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Association between oxidized nucleobases and mitochondrial DNA damage with long-term mortality in patients with sepsis

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A R T I C L E I N F O

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A B S T R A C T

Background: Sepsis not only leads to short-term mortality during hospitalization, but is also associated with increased mortality during long-term follow-up after hospital discharge. Metabolic stress during sepsis may cause oxidative damage to both nuclear and mitochondrial DNA (mtDNA) and RNA, which could affect long-term health and life span. Therefore, the aim of this study was to assess the association of sepsis with oxidized nucleobases and (mt)DNA damage and long-term all-cause mortality in septic patients.

Methods: 91 patients with sepsis who visited the emergency department (ED) of the University Medical Center Groningen between August 2012 and June 2013 were included. Urine and plasma were collected during the ED visit. Septic patients were matched with 91 healthy controls. Death rate was obtained until June 2020.The degree of oxidation of DNA, RNA and free nucleobases were assessed in urine by mass-spectrometry. Lipid peroxidation was assessed in plasma using a TBAR assay. Additionally, plasma levels of mtDNA and damage to mtDNA were determined by qPCR.

Results: Sepsis patients denoted higher levels of oxidated DNA, RNA, free nucleobases and lipid peroxidation than controls (all $p < 0.01$). Further, sepsis patients displayed an increase in plasma mtDNA with an increase in mtDNA damage compared to matched controls ($p < 0.01$). Kaplan meier survival analyses revealed that high degrees of RNA- and nucleobase oxidation were associated with higher long-term all-cause mortality after sepsis ($p < 0.01$ and $p = 0.01$ respectively). Of these two, high RNA oxidation was associated with long-term all-cause mortality, independent of adjustment for age, medical history and sepsis severity (HR 1.29 [(1.17–1.41, 95% CI] $p < 0.01$).

Conclusions: Sepsis is accompanied with oxidation of nuclear and mitochondrial DNA and RNA, where RNA oxidation is an independent predictor of long-term all-cause mortality. In addition, sepsis causes mtDNA damage and an increase in cell free mtDNA in plasma.

1. Introduction

Sepsis is a life-threatening syndrome of organ dysfunction subsequent to a dysregulated host response to infection. It is one of the most common causes of death among hospitalized patients [1,2]. In-hospital mortality rate is around 25%, rising to 80% for patients with severe sepsis and multi organ failure (MOF) [3]. In addition, mortality rates after surviving the initial sepsis episode remain high, with reported one-year mortality rates post-discharge between 7 and 43% [4] and a reported five-year mortality rate after severe sepsis of even 82% [5]. Sepsis is a complex and heterogeneous syndrome and therefore difficult

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Abbreviations

mtDNA	Mitochondrial DNA
MOF	Multi organ failure
8-oxodGuo	8-Oxo-7,8-dihydro-2′-deoxyguanosine
8-oxoGua	8-oxoguanine
8-oxoGuo	8-oxo-7,8-dihydroguanosine
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
qPCR	Quantitative polymerase chain reaction

to treat, leaving clinicians with limited therapeutic options. As a result, care is usually focused on source control (i.e. antibiotics, drainage) and organ support [1,2,6].

Sepsis is accompanied by a high oxidative burden and increased production of reactive oxygen species (ROS) by mitochondria [7,8]. Accumulating levels of ROS during sepsis cause oxidative damage to both nuclear and mitochondrial (mt) DNA and RNA [9]. Oxidative damage interferes with normal cell function, causing loss of cellular homeostasis, mitochondrial dysfunction and cell death [7,8,10]. Guanine is a nucleobase which is most prone to oxidation, due to its low redox potential [11]. Oxidation of guanine in nuclear and mitochondrial DNA and RNA has been linked to a variety of diseases, including sepsis [10,12–14]. Whether oxidized nucleobases and mitochondrial DNA damage in sepsis survivors is associated with long-term all-cause mortality is unknown. We hypothesize that oxidative damage to DNA and RNA during sepsis is associated with long-term all-cause mortality. We therefore measured oxidation of guanine in DNA and RNA and quantified mitochondrial DNA (mtDNA) damage and integrity in septic patients and compared this with matched controls. Next, using survival analyses, we associated oxidative RNA and DNA damage with long-term all-cause mortality.

2. Methods

2.1. Study population and study design

Data of adult non-trauma patients (>18 years of age) who presented at the ED of the University Medical Center Groningen (UMCG), and diagnosed with sepsis were collected prospectively from 16th of August 2012 to 1st of June 2013. Mortality status was registered until 1 June 2020. Written (deferred) informed consent was obtained from all participating patients. The study protocol was approved by the medical ethical committee of the University of Groningen (METc 2012.077). In total 94 patients were included in this study. When patients were admitted more than once during the study period, only data from the first hospital admission were included in the analyses, resulting in a total study population of 91 patients. Matched controls were selected from the Prevention of Renal and Vascular Endstage Disease (PREVEND) database. Patients were matched on sex, age, cardiovascular diseases, malignancy, diabetes, creatinine levels in blood and estimated Glomerular Filtration Rate (eGFR).

