white blood cells may serve as a surrogate for mitochondrial dysfunction caused by systemic oxidative stress.[15] However, only a few studies have investigated the association between mtDNA copy numbers and clinical outcomes in CKD patients.[16–19] These studies are characterized by small sample sizes and have led to conflicting results. We therefore explored the association of mtDNA copy numbers with prevalence of cardiovascular disease (CVD), as well as all-cause and cause-specific mortality in 4812 patients of the German Chronic Kidney Disease (GCKD) study, a prospective national CKD cohort study, during a 4-year follow-up.

RESULTS

Baseline characteristics of the study population

The characteristics of the 4812 patients available for analysis can be found in Table 1. Supplementary Table S1 gives these data for the quartiles of mtDNA copy number as a basis for the later statistical model building. Patients with prevalent CVD at baseline (n = 1235) had significantly lower mtDNA copy numbers than did patients without CVD (101.9, 25th–75th percentile: 77.4–120.5; vs. 109.0, 25th–75th percentile: 82.5–128.8; for detailed characteristics, see Supplementary Table S2). Logistic regression models applying different adjustments (Table 2) showed a significant association between a low mtDNA copy number and the odds of CVD, which remained significant after an extended adjustment for CVD risk factors as well as estimated glomerular filtration rate (eGFR) and urine albumin–creatinine ratio at baseline. Given that there was no indication for nonlinearity, an odds ratio was calculated per each 10 lower copies of mtDNA, resulting in 1.03-fold higher odds for prevalent CVD (odds ratio = 1.03 [95% CI 1.01–1.05], $P = 0.0084$). The model comparing quartile 1 against the combined quartiles 2–4 also showed significant associations with prevalent CVD (odd ratio = 1.21 [95% CI 1.03–1.43], $P = 0.0203$). Details for each quartile and each adjustment model can be found in Supplementary Table S3.

Prospective follow-up and mortality

During a median follow-up of 4 years, 342 deaths (7.1% of all patients) were recorded: 78 (22.8% of all deaths) from CVD causes; 64 (18.7%) due to infections; 34 (10%) due to other cardiac causes such as congestive heart failure, pulmonary embolism, or cardiac valve disease; 17 (5%) due to cerebrovascular causes; 7 (2%) due to peripheral vascular diseases; 7 (2%) due to kidney failure; 97 (28.4%) due to various causes; and 38 (11.1%) due to unknown causes. A detailed characterization of those patients who died and those who survived is provided in

Table 1 | Characteristics of all patients available for analysis (n = 4812)

Parameter	Mean ± SD or n (%)	[25th, 50th, 75th percentile]
Age (y)	60 ± 12	[53, 63, 70]
Sex (female)	1908 (39.7)	
Body mass index, kg/m^2	29.8 ± 6.0	[25.7, 28.9, 33.1]
Current smokers	767 (15.9)	
Causes of CKD		
Vascular nephropathy	1117 (23.2)	
Diabetic nephropathy	719 (14.9)	
Systemic disease	370 (7.7)	
Primary glomerulopathy	915 (19.0)	
Interstitial nephropathy	204 (4.2)	
Hereditary kidney disease	198 (4.1)	
Others	346 (7.2)	
Undetermined	943 (19.6)	
Immunosuppressive drugs including glucocorticosteroids	744 (15.5)	
Antihypertensive drugs		
ACE inhibitors	2265 (47.1)	
A-II receptor blockers	2006 (41.7)	
Diuretics	2934 (61.0)	
Calcium-channel blockers	1906 (39.6)	
Beta-blockers	2650 (55.1)	
Systolic blood pressure (mm Hg)	139.5 ± 20.2	[126, 138, 152]
Diastolic blood pressure (mm Hg)	79.1 ± 11.7	[71, 79, 87]
Hypertension	4630 (96.3)	
Diabetes mellitus	1726 (35.9)	
Cardiovascular disease[a]	1235 (25.7)	
eGFR (CKD-EPI formula; ml/min per 1.73 m^2)	49 ± 18	[37, 46, 58]
UACR (mg/g)[b]	432 ± 970	[10, 51, 391]
mtDNA copy number	**107.2 ± 36.4**	**[81.2, 101.9, 126.6]**
HDL cholesterol (mg/dL)	51.8 ± 18.0	[39.3, 48.3, 61.2]
LDL cholesterol (mg/dL)	118 ± 44	[89, 113, 143]
Albumin (mg/L)	38.3 ± 4.3	[36.2, 38.7, 40.8]
High-sensitivity C-reactive protein (mg/L)	4.76 ± 8.47	[1.02, 2.27, 5.01]

ACE, angiotensinconverting enzyme; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; EPI, Epidemiology Collaboration, HDL, high-density lipoprotein; LDL, low-density lipoprotein; mtDNA, mitochondrial DNA; UACR, urine albumin–creatinine ratio

[a]Cardiovascular disease was defined as myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, stroke, or interventions at the carotid arteries.

