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Original article

Indicator of RNA oxidation in urine for the prediction of mortality in patients with type 2 diabetes and microalbuminuria: A post-hoc analysis of the Steno-2 trial

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ABSTRACT

Objective: The RNA oxidation product, 8-oxo-7,8-dihydroguanosine (8-oxoGuo), has been associated with mortality in patients with type 2 diabetes (T2D). However, the identification and the potential effect of approved treatments decreasing urine 8-oxoGuo level remain unraveled. In the Steno-2 study intensified multifactorial treatment compared with conventional multifactorial treatment reduced mortality in T2D patients with microalbuminuria by 45%. We assessed association between 8-oxoGuo at advanced baseline and total mortality with up to 19.9 years follow-up and from end of intervention to end of follow-up up to (up to 13.9 years).

Materials and methods: In 1993, 160 T2D patients with microalbuminuria were included in the Steno-2 trial. Urine samples from baseline were not available, but samples were available from 155 patients (97%) in 1995 (advanced baseline) and from 125 patients (96%) in 2001 (end of intervention). Hazard ratios (HR) for log2-transformed 8-oxoGuo and dichotomized (cut-off at median; low vs. high RNA oxidation) were estimated using Cox regressions.

Results: During follow-up of 19.9 years after advanced baseline, 89 died and no association between 8-oxoGuo and mortality was found ($p = 0.40$). From the end of 7.8 years of intervention and during remaining 13.9 years of observation, 61 died and doubling the urine 8-oxoGuo level was associated with mortality with a HR 3.08 (95% CI [1.86 − 5.12]; $p < 0.001$) after multiple adjustments. Patients with low 8-oxoGuo in the intensified-treatment had the lowest risk of dying compared with high 8-oxoGuo in the conventional-treatment both from advanced baseline onwards, adjusted HR 0.40 (95% CI [0.21 − 0.75]; $p = 0.004$), and from end of intervention onwards, adjusted HR 0.28 (95% CI [0.13 − 0.61]; $p = 0.001$).

Conclusions: In T2D patients with microalbuminuria, high levels of urine 8-oxoGuo after 7.8 years of multifactorial intervention was associated with higher mortality during 13.9 years of post-trial follow-up. Patients with low 8-oxoGuo in the intensified treatment group had the lowest risk of dying.

1. Introduction

According to the American Diabetes Association one in 11 Americans has diabetes [1]. In the Danish population about one in 18 has diabetes and type 2 diabetes is responsible for 80% of the cases [2]. Not only do patients with type 2 diabetes have decreased life

Abbreviations: UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry

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expectancy but the disease leads to severe complications, negatively affecting quality of life [3]. Even though mortality and morbidity has improved over the last decades [4], the increased diabetes prevalence warrants continued improvement of diabetes care [3].

The Steno-2 study has had considerable effect on type 2 diabetes treatment worldwide [5,6]. In the Steno-2 study intensified multifactorial treatment compared with conventional multifactorial treatment cut by half morbidity and mortality in T2D patients with microalbuminuria and increased complications free length of life by approximately eight years [5,7].

However, current treatment strategies are limited by means of not being able to predict which patients will need intensified treatment [8]. Thus, despite an agreement of intensified multifactorial treatment in type 2 diabetes, the remedies to tailor treatment for the individual patient await. In our attempt of pursuing this personal medicine approach, we have previously shown that RNA oxidation product 8-oxo-7,8-dihydroguanosine (8-oxoGuo) is prognostic for mortality and cardiovascular death in patients with type 2 diabetes [9], but at present no approved treatments lowering urine 8-oxoGuo level exist. The biomarkers of DNA and RNA oxidation 8-oxoGuo and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) are considered as reliable biomarkers of oxidative stress in several human diseases [10,11]. Oxidative stress is defined as an oxidants/antioxidants imbalance resulting in oxidants overproduction and causes cell signaling disruption and molecular damage depending on the extent [12,13]. Accumulating evidence shows that oxidative stress is linked to the pathogenesis of diabetic vascular complications [14–17], and involves oxidative damage to e.g. DNA and RNA [18,19]. Thus, altering nucleic acid oxidation levels could possibly better disease progression.

The objective of this study was to assess, in a post-hoc analysis, the association between urinary levels of 8-oxoGuo and all-cause mortality from advanced baseline to end of follow-up; up to 19.9 years, and from end of 7.8 years of intervention to end of follow-up; up to 13.9 years. For comparison, same analyses with DNA oxidation product 8-oxodG were performed since increased urinary 8-oxodG has been correlated with hyperglycemia and implicated in diabetic complications [20].

2. Material and methods

2.1. Study population and design

Detailed information from the Steno-2 study on patient inclusion, randomisation, intervention and follow-up including anthropometric, physiological and biochemical measurements have previously been published [21].

In short, in 1993, 160 patients with type 2 diabetes and microalbuminuria were randomised 1:1 to eight years of either intensified multifactorial treatment or conventional multifactorial treatment (control group).

At the advanced baseline (1995), urine samples from 155 subjects (97% of the patients) were available (Fig. 1). At the end of 7.8 years intervention (2001), 125 urine samples (96% of the patients alive) were available (Fig. 1).