Collected data of both the sepsis and the control patients included patient characteristics, vital parameters, laboratory measurements, blood and urine at admission and mortality at follow-up. Blood and urine was collected either at the ED (sepsis patients) or outpatient clinic (control patients), followed by prompt transportation to the central lab via pneumatic post where samples were processed and immediately stored at −80 °C. As directed for the MDA measurements, samples were slowly tawed at 4 °C and measurements were performed immediately after tawing. Mortality status was obtained from the Municipal Personal Records Database (BRP), containing reliable and complete registration all Dutch citizens. Patient characteristics consisted of age, sex, race, presence of diabetes mellitus, chronic kidney disease, cardiovascular disease, liver disease and cancer. Vital parameters consisted of heart frequency, systolic and diastolic blood pressure and mean arterial pressure. In septic patients also body temperature (tympanic), oxygen saturation, and respiratory rate at admission were obtained. Laboratory measurements at admission included levels of leucocytes, thrombocytes, lactate, creatinine, hemoglobin, CRP, and ureum. eGFR was estimated with the MDRD method: estimated GFR (ml/min/1.73m2) = 186 x (Creat/88.4)-1.154 x (Age)-0.203 x (0.742 if female) x (1.210 if black) [15]. Other data included the source of infection, and length of hospital and intensive care unit (ICU) stay.

2.2. Sepsis definition

Sepsis was defined as suspected or confirmed infection and the presence of two or more systemic inflammatory response syndrome (SIRS) criteria [16]. Severe sepsis was defined as sepsis with organ dysfunction, while septic shock was defined as persisting hypotension despite adequate fluid resuscitation (>2 L) [16]. The SIRS criteria used were body temperature >38 °C or <36 °C, heart frequency >90 beats/min, respiratory rate >20 breaths/min or $PaCO_2$ <32 mmHg, and white blood cell count >12,000 cells/mm^3, <4,000 cells/mm^3 or >10% immature (band) forms [16].

2.3. DNA isolation

Total DNA from plasma was isolated to measure mitochondrial DNA (mtDNA) levels and mtDNA damage. DNA was isolated from 50ul of plasma using the Maxwel RSC ccfDNA plasma kit (Promega, Madison, USA) and Maxwell 16 MDx AS3000 (Promega) according to the manufacturer's protocol.

2.4. Oxidative stress measurement

Reactive oxygen damage was measured with the Thiobarbituric Acid Reactive Substances (TBARS) assay Kit (Cell Ciolabs Incl, San Diego, CA, USA). This kit is well-established for screening and monitoring lipid peroxidation, which indicates oxidative stress in cells. In short, the assay screens lipid peroxidation via quantitative measurement of MDA (Malondialdehyde). MDA forms a 1:2 adduct with TBA and produces a new chemical compound which we measured by fluorimetry and spectrophotometry with the Synergy H4 Hybrid Microplate Reader (BioTek, Vermont, USA). In total 6.5ul plasma was used, diluted (1:10) in destilated water.

2.5. Measurement of DNA, RNA and free nucleobase oxidation in urine

8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxoguanine (8-oxoGua) concentrations in urine were measured to define DNA-, RNA- and free nucleobase oxidation, respectively. A validated method of LC–MS/MS with online SPE as previously reported by Shih et al. was used (17). Briefly, 20 μL of urine was diluted 10 times with a solution containing 5 ng of [15N5]-8-oxoGua, 0.5 ng of [15N5]-8-oxodGuo and 0.5 ng of [13C, 15N2]-8-oxoGuo as internal standards in 5% (v/v) methanol/1 mM ammonium acetate. A 50 μL of prepared urine sample was directly injected into the online SPE LC–MS/MS. After automated sample cleanup, LC–MS/MS analysis was performed using an Agilent 1100 series HPLC system (Agilent Technology) interfaced with an API 4000 QTrap hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a TurboIonSpray source. Samples were analysed in the positive ion multiple reaction monitoring mode, and the transitions of the precursors to the product ions were as follows: *m/z* 168 → 140 (quantifier ion) and 168 → 112 (qualifier ion) for 8-oxoGua, *m/z* 173 → 145 for [15N5]-8-oxoGua; *m/z* 284 → 168 (quantifier ion) and 284 → 140 (qualifier ion) for 8-oxodGuo, *m/z* 289 → 173 (quantifier

ion) for [15N5]-8-oxodGuo; m/z 300 → 168 (quantifier ion) and 300 → 140 (qualifier ion) for 8-oxoGuo, m/z 303 → 171 (quantifier ion) for [15N5]-8-oxoGuo. Urinary creatinine was determined using a HPLC–UV method [18].