[b]UACR was calculated according to the following equation: albumin in urine (mg/L) x 100 / creatinine in urine (mg/dL) and is given in mg/g.

Table 2 | Results of logistic regression model on prevalent cardiovascular disease risk for each decrease in 10 mtDNA copies, as well as the first quartile of mtDNA copy number versus quartiles 2 to 4 (as reference category)

	For each decrease of 10 mtDNA copies		Quartile 1 versus quartiles 2–4	
Adjustment model[a]	OR [95% CI]	*P* value	OR [95% CI]	*P* value
Model 1	1.04 [1.02, 1.06]	5.93e-05	1.31 [1.13, 1.53]	0.0004
Model 2	1.03 [1.01, 1.05]	0.0026	1.23 [1.05, 1.44]	0.0088
Model 3	1.03 [1.01, 1.05]	0.0084	1.21 [1.03, 1.43]	0.0203

CI, confidence interval; mtDNA, mitochondrial DNA; OR, odds ratio.

[a]Model 1: adjusted for age and sex; Model 2: same as model 1, plus estimated glomerular filtration rate, urine albumin/creatinine ratio, and diabetes; Model 3: same as model 2, plus low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, body mass index, systolic blood pressure, diastolic blood pressure, C-reactive protein, and albumin.

Supplementary Table S4. Kaplan-Meier curves and log-rank tests (Figure 1) indicated that mtDNA categories divided into quartiles differed significantly with respect to mortality risk, with the exception of CVD death ($P = 7 \times 10^{-09}$ for all-cause mortality, $P = 0.20$ for CVD death, and $P = 0.0004$ for death due to infections). There was a clear separation for patients within the 1st quartile (<81.2 mtDNA copies) compared to quartiles 2–4, especially for all-cause mortality. In line with this observation, nonlinear splines showed increased risk for patients within the first quartile of mtDNA copies, compared with quartiles 2–4 for all 3 outcome variables (Figure 2). In quartiles 2–4, confidence bands overlapped with the reference line (hazard ratio [HR] = 1, centered at the median value of mtDNA copies), indicating no significant difference. This was in line with the results from Cox regression analyses for each of the quartiles 2, 3, and 4 compared with quartile 1, which showed similar estimates for the 3 quartiles (Supplementary Table S5). Because of this, and for reasons of statistical power, we collapsed quartiles 2–4 as a reference and compared it to quartile 1 in all subsequent analyses. Cox regression analyses results in Table 3 show that lower mtDNA content (<25th percentile) was significantly associated with a 1.81-fold higher risk of overall mortality (HR = 1.81 [95% CI 1.46–2.26], $P = 7.98 \times 10^{-08}$). The association was attenuated but remained significant in all adjustment models (model 3: HR = 1.37 [95% CI 1.09–1.73], $P = 0.0073$). Copy number of mtDNA was not significantly associated with death due to CVD (Table 3). The highest hazards for cause-specific death were found for deaths due to infections (HR = 2.60 [95% CI 1.59–4.26], $P = 0.0001$ in the age- and sex-adjusted model) which remained significant in the fully adjusted model (HR = 1.82 [95% CI 1.08–3.08], $P = 0.0250$; Table 3). Whether adjustment for baseline CVD was performed or not did not markedly alter the results, regardless of the outcome. In any of the models of cause-specific death, competing risks regression was performed. Corresponding subdivision HRs were diminished only marginally in comparison to the cause-specific HRs.

Hospitalizations due to infections during prospective follow-up

Prompted by the association between low mtDNA copy numbers and death due to infections, we analyzed the time to the first hospitalization due to an infection. In total, 644 patients experienced 966 hospitalizations due to infections. Cox regression analyses in Table 4 show that lower mtDNA copy numbers were significantly associated with higher risk for hospitalizations due to infections (HR = 1.34 [95% CI 1.13–1.59], $P = 0.0007$) in the age- and sex-adjusted model). This association remained significant in the fully adjusted model (HR = 1.19 [95% CI 1.00–1.42], $P = 0.0483$). The results for each quartile can be found in Supplementary Table S6.