2.2. Study endpoints

For the assessment of the urinary nucleic acid oxidation biomarker risk analyses, we used all-cause mortality as outcome measure in pooled-cohort analyses from 1995 with up to 19.9 years of follow-up and from 2001 with up to 13.9 years of follow-up, respectively.

2.3. Measurement of urinary nucleic acid oxidation biomarkers

8-oxoGuo levels and 8-oxodG levels in urine samples were quantified by a validated method based on ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and corrected for urinary creatinine concentration [22].

Previously published data from our laboratory showed a lower limit of quantification of 1.0 nM for both 8-oxoGuo and 8-oxodG, with an accuracy of 98.7% for 8-oxoGuo and 95.7% for 8-oxodG [22]. Average within-day precision was 2.9% for 8-oxoGuo and 3.7% for 8-oxodG; average between-day precision was 1.5% for 8-oxoGuo and 3.4% for 8-oxodG [22]. Specificity was determined by measuring quantifier and qualifier ions and using the relevant acceptance criteria for the response ratio between the two ions.

The analyses were performed in June 2017. The urine samples were stored at − 80 °C until the time of analyses. At this time the urine samples were stored at − 20 °C, which secure stable amount of the nucleic acid oxidation markers throughout 15 years [23].

2.4. Ethics

The original Steno-2 study was in accordance with the declaration of Helsinki and approved by the local ethics committee (Ethics committee, Capital Region of Denmark; protocol ID number: H-KA-99035-GS, add. 41104) and the Danish Data Protection Agency (J.Nr. 2015-41-4042). The original trial was registered at Clinicaltrials.gov, number NCT00320008. This post-hoc study was, in addition to the above, approved by the local ethics committee (H-16029199). All participants gave their informed consent before randomisation and again at each follow-up visit.

2.5. Statistics

For variables with Gaussian distribution, means were compared using *t*-test. For dichotomous variables, Chi-squared test were used to compare groups. Based on the Kaplan-Meier estimates, survival probability plots were drawn. Cox regression analyses were performed for the adjusted and stratified all-cause mortality estimations. 8-oxoGuo and 8-oxodG were used both as continuous variables (log2-transformed) and as categorical (dichotomized, cut-off at median; low vs. high RNA oxidation). Based on previous literature and availability of covariates, the final fully adjusted Cox regression model included adjustment for age, sex, treatment allocation, urinary albumin excretion ratio (AER), systolic blood pressure, total plasma cholesterol, and due to availability either waist-hip-ratio (WHR) in 1995 and body mass index (BMI) in 2001, respectively. Complete case analyses were conducted for log2-transformed models since one was missing in the multiple adjusted models (1 missing for WHR in 1995 and 1 missing for BMI in 2001, respectively). To ensure stable models, all models were fitted with 1000 bootstrap samples and considered stable. We tested the added value of RNA oxidation in the Cox regressions models with the area under the curve (AUC) and prediction error with the Brier score.

Furthermore, for the assessment of an intensified multifactorial treatment compared with conventional treatment on RNA oxidation in patients with type 2 diabetes and microalbuminuria on mortality, patients were stratified according to levels of RNA oxidation and intervention in a Cox regression model adjusted for age and sex.

R version 3.4.1 was used to perform the statistical analyses [24].

3. Results

3.1. Patients' characteristics according to nucleic acid oxidation marker levels

In 1995, median RNA oxidation level was 2.4nmol/mmol creatinine and median DNA oxidation level was 1.3nmol/mmol creatinine. No difference in nucleic acid oxidation marker levels was seen between the intensified and conventional treatment group.

In 1995, patients' characteristics were similar in the low and high RNA oxidation groups except for more women (33.8% vs. 17.9%, $p = 0.04$), higher HbA_{1c} (74.1 mmol/mol vs. 68.2 mmol/mol, $p = 0.04$)