2.6. Quantification of mitochondrial DNA levels

Mitochondrial DNA levels were determined using quantitative polymerase chain reaction (qPCR). Therefore, we determined the cycle threshold (ct) of NADH dehydrogenase 1 (*ND1)* and nuclear gene Beta-2 Microglobulin (*β2M)*. Oligonucleotide primers (Sigma Aldrich, Darmstadt, Germany; Table 1) were designed using Clone Manager 9 software and were validated by assessing efficiency, melting- and temperature curves using CFX384- Real-Time system (Biorad, California, USA). Amplification of the DNA was performed using the following thermal profile: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 61 °C for 60 s. All reactions were carried out in duplicate and obtained threshold cycles (ct) values were averaged. A standard curve was used to determine the efficiency, linear range, and reproducibility of the qPCR assay. The difference in the ct value between *ND1* and *B2M* was used to quantify the relative abundance of the mitochondrial genome.

2.7. Quantification of mitochondrial DNA damage

MtDNA damage was assessed by long-range PCR, by which we measured gene-specific damage by quantifying the decrease in amplification of DNA extracted from plasma and visualized this on an agarose gel [19]. The long-range PCR was performed using the TaKaRa LA Taq DNA polymerase kit (Takarabio, Kusatsu, Japan). Therefore, a 10 kb mtDNA template, stretching from *ND5* to *ND1* and thereby consisting more than two-third of the mitochondrial genome, was amplified by long-range PCR (Biorad, California, USA), while a short mtDNA fragment of approximately 200bp (*D-loop;* a regulative region) was amplified by qPCR to serve as reference. In total, 5ul of DNA was used. For the amplification of the long 10 kb mtDNA part, the following thermal profile was used: 94 °C for 1 min, followed by 18 cycles of 15 s at 94 °C and 12 min at 64 °C, and ended with 10 min at 72 °C. The short fragment was amplified using the thermal profile: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 61 °C for 60 s. Both PCR products were ran separated on a 1% agarose gel (45 min, 100 V). The intensity of the bands on the gel was analysed using Image Lab (Biorad, California, USA). The ratio of the intensity of the short stable fragment to the long unstable fragment was calculated to quantify mtDNA damage.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 23.0.0.3 (IBM Corp. SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA.). Patient demographics were analysed using a Chi-Square test for categorical variables, an independent sample T-test for continuous normally distributed variables, and a Mann-Whitney U test for continuous not normally distributed variables. The Shapiro Wilk test was used to test normality. Correlations between continues and categorical variables were tested with either Pearson or Spearmen's Rho, depending on distribution. Kaplan-Meier survival curves were created with GraphPad Prism 8 (GraphPad Software, La Jolla California USA), and differences between groups were analysed by a Mantel-Cox log rank test. Univariable Cox-regression analysis (cut-off $p < 0.10$), followed by a multivariable Cox regression analysis was used to calculate the associations between age, sex, sepsis severity, diabetes, cardiovascular disease, cancer, MDA, RNA-, DNA-, and nucleobase oxidation and mtDNA damage with long-term all-cause mortality. A receiver operating characteristic (ROC) curve analysis with nonparametric assumption was made with MedCalc Statistical Software version 19.2.6 (MedCalc Software, Ltd, Ostend, Belgium). This was performed using all-cause mortality as the classification variable and RNA- and nucleobase oxidation as the prognostic variable. The area under the curve (AUC) was used to compare the discriminatory power. An AUC of 1.0 is considered to be perfect in discrimination and 0.5 is considered equal to chance. A p-value of <0.05 was considered significantly different (* means $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Table 1
List of primers used for DNA amplification.

Gene		Primer sequence
ND1 (mtDNA)	Forward:	TGGCTCCTTTAACCTCTCCA
	Reverse:	GGTTCGGTTGGTCTCTGCTA
Short fragment (*D-loop,* mtDNA)	Forward:	AACCTACCCACCCTTAACAG
	Reverse:	CACTCTTGTGCGGGATATTG
β2M (nDNA)	Forward:	CTGGGTAGCTCTAAACAATGTATTCA
	Reverse:	CATGTACTAACAAATGTCTAAAATGGT
Long fragment (mtDNA)	Forward:	TCTAAGCCTCCTTATTCGAGCCGA
	Reverse:	TTTCATCATGCGGAGATGTTGGATGG

mtDNA mitochondrial DNA, nDNA nuclear DNA.

3. Results

3.1. Study population

Data were obtained in 91 patients with sepsis and 91 matched controls (Table 2). Foci of infection were identified as respiratory tract infection (36%), urinary tract infection (34%), intra-abdominal infection (12%), other infection focus (8%) or no obvious focus could be found (10%). The median (interquartile range) age of the patients with sepsis was 62 (21) years and for the matched controls the median age was 66 (16) years. 55% was male in the septic group and 57% in the matched controls. Median follow-up was seven years for both non-severe septic patients and severe septic patients and four months for patients with septic shock. Median follow up for matched controls was almost six years. Patient characteristics are listed in Table 2.

Table 2
Patient characteristics.