Sensitivity analyses and interaction analysis

The sensitivity analysis within the subgroup of 532 participants with available Houseman variables that reflect estimated blood cell type proportions showed only very marginal influence of these variables on the association of mtDNA copy number with

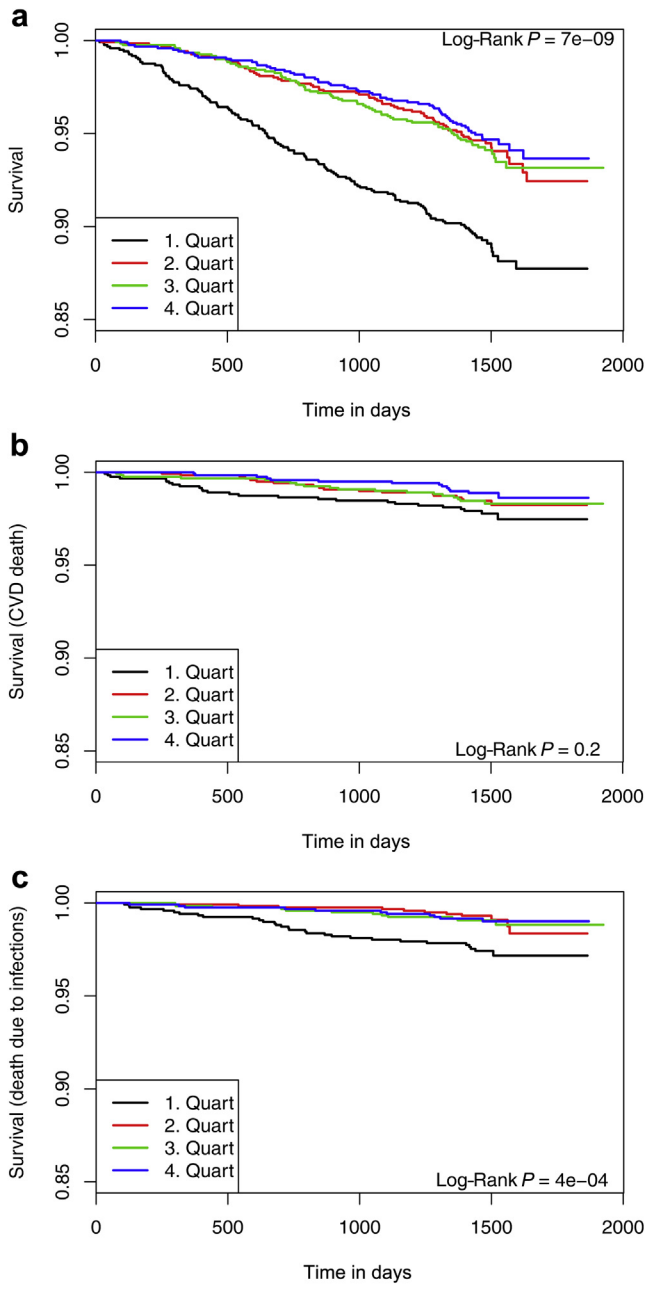

Figure 1 | Kaplan-Meier curves for all-cause mortality (a), cardiovascular disease (CVD) mortality (b), and death due to infections (c) by quartiles (Quart) of mitochondrial DNA.

hospitalization: HR = 1.98 [95% CI 1.16–3.37], $P = 0.0123$ without Houseman-variables; HR = 1.82 [95% CI 1.02–3.23], $P = 0.0423$ after adjustment for Houseman-variables. The Houseman-variables were also not significantly associated with mtDNA copy numbers (all P-values >0.2).

A total of 744 patients received immunosuppressive drugs, including glucocorticoids, at baseline. If we adjusted our analysis on death as well as hospitalizations due to infections for immunosuppressive drugs taken at baseline, the results did not change for the former (HR = 1.78 [95% CI 1.06–2.98], $P = 0.029$) but was no longer significant for the latter (HR = 1.14