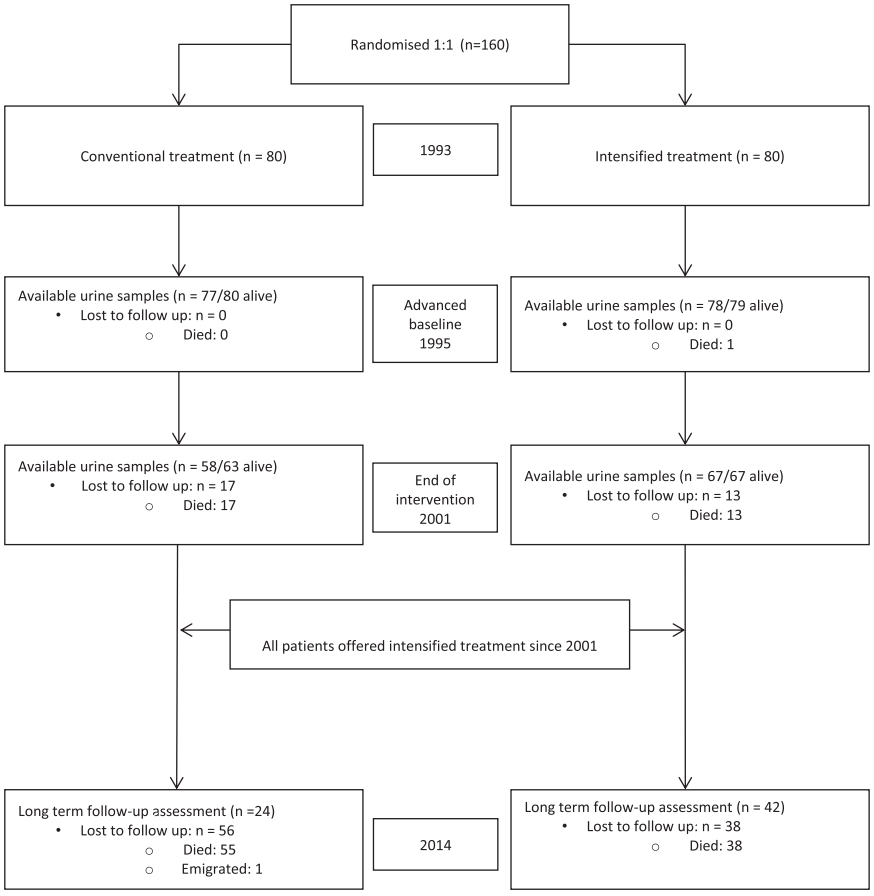

Fig. 1. The consort diagram shows the patient flow of the original Steno-2 study design and available urine analyses at the advanced baseline in 1995 and 2001.

Table 1a
Advanced baseline variables (1995) according to low and high RNA oxidation.

Advanced baseline variables (1995)	Level	8-oxoGuo low (n = 78)	8-oxoGuo high (n = 77)	Total (n = 155)	p-value
Age (years)	mean (sd)	55.8 (7.3)	57.3 (7.0)	56.5 (7.2)	0.18
Sex	Women	14 (17.9)	26 (33.8)	40 (25.8)	
	Men	64 (82.1)	51 (66.2)	115 (74.2)	0.04
Allocation	Conventional	35 (44.9)	42 (54.5)	77 (49.7)	
	Intensified	43 (55.1)	35 (45.5)	78 (50.3)	0.30
HbA_{1c} (mmol/mol)	mean (sd)	68.2 (16.2)	74.1 (18.9)	71.1 (17.8)	0.04
Years with diabetes	mean (sd)	8.2 (6.0)	10.0 (6.3)	9.1 (6.2)	0.06
Urinary AER	median [IQR]	70.9 [36.4, 136.3]	71.5 [33.8, 165.0]	71.5 [34.1, 158.7]	0.73
Macroalbuminuria	No	75 (96.2)	65 (84.4)	140 (90.3)	
	Yes	3 (3.8)	12 (15.6)	15 (9.7)	0.03
eGFR	mean (sd)	113.6 (24.4)	106.8 (24.4)	110.2 (24.6)	0.08
	missing	1	0	1	
Cholesterol	mean (sd)	5.1 (1.0)	5.2 (1.5)	5.2 (1.3)	0.48
Hdl	mean (sd)	1.0 (0.3)	1.0 (0.3)	1.0 (0.3)	0.86
Ldl	mean (sd)	3.2 (0.8)	3.2 (0.8)	3.2 (0.8)	0.60
	missing	2	5	7	
Triglycerides	median [IQR]	1.6 [1.1, 2.4]	1.5 [1.0, 3.0]	1.5 [1.0, 2.6]	0.87
Systolic BP	mean (sd)	143 (20.1)	144 (21.3)	143 (20.6)	0.67
Diastolic BP	mean (sd)	80 (8.3)	81 (9.6)	81 (8.9)	0.77
Bodyweight	mean (sd)	90.0 (14.2)	90.7 (15.3)	90.4 (14.7)	0.75
WHR	mean (sd)	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)	0.58
	Missing	0	1	1	
BMI (1993)	mean (sd)	29.8 (4.5)	29.7 (4.3)	29.8 (4.4)	0.90
	Missing	12	16	28	
Smoking (1993)	No	51 (65.4)	47 (61.0)	98 (63.2)	
	Yes	27 (34.6)	30 (39.0)	57 (36.8)	0.69

Abbreviations: IQR, interquartile range.

Table 1b
Advanced baseline variables (1995) according to low and high DNA oxidation.