	Sepsis ($n = 91$)	Control ($n = 91$)
Mean age (years)	62 (21)	66 (16)
Male sex	50 (55%)	54 (57%)
Comorbidities		
Cardiovascular disease	46 (51%)	45 (49%)
Malignancy	26 (29%)	18 (20%)
Diabetes	23 (25%)	24 (26%)
Serum ceatinine (μmol/L)	85 (65)	81 (34)
eGFR (m/min/1,73m^2)		
<15	1 (1%)	1 (1%)
15-29	9 (10%)	3 (4%)
30-44	8 (9%)	13 (14%)
45-59	17 (18%)	13 (14%)
60-90	29 (32%)	33 (36%)
≥90	27 (30%)	28 (31%)
Sepsis severity		N/A
Sepsis	39 (43%)	
Severe sepsis	49 (54%)	
Septic Schock	3 (3%)	
Sepsis focus		N/A
Respiratory tract	33 (36%)	
Urinary tract	31 (34%)	
Intra abdominal	11 (12%)	
E.C.I	9 (10%)	
Other	7 (8%)	

Data are presented as median with interquartile range or percentages. There was no significant difference between sepsis and matched controls in any characteristic. Malignancy was defined as a solid tumor with/without metastases (no squamous cell carcinoma/basal cell carcinoma). eGFR; estimated glomerular filtration rate, E.C.I; e causa ignota, N/A; not applicable.

3.2. Sepsis causes oxidative stress

To assess whether sepsis leads to oxidative damage, we determined oxidation of DNA, RNA and free nucleobases in urine and lipid peroxidation in plasma. Septic patients had a significantly higher amount of oxidized DNA, RNA, and free nucleobase found in urine than matched controls (Fig. 1A-C, $p < 0.001$). In addition, septic patients had significantly higher lipid peroxidation in plasma than matched controls (Fig. 1D, $p < 0.001$). In septic patients, levels of RNA oxidation were positively correlated with DNA oxidation (Spearman's Rho $r = 0.607$, $p < 0.001$), free nucleobase oxidation (Spearman's Rho $r = 0.543$, $p < 0.001$), sepsis severity (i.e. sepsis, severe sepsis, shock) (Spearman's Rho $r = 0.299$, $p = 0.006$) and amount of days spent in the hospital (Spearman's Rho $r = 0.327$ $p = 0.002$). Nucleobase oxidation positively correlated with sepsis severity (Spearman's Rho $r = 0.292$ $p = 0.007$), lactate levels (Spearman's Rho $r = 0.280$, $p = 0.028$), Multi Organ Failure (MOF) (Spearman's Rho $r = 0.316$, $p = 0.003$), the amount of days spent in the ICU (Spearman's Rho $r = 0.238$ $p = 0.029$) and in-hospital deaths (Spearman's Rho $r = 0.247$ $p = 0.023$). Collectively, these data suggests a high oxidative burden during sepsis reflected by oxidation of lipids, nucleobases, DNA and RNA, which were positively correlated with sepsis severity.

3.3. Sepsis increases mtDNA levels in plasma and mtDNA damage

To estimate mtDNA levels, the relative amount of mitochondrial gene *ND1* to nuclear gene *B2M* was determined in plasma. MtDNA levels were increased in plasma of septic patients compared to control subjects (Fig. 2A, $p < 0.001$). Next, we examend mtDNA damage as intact mitochondrial DNA is important for normal function of the mitochondria [8]. To determine if sepsis leads to mtDNA damage in human plasma, we performed a long-range PCR, whereby the long fragment of 10 kb is relatively more prone to damage as compared to the short fragment of 200 bp. Consequently, mtDNA damage can be measured by a relative decrease in the amount of the long fragment as compared to the short mtDNA fragment (Supplemental Fig. 1). Sepsis patients had higher levels of mtDNA damage than matched controls (Fig. 2B, $p < 0.001$). Collectively these data shows that sepsis causes an increase in mtDNA levels in plasma and an increase in mtDNA damage.

3.4. RNA and nucleobase oxidation are associated with mortality

To study whether increased oxidation and mitochondrial damage identifies septic patients at risk for long-term all-cause mortality, we first stratified patients in tertiles according to oxidative- and mitochondrial DNA damage to generate Kaplan-Meier survival curves. Kaplan-Meier analyses showed no difference in long-term all-cause mortality with low, medium or high amounts of DNA oxidation. Furthermore, it showed