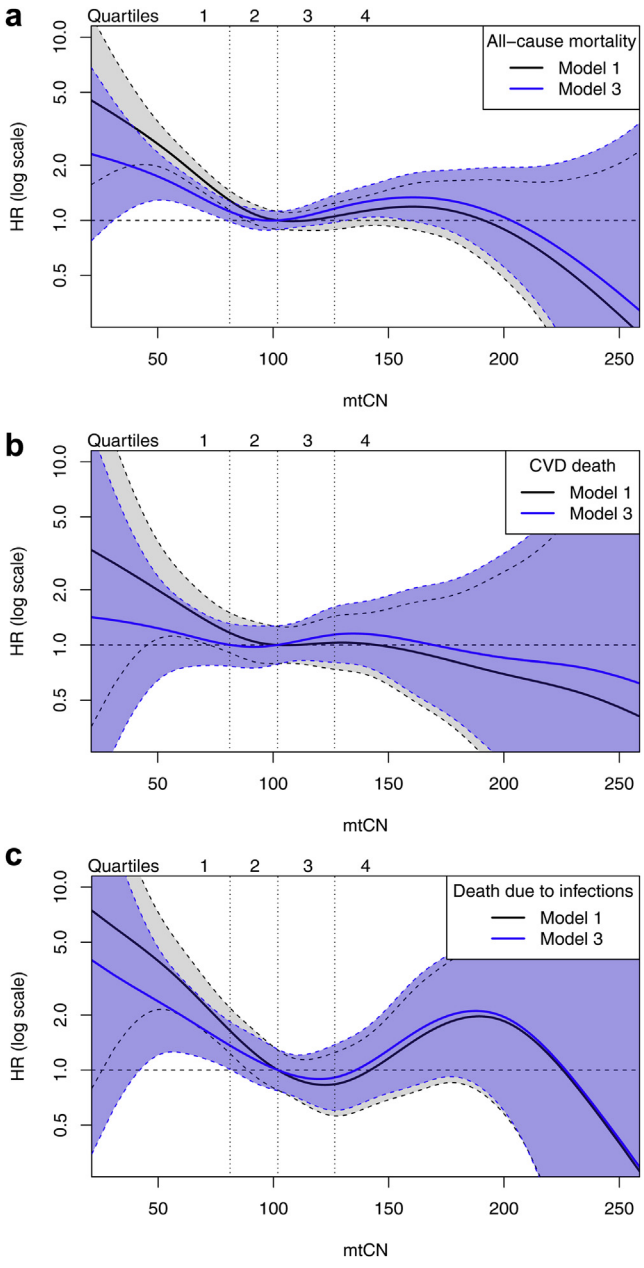

Figure 2 | Adjusted nonlinear splines (and 95% confidence bands) for the association between increasing mitochondrial (mt)DNA copy number (CN) and hazard ratio (HR) of all-cause mortality (a), cardiovascular disease (CVD) mortality (b), and death due to infections (c). HR is given as log-scale on the y-axes. Black line: adjustment model 1 (adjusted for age and sex). Blue line: adjustment model 3 (adjusted for age, sex, estimated glomerular filtration rate, urine albumin/creatinine ratio, diabetes mellitus, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, high-sensitivity C-reactive protein, albumin, smoking, body mass index, systolic blood pressure, diastolic blood pressure [and CVD in panels **a** and **b**]). Vertical dotted lines indicate the thresholds of mtDNA quartiles; the median value of mtDNA (101.9) is set as a reference (HR = 1; horizontal dashed line).

[95% CI 0.95–1.35], $P = 0.15$). A further sensitivity analysis for the main causes of CKD did not change any of our findings.

We examined whether eGFR is an effect modifier of our main findings on prevalent CVD and all-cause mortality by grouping patients into categories of eGFR >45 ml/min per 1.73 m^2 and eGFR ≤45 ml/min per 1.73m^2. In general, there was no indication of a significant interaction. However, stratified analyses revealed stronger associations for those with eGFR ≤45 ml/min per 1.73m^2 when quartile 1 was compared against quartiles 2–4 of mtDNA copy numbers for both outcomes (Supplementary Tables S7 and S8).

Table 3 | Results of Cox model on all-cause mortality, death due to cardiovascular disease, and death due to infection for first quartile of mitochondrial DNA versus quartiles 2 to 4 (as reference category); for death due to cardiovascular disease and infection, both cause-specific hazard ratio (HR) and subdivision HR (SHR) derived from a competing risk regression are given

Outcome	Quartile 1 versus quartiles 2-4			
Adjustment model[a]	HR [95% CI]	*P* value	SHR [95% CI]	*P* value
All-cause mortality, n = 342				
Model 1	1.81 [1.46, 2.26]	7.98e-08		
Model 2	1.56 [1.25, 1.95]	1.05e-04		
Model 3	1.37 [1.09, 1.73]	0.0073		
Cardiovascular death, n = 78				
Model 1	1.41 [0.88, 2.25]	0.1565	1.35 [0.84, 2.16]	0.2113
Model 2	1.19 [0.74, 1.92]	0.4597	1.14 [0.71, 185]	0.5823
Model 3	1.04 [0.64, 1.70]	0.8757	1.00 [0.60, 1.66]	1.000
Death due to infections, n = 64				
Model 1	2.60 [1.59, 4.26]	0.0001	2.51 [1.53, 4.12]	0.00025
Model 2[b]	2.18 [1.32, 3.62]	0.0025	2.12 [1.28, 3.50]	0.0034
Model 3[b]	1.82 [1.08, 3.08]	0.0250	1.78 [1.04, 3.04]	0.0354

[a]Model 1: adjusted for age and sex; Model 2: same as model 1 plus eGFR, urine albumin/creatinine ratio, diabetes, prevalent cardiovascular disease; Model 3: same as model 2 plus low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, body mass index, systolic blood pressure, diastolic blood pressure, C-reactive protein and albumin.
[b]Same as former models 2 and 3, but not adjusted for prevalent cardiovascular disease.