Baseline variables in 1995	Level	8-oxodG low (n = 78)	8-oxodG high (n = 77)	Total (n = 155)	p-value
Age (years)	mean (sd)	56.1 (7.2)	57.0 (7.2)	56.5 (7.2)	0.42
Sex	Women	18 (23.1)	22 (28.6)	40 (25.8)	
	Men	60 (76.9)	55 (71.4)	115 (74.2)	0.55
Allocation	Conventional	38 (48.7)	39 (50.6)	77 (49.7)	
	Intensified	40 (51.3)	38 (49.4)	78 (50.3)	0.94
HbA_{1c} (mmol/mol)	mean (sd)	70.3 (17.1)	72.0 (18.5)	71.1 (17.8)	0.54
Years with diabetes	mean (sd)	8.9 (5.8)	9.3 (6.6)	9.1 (6.2)	0.74
Urinary AER	median [IQR]	72.3 [38.4, 164.3]	70.4 [33.8, 133.0]	71.5 [34.1, 158.7]	0.55
Macroalbuminuria	No	71 (91.0)	69 (89.6)	140 (90.3)	
	Yes	7 (9.0)	8 (10.4)	15 (9.7)	0.98
eGFR	mean (sd)	109.3 (24.9)	111.0 (24.4)	110.2 (24.6)	0.67
	missing	1	0	1	
Cholesterol	mean (sd)	5.1 (1.0)	5.3 (1.5)	5.2 (1.3)	0.40
Hdl	mean (sd)	1.0 (0.3)	1.0 (0.3)	1.0 (0.3)	0.17
Ldl	mean (sd)	3.2 (0.8)	3.2 (0.8)	3.2 (0.8)	0.61
	missing	2	5	7	
Triglycerides	median [IQR]	1.5 [1.0, 2.3]	1.7 [1.1, 3.0]	1.5 [1.0, 2.6]	0.14
Systolic BP	mean (sd)	145 (21.4)	142 (19.9)	143 (20.6)	0.29
Diastolic BP	mean (sd)	82 (8.7)	80 (9.1)	81 (8.9)	0.17
Bodyweight	mean (sd)	90.0 (15.2)	90.8 (14.3)	90.4 (14.7)	0.74
WHR	mean (sd)	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)	0.43
	missing	0	1	1	
BMI (1993)	mean (sd)	29.8 (4.7)	29.8 (4.1)	29.8 (4.4)	0.99
	missing	21	7	28	
Smoking (1993)	No	54 (69.2)	44 (57.1)	98 (63.2)	
	Yes	24 (30.8)	33 (42.9)	57 (36.8)	0.16

Abbreviations: IQR, interquartile range.

and macro-albuminuria (15.6% vs. 3.8%, p = 0.03) in patients with high RNA oxidation (Table 1a). Treatment allocation was not significantly different for high and low RNA oxidation group (p = 0.30). For DNA oxidation, no differences were seen between the low and high DNA oxidation group (Table 1b).

In 2001, median RNA oxidation level was 2.8nmol/mmol creatinine and median DNA oxidation level was 1.7nmol/mmol creatinine. No difference in levels or changes in nucleic acid oxidation marker levels was seen between the intensified and conventional treatment group.

In 2001, patients in the high versus in the low RNA oxidation group were older (mean = 63 years vs. 61 years, p = 0.049), more were women (38.7% vs. 12.7%, p = 0.002) and diastolic blood pressure was slightly lower (mean = 73 mmHg vs. 77 mmHg, p = 0.04; Table 2a). Still, allocation was not significantly different between high and low RNA oxidation group (p = 0.33).

For DNA oxidation, no differences were seen except for higher body weight in the high than in the low DNA oxidation group (mean = 96.9 kg vs. 90.8 kg, p = 0.04; Table 2b).

3.2. All-cause mortality pooled analyses for RNA oxidation

Urinary levels of RNA oxidation in 1995, after 2 years of intervention with following 19.9 years of follow-up, were not associated with mortality (Fig. 2a and Table 3).

From end of intervention in 2001, 7.8 years after baseline, with 13.9 years of follow-up, 38.1% compared with 59.7% in the low versus high

Table 2a
Descriptive variables (2001) according to low and high RNA oxidation.

Descriptive variables in 2001	Level	8-oxoGuo low (n = 63)	8-oxoGuo high (n = 62)	Total (n = 125)	p-value
Age	mean (sd)	60.9 (6.7)	63.4 (7.2)	62.2 (7.1)	0.049
Sex	Women	8 (12.7)	24 (38.7)	32 (25.6)	
	Men	55 (87.3)	38 (61.3)	93 (74.4)	0.002
Allocation	Conventional	26 (41.3)	32 (51.6)	58 (46.4)	
	Intensified	37 (58.7)	30 (48.4)	67 (53.6)	0.33
HbA_{1c} (mmol/mol)	mean (sd)	68.3 (14.9)	66.9 (18.9)	67.6 (16.9)	0.66
Urinary AER	median [IQR]	66.5 [25.1, 268.3]	63.9 [23.9, 268.0]	66.5 [24.7, 271.3]	0.83
Macroalbuminuria	No	44 (69.8)	44 (71.0)	88 (70.4)	
	Yes	19 (30.2)	18 (29.0)	37 (29.6)	1.00
eGFR	mean (sd)	93.1 (28.5)	83.8 (28.6)	88.5 (28.8)	0.07
	missing	2	2	4	
Cholesterol	mean (sd)	4.8 (1.3)	4.9 (1.4)	4.8 (1.3)	0.66
Hdl	mean (sd)	1.2 (0.4)	1.2 (0.4)	1.2 (0.4)	0.52
Ldl	mean (sd)	2.7 (1.1)	2.6 (0.9)	2.7 (1.0)	0.63
	missing	4	6	10	
Triglycerides	median[IQR]	1.4 [1.1, 2.3]	1.8 [1.1, 2.8]	1.5 [1.1, 2.5]	0.16
Systolic BP	mean (sd)	139 (16.1)	137 (17.9)	138 (16.9)	0.61
Diastolic BP	mean (sd)	77 (11.4)	73 (9.4)	75 (10.6)	0.04
Bodyweight	mean (sd)	94.3 (14.2)	93.5 (19.0)	93.9 (16.7)	0.80
	missing	0	1	1	
BMI	mean (sd)	30.5 (4.9)	30.8 (5.4)	30.6 (5.1)	0.75
	missing	0	1	1	

Abbreviations: IQR, interquartile range.