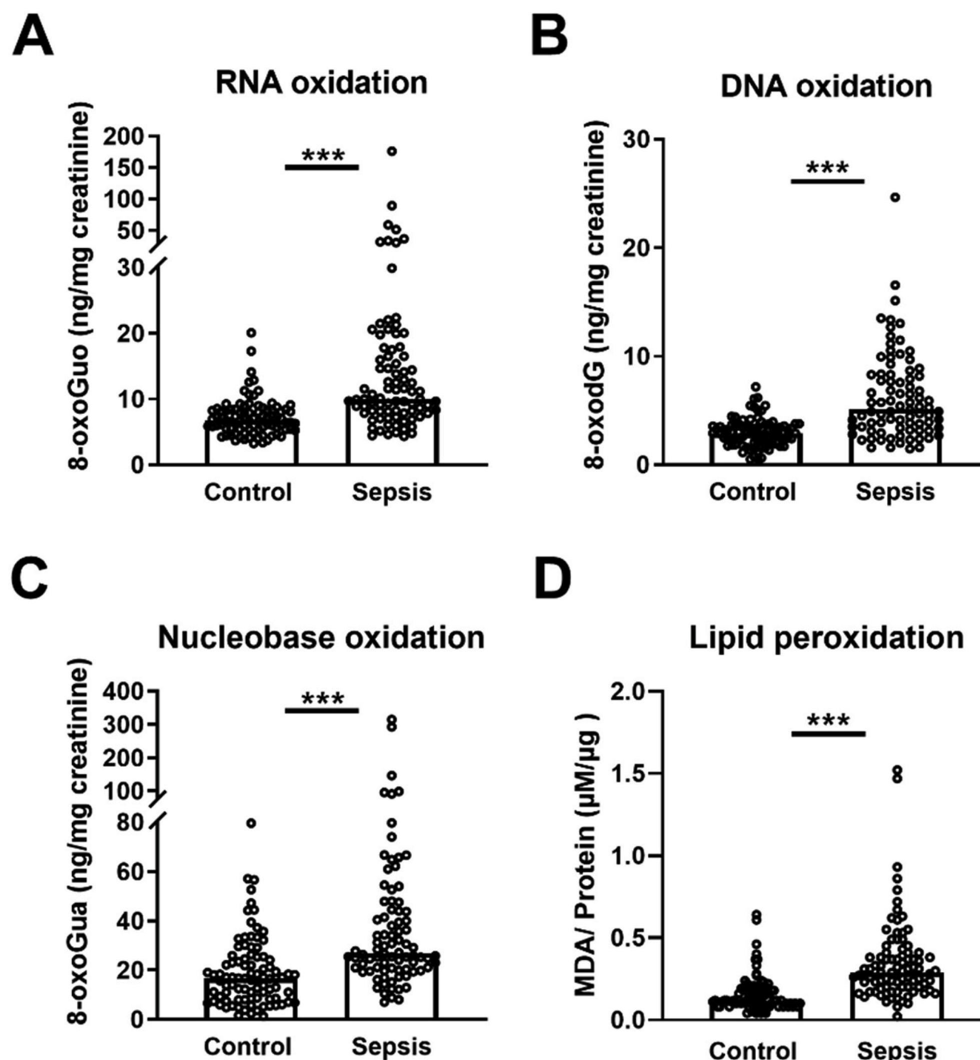

Fig. 1. Oxidation in urine and plasma. DNA, RNA and Nucleobase oxidation in urine was quantified by LC–MS/MS. Lipid peroxidation was quantified in plasma with the TBARS assay kit. A) DNA oxidation ($n = 91$ for controls and $n = 84$ for sepsis) B) RNA oxidation ($n = 91$ for controls and $n = 84$ for sepsis C) Nucleobase oxidation ($n = 91$ for controls and $n = 84$ for sepsis) D) Lipid peroxidation ($n = 91$ for controls and sepsis). Statistical analysis was performed using a Mann-Whitney u test. Bars represent the median, dots represent individual levels. *** means $p < 0.001$.

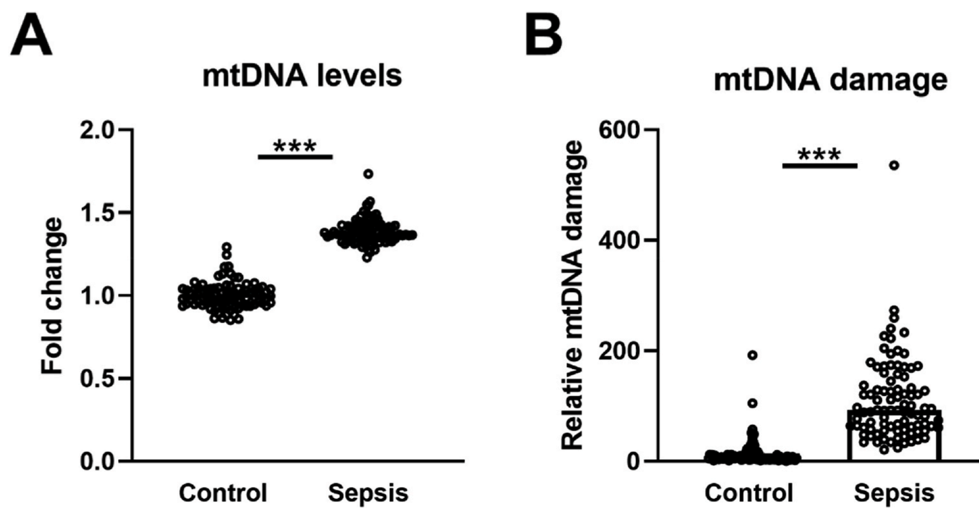

Fig. 2. Mitochondrial DNA levels and damage in plasma. Relative mitochondrial DNA levels were calculated per nuclei and mtDNA- and nuclear DNA were determined with RT-qPCR. mtDNA damage was determined with long-range PCR. A) mtDNA levels in plasma ($n = 91$) B) mtDNA damage in plasma ($n = 91$). Statistical analysis was performed using a Mann-Whitney u test. Bars represent the median, dots represent individual levels. *** means $p < 0.001$. mt: mitochondrial.

an increase in overall long-term all-cause mortality in the tertiles with the highest RNA- and nucleobase oxidation compared to those with intermediate or low oxidation (Fig. 3A-C). Lastly, Kaplan-Meier analyses showed no difference in long-term all-cause mortality with low, medium or high amounts of mtDNA damage (Fig. 3D).