DISCUSSION

Main findings

We found that low numbers of mtDNA copies were significantly associated with CVD at baseline and with all-cause mortality and death due to infections, as well as with hospitalizations due to infections during 4 years of follow-up. The associations remained significant after adjusting for kidney function and traditional CVD risk factors at study baseline.

Table 4 | Results of Cox model for first hospitalization due to an infection (644 events among 4812 patients)

	Quartile 1 versus quartiles 2-4	
Adjustment model[a]	HR [95% CI]	*P* value
Model 1	1.34 [1.13, 1.59]	0.0007
Model 2	1.27 [1.07, 1.51]	0.0057
Model 3	1.19 [1.00, 1.42]	0.0483

CI, confidence interval; HR, hazard ratio.
[a]Model 1: adjusted for age and sex; Model 2: same as model 1 plus estimated glomerular filtration rate, urine albumin/creatinine ratio, and diabetes; Model 3: same as model 2 plus low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, body mass index, systolic blood pressure, diastolic blood pressure, C-reactive protein, and albumin.

All-cause mortality

Up to now, studies investigating mtDNA copy numbers and mortality in CKD patients have been performed only in dialysis patients. These small-sized studies reported conflicting results. Rao *et al.*[16] found a decreased mtDNA copy number to be associated with poor outcome in a cohort of 173 patients, 102 of whom died during 2.7 years of follow-up. On the contrary, Wang and colleagues[18] found a higher mtDNA copy number to be associated with increased all-cause mortality risk in 95 hemodialysis patients, 10 of whom died during 3 years of follow-up. A more recent study did not find any association with mortality in 120 peritoneal dialysis patients, of whom 24 died.[19] Our study in patients with moderate CKD shows a clear association with a 1.81-fold increased mortality risk in the quartile of patients with the lowest numbers of mtDNA copies. This risk remained significant after extended adjustment for kidney function and CVD risk factors.

The relationship between mtDNA copy number and mortality is much clearer in the general population, for which large studies are available, the results of which are in line with our findings. Ashar *et al.*[20] described that a low mtDNA copy number was inversely associated with prevalent frailty and was an independent predictor of all-cause mortality in 16,401 participants from 2 population-based cohorts. Similarly, the results obtained by Mengel-From *et al.*[21] showed an association of low mtDNA content with higher mortality risk and worse self-reported health conditions in elderly individuals.

Cardiovascular disease

The literature on mtDNA copy number and CVD in the context of CKD is sparse. Only 2 studies in peritoneal dialysis patients have been published. Yoon *et al.*[19] found no significant association between mtDNA copy numbers and cardiovascular events for 24 patients with events, out of 120 patients during 3 years of follow-up. The second study indicates that an increased circulating free-mtDNA content (which might be different from cell-derived mtDNA content) correlates with a systemic inflammatory state and is a strong predictor of cardiovascular events.[22] We observed a highly significant and linear inverse relationship between mtDNA copy number and prevalent CVD, which was attenuated but remained significant with additional adjustment for kidney function and CVD risk factors (Table 2). Interestingly, we did not observe a significant association with cardiovascular deaths during the 4 years of follow-up. Studies published in non-CKD populations showed consistent results in case–control[23–25] as well as prospective observations, revealing associations between lower mtDNA copy number and stroke, coronary heart disease, CVD in general,[26] sudden cardiac death,[27] and cardiovascular mortality.[28] Our findings of an association between mtDNA copy number variation and prevalent CVD are consistent with these observations, but we did not observe an association with CVD death. A potential explanation is that the prospective 4-year observation period might have been too short, and the copy numbers measured at baseline might have already been confounded by subclinically developing stages of CVD.

Death and hospitalization due to infections

To our knowledge, this is the first study in CKD patients investigating an association between mtDNA copy number and infectious diseases. This is surprising considering that infectious diseases are among the leading causes of death in CKD patients and that hospitalizations related to infections are very common. Almost 15% of the patients in our study experienced a hospitalization for infection, many of them several times, and 64 patients died due to infections. The association with death due to infections was among the strongest associations observed, with roughly a doubling of risk in patients within the lowest quartile of mtDNA copy numbers. It was also significant after adjustment for high-sensitivity C-reactive protein and serum albumin, which are markers of inflammation and malnutrition. It has been shown by Knez *et al.*[29] in a general population sample that inflammatory markers, such as C-reactive protein, are inversely associated with mtDNA content in peripheral blood. Hence, mtDNA copy number might be an independent predictor of deaths and hospitalizations due to infections. This possibility is in line with the immune-modulatory involvement of mitochondria with innate immunity and immune response to bacterial and viral infections.[30]