Table 2b
Descriptive variables (2001) according to low and high DNA oxidation.

Descriptive variables in 2001	Level	8-oxodG low (n = 63)	8-oxodG high (n = 62)	Total (n = 125)	p-value
Age	mean (sd)	62.6 (7.1)	61.8 (7.0)	62.2 (7.1)	0.53
Sex	Women	14 (22.2)	18 (29.0)	32 (25.6)	
	Men	49 (77.8)	44 (71.0)	93 (74.4)	0.50
Allocation	Conventional	30 (47.6)	28 (45.2)	58 (46.4)	
	Intensified	33 (52.4)	34 (54.8)	67 (53.6)	0.92
HbA_{1c} (mmol/mol)	mean (sd)	69.1 (14.2)	66.1 (19.3)	67.6 (16.9)	0.33
Urinary AER	median [IQR]	70.1 [28.0, 284.7]	58.2 [19.6, 262.1]	66.5 [24.7, 271.3]	0.33
Macroalbuminuria	No	44 (69.8)	44 (71.0)	88 (70.4)	
	Yes	19 (30.2)	18 (29.0)	37 (29.6)	1.00
eGFR	mean (sd)	88.8 (31.6)	88.2 (25.7)	88.5 (28.8)	0.90
	missing	1	3	4	
Cholesterol	mean (sd)	4.7 (1.2)	5.0 (1.4)	4.8 (1.3)	0.21
Hdl	mean (sd)	1.2 (0.4)	1.2 (0.4)	1.2 (0.4)	0.77
Ldl	mean (sd)	2.6 (1.0)	2.7 (1.1)	2.7 (1.0)	0.51
	missing	5	5	10	
Triglycerides	median [IQR]	1.4 [1.1, 2.2]	1.7 [1.1, 3.1]	1.5 [1.1, 2.5]	0.21
Systolic BP	mean (sd)	140 (17.6)	136 (16.1)	138 (16.9)	0.18
Diastolic BP	mean (sd)	76 (10.9)	74 (10.3)	75 (10.6)	0.31
Bodyweight	mean (sd)	96.9 (16.1)	90.8 (16.8)	93.9 (16.7)	0.04
	missing	0	1	1	
BMI	mean (sd)	31.4 (5.0)	29.8 (5.2)	30.6 (5.1)	0.09
	missing	0	1	1	

Abbreviations: IQR, interquartile range.

RNA oxidation group had died (log-rank test p = 0.02, Fig. 2c). Both the unadjusted, age- and sex adjusted, and multiple adjusted cox regression analyses showed significantly higher hazard ratio (HR) for log2-transformed RNA oxidation (multiple adjusted HR 3.08 [1.86–5.12]; p-value < 0.001, Table 3).

3.3. All-cause mortality for RNA oxidation stratified for intervention

When stratifying according to intervention group in 1995 (Fig. 3a), the lowest HR was seen for low RNA oxidation group receiving intensified treatment (age- and sex adjusted HR 0.40, 95% CI [0.21–0.75], p = 0.004; Fig. 4a).

From 2001 to end of follow-up (Fig. 3b), the low RNA oxidation group receiving intensified treatment had significantly lower HR compared to conventional treatment with high RNA oxidation (age- and sex adjusted HR 0.28, 95% CI [0.13 −0.61], p = 0.001; Fig. 4b).

3.4. DNA oxidation survival analyses

For DNA oxidation, no difference in mortality was seen between low and high DNA oxidation (log-rank test p = 0.6, Figs. 2b and 2d).

3.5. Additional analyses

The added value of the urinary RNA oxidation biomarker 8-oxoGuo in the fully adjusted Cox regression model (2001) was evaluated by the AUC. The AUC was 88.7% for the full model versus a full model without 8-oxoGuo 86.1%, but was not statistical significant (p = 0.17). The prediction error was evaluated by the Brier score and was 14.1% with 8-oxoGuo versus 15.2% in a full model without 8-oxoGuo, but not statistical significant.