Next, we performed a Cox regression survival analysis to assess the association between markers of oxidation and mtDNA with mortality among the sepsis patients. Univariate Cox regresson analyses revealed that sepsis severity (HR 1.86 [1.04–3.32, 95%CI] $p = 0.037$), age (HR 1.65 [1.31–2.06, 95%CI] per 10 years, $p < 0.001$), cancer (HR 2.24 [1.24–4.06 95%CI] $p = 0.011$), cardiovascular disease (HR 2.07 [1.14–3.75, 95%CI] $p = 0.017$), nucleobase oxidation (HR 1.07 [1.03–1.11, 95%CI] $p = 0.001$) and RNA oxidation (HR 1.18 [1.08–1.29, 95%CI] $p < 0.001$) were associated with long-term all-cause mortality. Sex, diabetes, lipid peroxidation, DNA oxidation and mtDNA damage were not associated with long-term all-cause mortality. Next,

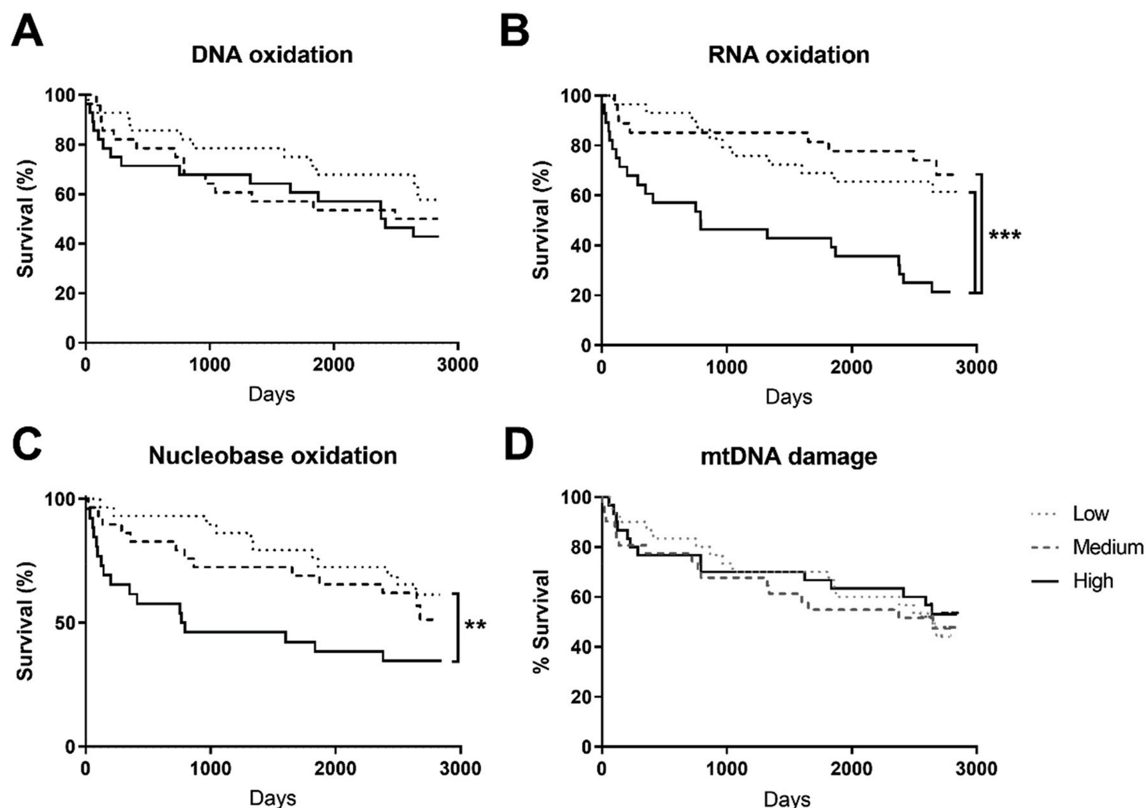

Fig. 3. Overall survival in septic patients with low, medium or high oxidation and mtDNA damage. Survival is expressed with Kaplan-Meier curves and differences were defined with logrank test. A) overall survival duration with different DNA oxidation levels in urine ($n = 84$) B) overall survival duration with different RNA oxidation levels in urine ($n = 84$) C) overall survival duration with different nucleobase oxidation levels in urine ($n = 84$ D) overall survival duration with different mtDNA damage levels in plasma ($n = 91$). **/*** means $p < 0.01/< 0.001$. mt: mitochondrial.

multivariate Cox regression analyses revealed that RNA oxidation is independently associated with long-term all-cause mortality (HR 1.22 [1.12–1.34, 95%CI] per 10 ng/mg, $p < 0.001$) after adjustment for age, cardiovascular disease, malignancy, sepsis severity, and nucleobase oxidation (Table 3). Other predictors of long-term all-cause mortality were age (HR 1.86 [1.45–2.39, 95% CI] per 10 years, $p < 0.001$), and malignancy (HR 3.88 [2.05–7.37, 95% CI] $p < 0.001$) (Table 3).