Oxidative stress as a possible explanation of the findings

The precise mechanism underlying the relationship between mtDNA copies and adverse outcomes in CKD patients remains largely elusive. It might be explained by the involvement of mitochondrial ROS produced by oxidative phosphorylation. At least in healthy conditions, this process is essential for a variety of cellular processes, such as regulation of differentiation and autophagy, metabolic adaptation, immune cell activation, and inflammation.[31] Mitochondrial ROS have not only a direct role in immunity through the cytotoxic effect on pathogens by oxidative damage, but also can stimulate innate immune response. In particular, mitochondrial ROS appear to play a crucial role in activation and modulation of T-cell proliferation.[32] On the contrary, increased ROS levels have been implicated in many pathologic conditions, from cancer to degenerative disease and aging.[33] It has been shown that CKD is also characterized by deregulation of ROS production.[34] The maintenance of intracellular redox balance is essential to modulate a proper T-cell-mediated immunity response.[35] When the antioxidant defense system of the cell is not able to recover the physiological redox state, the resulting oxidative stress can cause damage to mitochondrial proteins, membranes, and DNA, and gradually lead to fragmentation and dysfunction.[36] A low mtDNA copy number in cells, indicative of a concomitant decrease in cellular energy production, might indicate an insufficient oxidative stress response.[37] This process could result in a vicious cycle because the exposure to ROS, especially hydrogen peroxide, might cause damage to the DNA replication

enzymes of the mitochondria and thereby aggravate the reduction of the mtDNA copy number.[38,39]

Differences compared with studies investigating cell-free circulating mtDNA copy number

The measurement of mtDNA copy numbers derived from peripheral blood mononuclear cell or whole blood (as in our study) or from cell-free circulating mtDNA copy numbers from plasma might be very different and yield opposite associations with clinical outcomes. The measurement of mtDNA copy numbers from nuclear cells is an attempt to assess relative mitochondrial abundance within cells, and therefore a higher mtDNA copy number is expected to be associated with beneficial outcomes. This was indeed the case for the investigated outcomes in our and other studies.[16,21,23] In contrast, the cell-free circulating mtDNA copy numbers might indicate mtDNA copies that have been released into circulation from damaged cells. This damage-associated pattern can activate toll-like receptors leading to a systemic inflammatory response syndrome.[40–42] Studies in various patients indeed revealed a higher cell-free mtDNA copy number in patients with clinical outcomes.[22,43,44]

Strengths and limitations of the study

To our knowledge, this is largest reported study of mtDNA copy numbers in CKD patients using prespecified and centrally adjudicated outcome measures. Moreover, it is the first study demonstrating a strong association between mtDNA copy numbers and risk of mortality and infections in patients with moderately severe CKD. Further strengths include the use of the same DNA extraction and quantification method for all samples using a plasmid normalization approach for thorough standardization of the assay. This is of utmost importance as we recently determined that DNA extraction methods have a strong influence on mtDNA quantification results.[45]

Limitations of this study include the fact that the mtDNA copy number measurement was performed from blood DNA. Therefore, blood cell type composition is a potential confounder. Unfortunately, Houseman-variables reflecting blood cell type composition were available for adjustment in only a subset of patients. Therefore, the possibility that platelet count and white blood cell distribution had an influence on the association of mtDNA content with the endpoints cannot be ruled out completely. However, many of these patients were suffering from immunological diseases, and we would have expected stronger effects of confounding by cell type composition, which did not occur. Second, mtDNA copy numbers were measured only once at baseline, which does not necessarily reflect possible adaptations during the observation period. Third, our data were generated from CKD patients under specialist care, so the observations may not be generalizable to other patient populations. Fourth, the reasons for hospitalization due to infection are very heterogeneous, and we currently do not have specifics on the etiologic reasons for this type of hospitalization. In addition, the hospitalizations might reflect only the most severe infections, and therefore, subsequent analyses triggered by the secondary outcome analyses (various death reasons) have to be interpreted with caution. Furthermore, residual confounding cannot be excluded, and causality cannot be deduced from the observed associations.

Conclusions

This study demonstrates an independent association of low mtDNA copy numbers with prevalent CVD as well as all-cause and infection-related risk of mortality and hospitalization due to infections during 4 years of follow-up in patients with moderate CKD. Further studies are required to understand and characterize the mechanisms underlying this relationship.

METHODS

Study population and outcome definition

The German Chronic Kidney Disease (GCKD) study is an ongoing prospective multicenter observational national cohort study. Design and details of the study have been published previously.[46] Briefly, the study aimed to enroll patients with an eGFR of 30–60 ml/min per 1.73 m^2 (stage G3, A1-3) or an eGFR >60 ml/min per 1.73 m^2 in the presence of overt proteinuria (stage G1-2, A3) under regular care by nephrologists. Exclusion criteria were non-Caucasian ethnicity, solid organ or bone marrow transplantation, active malignancy within 24 months prior to screening, New York Heart Association Stage IV heart failure, legal custodianship, or inability to provide consent. Before enrollment, a screening phase identified potential patients under care of resident nephrologists according to the mentioned criteria. After this screening phase, patients were enrolled according to a standardized procedure. Their eGFR and proteinuria level were measured in a central lab; about 10% turned out to have an eGFR below 30 ml/min per 1.73 m^2, and about 6% had an eGFR >60 ml/min without having overt proteinuria. This reflects variability in either parameters between enrollment and the baseline visit or the measurement of kidney function in local laboratories.