In 2001, interaction analyses between RNA oxidation and

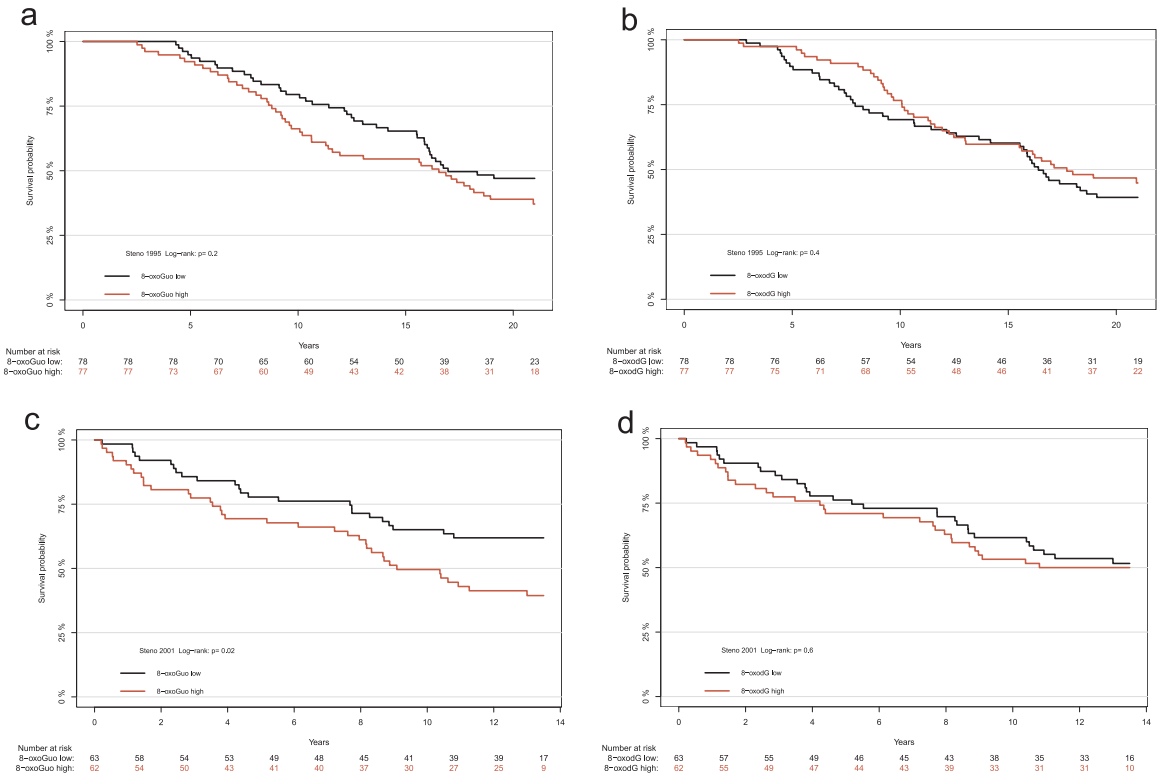

Fig. 2. Survival probability according to low and high nucleic acid oxidation markers in 1995 onwards and 2001 onwards. Kaplan-Meier plot shows pooled cohort analyses of survival probability, the black line shows low nucleic acid oxidation = below the median and the red line shows high nucleic acid oxidation = above the median.

Table 3
RNA oxidation and all-cause mortality risk.

Cox regression analyses	Total number of events	Unadjusted model according to log2(8-oxoGuo) with 95% CI	P-value	Age- and sex -adjusted model according to log2(8-oxoGuo) with 95% CI*	P-value	Multiple adjusted model according to log2(8-oxoGuo) with 95% CI**	P-value
Advanced baseline 1995	88	1.36 [0.97–1.92]	0.08	1.23 [0.86–1.75]	0.26	1.18 [0.80–1.73]	0.40
End of intervention 2001	60	2.84 [1.81–4.45]	< 0.001	2.88 [1.79–4.61]	< 0.001	3.08 [1.86–5.12]	< 0.001

* Age-, sex- and intervention- stratification gave similar results.
** Multiple models included adjustment for age, sex, albumin excretion rate (geometric mean), systolic blood pressure, allocation and either waist hip ratio (1995) or body mass index (2001).

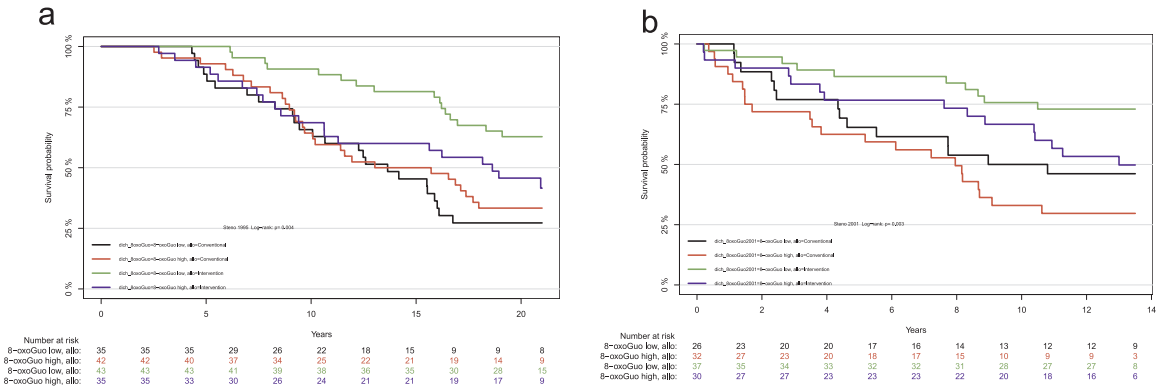

Fig. 3. Survival probability according to low and high RNA oxidation markers stratified for intervention. Kaplan-Meier plot shows survival probability in 1995 onwards and 2001 onwards; for the conventional treatment, the black line shows low RNA oxidation = below the median and the red line shows high RNA oxidation = above the median; for the intensified treatment, the green line shows low RNA oxidation = below the median and the blue line shows high RNA oxidation = above the median.

intervention in the fully adjusted Cox regression model were tested with ANOVA and was not significant (p = 0.18). Furthermore, we tested the same interactions in 1995 and found p = 0.03.