Lastly, ROC curves were constructed for predicting long-term all-cause mortality in patients with sepsis. Only oxidation markers and mitochondrial damage parameters that associated with long-term all-cause mortality were used to construct the ROC curve (i.e. RNA and nucleobase oxidation). The area under the ROC curve was 0.71 (0.60–0.82 95%BI, $p = 0.001$) for RNA oxidation and 0.67 (0.56–0.79 95%BI, $p = 0.007$) for nucleobase oxidation (Fig. 4).

4. Discussion

Sepsis is accompanied by a high oxidative burden and linked with damage to (mitochondrial) DNA and RNA [20,21]. However, the relationship between (mt) DNA and RNA damage and long-term all-cause mortality in sepsis survivors is unknown. In this study we showed higher levels of oxidated DNA, RNA and free nucleobases in urine from septic patients than matched controls. Moreover, septic patients had increased lipid peroxidation in plasma compared to matched controls. In addition, septic patients showed an increase in mtDNA levels and mtDNA damage in plasma compared to matched controls. Univariate Cox regresson analyses revealed that RNA oxidation and nucleobase oxidation in urine were associated with long-term all-cause mortality. Multivariate Cox Regression analysis showed that RNA oxidation was independently associated with long-term all-cause mortality, even after adjusting for age, nucleobase oxidation, cardiovascular disease, malignancy and sepsis severity.

Our data corroborates the increase in oxidative damage in sepsis [9, 22] evidenced by increased urinary DNA-, RNA- and free nucleobase levels compared to matched controls. Despite that oxidized DNA-, RNA- and free nucleobases are regarded as important biomarkers of oxidative stress [17], data in sepsis is scare. Our data are in keeping with Cheng et al. where nonsurvivors denoted higher DNA oxidation levels than critically ill sepsis patients survivors [23]. DNA oxidation levels in blood are as well higher in severe septic patients and patients with septic shock compared to controls [24]. However, in both studies data about RNA and nucleobase oxidation were lacking [23,24]. Another way to measure oxidative stress is via lipid peroxidation. When ROS are released it disrupts lipid membranes, causing a rise in reactive aldehydes, such as MDA which can be measured in plasma [25]. In agreement, we found a higher amount of plasma lipid peroxidation in septic patients than in matched controls, which corresponds with pervious findings [10,25]. In conclusion, sepsis causes oxidation of DNA, RNA and nucleobases with increased excretion of the oxidized species in urine, as well as lipid peroxidation in plasma, indicating an overall unbalanced oxidant state in the body.

Mitochondrial failure, which also comes with mtDNA damage, emerges as a keyplayer in the pathogenesis of organ failure in sepsis [20, 26]. Our data supports the link between sepsis, and mtDNA damage.

Table 3
Association of oxidation markers with long term-all-cause mortality.

	Adj. HR (95% CI)	*p*-value
Age (per 10 years)	1.86 (1.45–2.39)	<0.001
Malignancy	3.88 (2.05–7.37)	<0.001
RNA oxidation (per 10 ng/mg)	1.22 (1.12–1.34)	<0.001

In addition to the factors shown in the table, cardiovascular disease, sepsis severity and nucleobase oxidation were entered in the multivariable forward: conditional binary logistic regression analysis. Model characteristics: $X^2 = 46.65$, df 3, $p < 0.001$. HR: Hazard Ratio, 95% CI: 95% Confidence Interval.

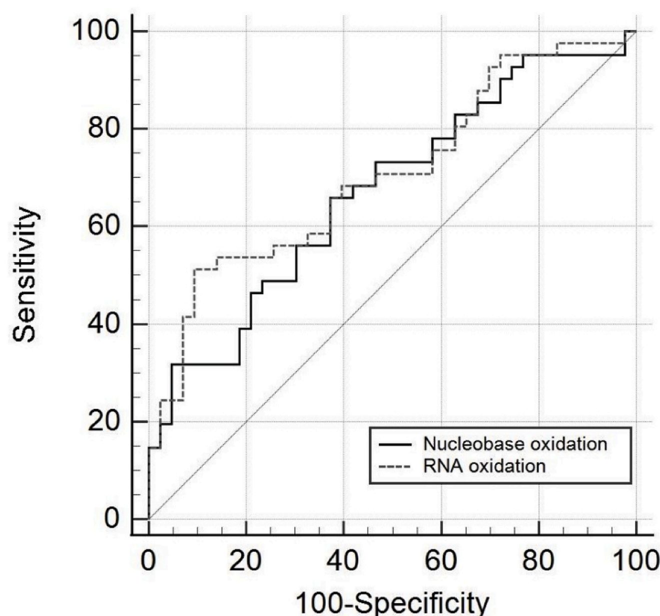

Fig. 4. Survival ROC curve. Receiver operating characteristic analysis using DNA and RNA as a predictor of long-term all-cause mortality in septic patients ($n = 84$).