In total, we enrolled 5217 patients between the years 2010 and 2012. The study was approved by the ethics committees of all participating institutions and registered in the national registry for clinical studies (DRKS 00003971). All methods were carried out in accordance with approved guidelines and the Declaration of Helsinki. Written informed consent was obtained from each study participant. Trained personnel obtained information on clinical data, socio-demographic factors, medical and family history, medications, and health-related quality of life. Prevalence of CVD at baseline was defined as a history of myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, stroke, or carotid artery interventions. Data are collected and managed using Askimed (https://www.askimed.com), a cloud-based web platform storing data in a MySQL database.

Patients are followed on a yearly basis by trained personnel, alternating face-to-face visits with telephone visits. During these visits, data on hospitalizations, outcome events, and medical history are updated during a structured interview. Any hospital discharge reports are collected from the treating physicians and/or hospitals. Endpoints are continually extracted from these reports by a trained and supervised endpoint committee composed of 3 independent physicians, according to a prespecified endpoint catalogue.

Information on cause of death is taken from these reports, as well as from death certificates collected from civil registry offices whenever study personnel are informed of the death of a study participant.

The current analysis includes all endpoints that occurred during the first 4 years of follow-up. The primary outcome was all-cause mortality. To evaluate whether identified associations could be attributed to a specific cause of death, death from cardiovascular causes and infectious diseases were considered as secondary outcomes. Other causes of death were also recorded, but these subgroups were not large enough to conduct further cause-specific analyses. To support results obtained for death due to infectious diseases, we further analyzed data on hospitalizations due to infectious diseases during the first 4 years of follow-up.

It is possible that mtDNA content is influenced by platelet count and white blood cell distribution,[47] which were not available in the GCKD study. However, in a subsample of 532 patients with specific diagnoses (focal segmental glomerulosclerosis, systemic lupus erythematosus, membranous nephropathy, autosomal dominant polycystic kidney disease), data from a DNA methylation array were available, which enabled the estimation of the proportion of the major white blood cell types (CD4+ and CD8+ T cells, natural killer cells, monocytes, granulocytes, and B cells) using a statistical method described by Houseman *et al.*[48]

Plasmid-normalized quantitative polymerase chain reaction for mtDNA copy number quantification

Biosamples were collected in a standardized way at the baseline visit and shipped frozen to a central laboratory for routine clinical chemistry and to a central biobank for future analyses. DNA was extracted from frozen ethylenediamine tetraacetic acid–blood samples using Chemagic Magnetic Separation Module I (PerkinElmer Chemagen Technologie GmbH, Baesweiler, Germany), an automated magnetic beads-based method.

The quantification of mtDNA copy number per diploid cell was performed as recently described[45] from a total of 5049 DNA extracts using a duplex quantitative polymerase chain reaction assay that allows for simultaneous targeting of a single copy nuclear gene (beta-2-microglobulin) and the transfer (t)RNA^{Leu} gene on the mtDNA. A plasmid containing both targets was included in all runs in order to correct for interassay variability. This method reduces the interassay variability dramatically, from 21% to 7%.[45] The mtDNA copy number was calculated using the Delta Delta Cq (quantification cycle) method:

$$2 \times E^{-(\Delta Cq\ \text{sample} - \Delta Cq\ \text{plasmid})}$$

where "E" is the averaged mean efficiency of the polymerase chain reactions of the 2 targets,[49] and "2" is used to account for the 2 copies of nuclear DNA in a cell.

The primer and probe sequences were modified from Bai and Wong.[50] In brief, a region of mtDNA–tRNA^{Leu} was amplified using the forward primer 5'- CACCCAAGAACAGGGTTTGT and the reverse primer 5'- TGGCCATGGGTATGTTGTTA; a region of beta-2-microglobulin (B2M) was amplified using the forward primer 5'-TGCTGTCTCCATGTTTGATGTATCT and the reverse primer: 5'-TCTCTGCTCCCCACCTCTAAGT. Probe sequences were:

FAM-5'-TTACCGGGCTCTGCCATCT

BHQ1 for mt-tRNA^{Leu} and

Yakima Yellow - 5'-CAGGTTGCTCCACAGGTAGCTCTAG-BHQ1 for beta-2-microglobulin. Primers and probes were synthesized by Microsynth AG (Balgach, Switzerland).