4. Discussion

In patients with type 2 diabetes and microalbuminuria we found that a high level of RNA oxidation after 7.8 years of intervention was associated with higher mortality after 13.9 years of follow-up.

For the first time, we have showed that low urinary RNA oxidation level after an intensified multifactorial treatment for 7.8 years is associated with lower mortality compared with high RNA oxidation after a conventional treatment. This was also seen after only 2 years intervention, but not as pronounced.

Type 2 diabetes is a multi-organ disease [25], and as such, a whole-body biomarker like urinary RNA oxidation is straightforward as both a prognostic and predictive biomarker. While we have established urinary RNA oxidation as a prognostic biomarker [9,26], we believe this study is the first evidence of urinary RNA oxidation as a predictive biomarker in patients with type 2 diabetes.

To our knowledge, this is the first step towards finding treatments that decrease urinary RNA oxidation and improve survival. This finding also merits future examination of more specific pharmacological treatment regimens than in this study. The Steno-2 study design is too limited in size to enable analyses of each single component of the intervention. Moreover, the intensified treatment regimen included lifestyle changes including smoking cessation which could have altered the urinary nucleic acid oxidation markers. Impact of lifestyle intervention is seen for heart failure markers like NT-proBNP in the Look AHEAD study [27], and a number of diabetic risk factors were decreased in the Diabetes Care in General Practice (DCGP) study which also included an intensified lifestyle intervention [28].

In the redox research field, DNA oxidation has been given the major attention over the past decades whereas less thought has been given on RNA oxidation [29,30]. Yet, RNA oxidation is increasingly being appreciated for its part in various diseases [29,30]. It is now evident that imbalance or rather disturbed oxidative damage to RNA can be measured in urine and used as a biomarker for disease complications; exemplified in diabetic, neurological and psychiatric diseases [30,31].

Oxidative damage to DNA measured as 8-oxodG was discovered in 1984 and later in vitro and in vivo experiments found that 8-oxoGuo was higher in RNA than 8-oxodG in DNA after inducement by agents [10,11]. Plausible reasons for this difference between DNA and RNA include the double-stranded structure of DNA versus the single-stranded structure of RNA, the lack of protective proteins such as histones in DNA and repair enzymes which provide prompt removal of 8-oxoGua in DNA or maybe higher reactivity of reactive oxygen species (ROS) with RNA in the cytoplasm versus nuclear DNA [11,32]. Moreover, the location of RNA in the cytoplasm in close proximity to the ROS-producing mitochondria versus nuclear DNA suggests that compartmentalization may also be of significance [31]. Although repair mechanisms may exist for RNA, degradation and elimination are currently the believed mechanisms [30,31,33]. We suggest that these reasons could explain the differences we and others see between RNA and DNA oxidation in patients with type 2 diabetes [9,19,26,34].

With this study, we indicate that a urinary RNA oxidation biomarker could be implemented in clinical trials to monitor treatment response and subsequent risk of diabetic late complications. Recently, an expert group emphasized the necessity of using the full biorepositories, highlighting the findings of new biomarkers, to achieve precision medicine onwards [35]. Perhaps this could contribute to transform the future diabetes care along with already improved reevaluations since FDA guidelines in 2008 prompted cardiovascular outcome safety trials [35].