This is primarily evidenced by the high amount of mtDNA damage found in plasma from septic patients compared to matched controls. The high mtDNA damage is in line with what we found in the human kidney from sepsis-AKI patients [20]. mtDNA damage is as well found in mononuclear cells from septic patients [27] and in preclinical septic mice models [21,28]. Furthermore, we showed an increase in mtDNA levels in plasma from septic patients compared to matched controls. This is in agreement with previous findings, as in several studies it was shown that mtDNA levels of the mitochondrial genes *ND1*, *ND2*, Cytochrome *c* oxidase subunit III (*COXIII)* and *Cytochrome b* in plasma are higher in septic patients than in controls [29–31]. The exact mechanism behind this elevated cell free mitochondrial DNA in plasma is not yet fully understood, but cell free DNA is released by cells and immune cells, such as neutrophils, eosoinphils and macrophages as a result of apoptosis or other forms of cellular damage [32,33]. Hence elevated mtDNA levels and mtDNA damage could be seen as a marker of cellular damage.

This is the first study to reveal that RNA oxidation is an independent predictor of long-term all-cause mortality in sepsis. The prognostic information of RNA oxidation was independent of other important factors that have been linked to mortality including, patient age, sepsis disease severity, and patient comorbidities like diabetes, cancer and cardiovascular disease. In accordance with our findings, RNA oxidation, but not DNA oxidation, was an independent predictor of mortality in patients with established and treated type 2 diabetes [34]. We did not find an independent association between DNA oxidation and long-term all-cause mortality in sepsis. Most likely this is because RNA is more prone to oxidative damage compared to DNA [35–37]. In keeping with our findings, the combined DNA-, RNA- and nucleobase oxidation in serum was associated with 30-day mortality in septic patients [10]. Although patients with sepsis had profoundly higher plasma levels of mtDNA damage as compared to matched controls, we did not find an association between mtDNA and mortality in sepsis. Likely, the long-range PCR method that was used to measure mtDNA damage, is only useful in distinguishing between control subjects without mitochondrial damage and sepsis patients with large amounts of mitochondrial damage, but not sensitive enough to find an association between mtDNA damage and long-term mortality in sepsis patients. Many biomarkers have been proposed to predict mortality in sepsis [38–41]. Yet,

none of these evaluated long-term all-cause mortality, thus hampering the comparison to the significance of oxidized nucleobases in urine above other biomarkers. The aim of the current study, however, was not to identify biomarkers predictive of long-term outcome after sepsis, but to test the hypothesis that sepsis leads to oxidation and damage of mtDNA, which may in turn predict long-term outcome. The association between levels of oxidated nucleic acids in urine and long-term mortality in this cohort, emphasizes the potential added value as a biomarker in predicting long-term mortality after sepsis.

Strengths of this studies are its prospective design, precise matching of the patients, long-term follow-up, complete and comprehensive patient demography and highly specific and sensitive analytical method for the measurement of oxidative stress in urine by LC/MS/MS. Potential limitations in this study include its single-center setting in a tertiary care hospital with referral of patients for academic specialist care, which may limit its generalizability. Another limitation is the use of TBAR assays to define lipid peroxidation in plasma, whereas high-performance liquid chromatography measurements is considered more specific and sensitive [42,43]. Lastly, the sepis-2 criteria were used to include patients since the study started in 2012 and sepsis-3 criteria were only used from 2016 on [44]. However, this would cause an underestimation in oxidation of nucleobases and mtDNA damage, since sepsis-3 criteria includes more severe sepsis cases. In addition, 87% of our population meets the sepis-3 criteria.

5. Conclusion

In conclusion, we revealed an increase in oxidation of lipids, DNA, RNA and nucleobases, a profound increase in mtDNA damage and an increase in relative mtDNA levels in septic patients compared to matched controls. RNA oxidation and nucleobase oxidation, were associated with long-term all-cause mortality, whereas RNA oxidation was an independent and strong indicator of long-term all-cause mortality, even after adjusting for age, cardiovascular disease, malignancy and sepsis. These findings are of clinical relevance, as sepsis is a major cause of early and late organ failure and death worldwide. Given the role of mitochondria in the pathophysiology of sepsis, pharmacologic strategies directed at limiting oxidative stress formation and mtDNA damage, might prevent or halt long-term health effects in sepsis.

Ethics approval and consent to participate

This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The Medical Ethics Review Committee (METC) of the UMCG reviewed and approved this study (METc 2012.077).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.12.305.

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