The quantitative polymerase chain reaction was performed on the Quantstudio 6 instrument (Thermo Fisher Scientific, Waltham, MA) using the following conditions: 95 °C for 3 minutes for initial polymerase activation, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. All samples were measured in triplicate. Less than 4% of the samples were excluded from the analysis as they had a coefficient of variation >2% within the triplet reactions. The average interassay coefficient of variation was 7.6 %. Equal polymerase chain reaction efficiencies for both targets were confirmed in 5-fold serial dilutions ranging from 5.0 x 10^{-6} to 320 mtDNA copies per reaction. Finally, a total of 4812 samples with mtDNA copy number and the other clinical and outcome variables were analyzed for this study.

Statistical analysis

The baseline characteristics for all patients are presented as mean ± SD and quartiles or N (%). Logistic regression analysis was used to investigate the association between mtDNA copy number and prevalent CVD. Survival analyses were performed for 4 different outcomes: (i) all-cause mortality; (ii) death due to CVD; (iii) death due to infections; and (iv) hospitalizations due to infections. First, Kaplan-Meier curves and log-rank tests were performed for quartiles of mtDNA. To adjust for possible confounders and evaluate the form of the relationship, Cox proportional hazards models were fitted using different adjustment parameters: model 1 adjusted for age and sex; model 2 additionally adjusted for eGFR, urine albumin/creatinine ratio, and diabetes; model 3 was the same as model 2 plus adjustment for low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, body mass index, smoking, hypertension, C-reactive protein, and albumin. We further tested for effect modification by eGFR >45 ml/min per 1.73 m^2 and ≤45 ml/min per 1.73 m^2. Further downstream analyses were based on visual inspection of the nonlinear relationships, determined by nonlinear P-splines. More specifically, quartiles 2 to 4 were collapsed into one category, representing the reference category, and were compared to quartile 1, as this was suggested by the nonlinear curves for the incident outcome variables. For the analyses of cause-specific mortality, competing risk regression was also performed, considering all deaths from other causes as competing events. Therefore, both cause-specific HRs and subdivision HRs are reported. For hospitalization due to infections, sensitivity analyses were conducted within the patient group for which the proportions of the white blood cell types (so called "Houseman-variables") were available, with and without accounting for these potential confounding factors. Due to limited sample size within that subgroup, this analysis could not be performed for the other endpoints and was also not adjusted for any other variables. Further sensitivity analyses were performed for additional adjustment for immunosuppressive drugs taken at the baseline, as well as main causes of CKD.

APPENDIX

Current GCKD investigators and collaborators with the GCKD study are as follows:

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DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

FK designed the study. FF and LF carried out experiments. CL performed the statistical analysis of the data. LF, UTS, FKo, HM, HW, LF, VK, KUE, AK, and FK were involved in recruitment, data acquisition, and data management the GCKD study. UTS, FKo, and AK were involved in the end point adjudication. FF, CL, and FK drafted the article. All authors revised the manuscript critically for important intellectual content and gave approval of the final version.

SUPPLEMENTARY MATERIAL

Table S1. Characteristics of patients grouped by mitochondrial DNA copy number (mtDNA CN) quartiles (Q). CKD-EPI, Chronic Kidney Disease-Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table S2. Characteristics of patients with and without cardiovascular disease (CVD) at baseline. CKD-EPI, Chronic Kidney Disease-Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; mtDNA, mitochondrial DNA; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table S3. Results of logistic regression model on prevalent cardiovascular disease (CVD) risk for quartiles of mitochondrial DNA (mtDNA) copies with quartile 1 as reference. BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OR, odds ratio.

Table S4. Characteristics of survivors and nonsurvivors of follow-up. CKD-EPI, Chronic Kidney Disease-Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; mtDNA, mitochondrial DNA; UACR, urine albumin–creatinine ratio.

Table S5. Results of Cox model on all-cause mortality, death due to cardiovascular disease, and death due to infections for first quartile of mtDNA (reference) versus quartiles 2, 3, and 4. CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein.

Table S6. Results of Cox-model for first hospitalization due to an infection (644 events among 4812 patients). CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein.

Table S7. Results of logistic regression model on prevalent cardiovascular disease (CVD) risk for each increase in 10 mitochondrial DNA (mtDNA) copies as well as the first quartile of mtDNA copy number versus quartiles 2 to 4 (as reference category) stratified for estimated glomerular filtration rate (eGFR) >45 ml/min per 1.73 m^2 and ≤ 45 ml/min per 1.73 m^2. Tests for interactions are provided. CI, confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OR, odds ratio.

Table S8. Results of Cox model on all-cause mortality for first quartile of mitochondrial DNA (mtDNA) versus quartiles 2 to 4 (as reference category). Tests for interactions are provided. CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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