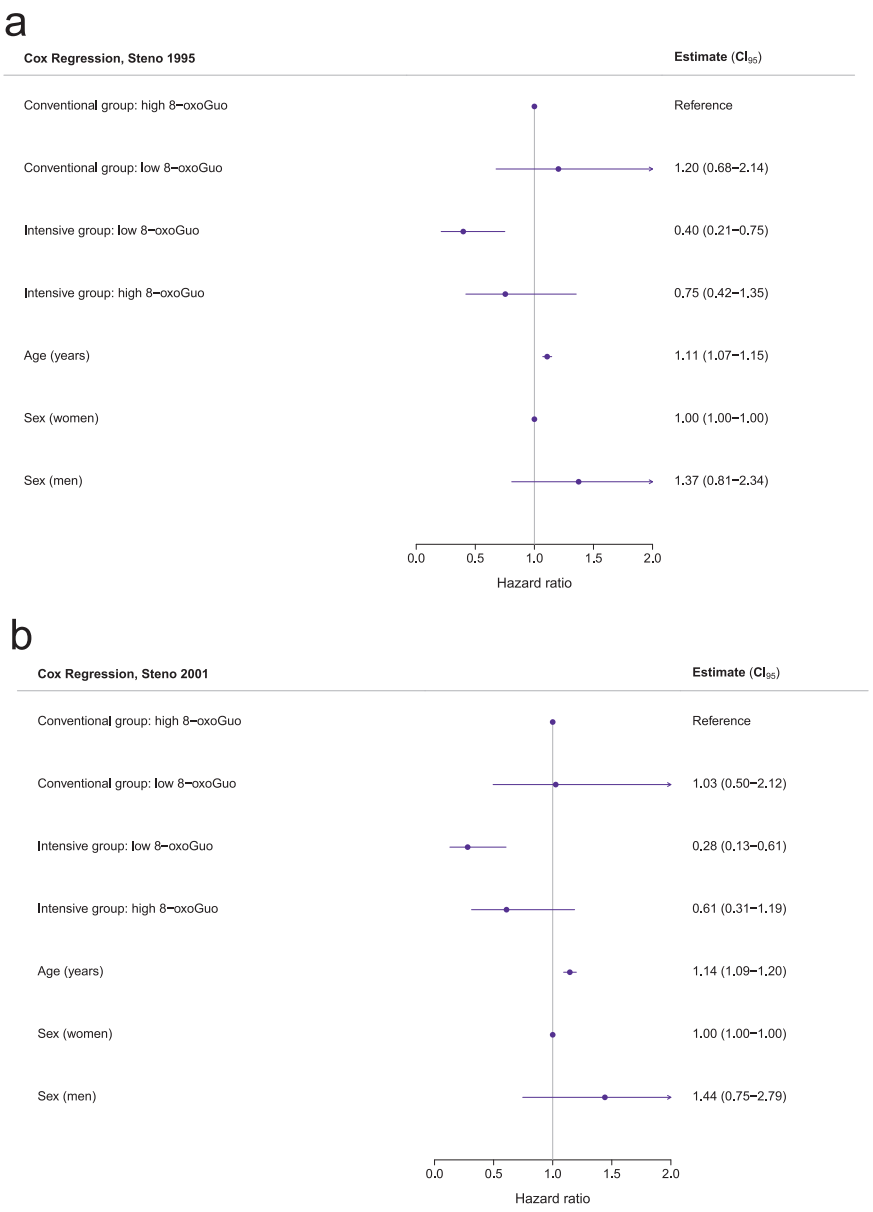

Fig. 4. Forest plot of hazard ratios according to low and high RNA oxidation in 1995 onwards and 2001 onwards stratified for intervention adjusted for age and sex.

5. Strengths and limitations

The Steno-2 study is a unique study in many ways. First of all, only one patient was lost to follow-up because of emigration. Second of all, detailed long-term follow-up for up till 19.9 years since advanced baseline in 1995 and 21.1 years since enrollment in 1993 was available. Third of all, care of the intensified treatment arm was primarily carried out at the Steno Diabetes Center facility ensuring minimally variation compared with multi-facility studies.

Importantly, the Steno 2 cohort was a high-risk group with pre-existing microalbuminuria. Therefore, these results may not be applicable to lower risk groups of patients with type 2 diabetes.

Due to availability, only urine samples for 155 patients (97%) in 1995 and urine samples for 125 patients (96% of subjects alive) in 2001 were used in this study. Since the Steno-2 study sample size was originally small further reductions in size warrant attention to the adjustments in the statistical models in this post-hoc study. We assured that our final models were stable; however, power issues or chance findings are likely to occur.

The median RNA oxidation and DNA oxidation was a little lower in this study compared with our previous cohort studies [9,26]. In part, this could be explained by the fact that the urine samples were not morning spot urine but a sample of 24-h urine collection and thus more diluted.

This was a post-hoc study and therefore was not designed to investigate the predictive potential of urinary RNA oxidation including the added value of the biomarker. These features should be investigated in a study with pre-specified power calculations.

Interestingly, we see that high RNA oxidation at the advanced baseline in 1995 was associated with diabetes risk factors such as macroalbuminuria and higher HbA_{1c}. These associations were attenuated in 2001. We speculate if this is due to the intervention, but this again require additional studies since no interactions were seen between RNA oxidation and intervention groups in our analyses in 2001. We did not see any difference between intervention groups in changes in RNA oxidation from available urine samples in 1995–2001, however, more patients died in the conventional treatment group than the intensified treatment group, and the patients who died plausible had higher RNA oxidation. The added value of the biomarker for RNA oxidation in our study showed a trend for better performance in the

prediction models, but was not statistically significant. Moreover, since the model already showed good prediction accuracy before adding RNA oxidation, performance enhancement of the model was more difficult. Although this is in agreement with a previous study [26], a larger study with high risk patients with type 2 diabetes is warranted to address the added value.

6. Conclusions

In T2D patients with microalbuminuria, urinary 8-oxoGuo level after 7.8 years of intensified multifactorial treatment was associated with total mortality during 13.9 years of post-trial follow-up. Patients allocated to intensified multifactorial treatment had lower risk of dying overall, and patients with low RNA oxidation in the intensified multifactorial treatment had the lowest risk of dying while patients with high RNA oxidation in conventional treatment had the highest risk of dying. These results are the first evidence of urine 8-oxoGuo as a predictive biomarker in patients with type 2 diabetes.

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Duality of interest

The authors declare no duality of interest. Since the conclusion of the 21 years follow-up of the Steno-2 study, JO has been a full-time employee of Novo Nordisk Scandinavia A/B, Region Denmark.

Contribution statement

OP conceived and designed the Steno-2 study and acquired all funding throughout the study and follow-up periods. In this post-hoc study, LKK performed all the statistical analyses including tables and figures, researched data, drafted the manuscript and critically revised the manuscript with the guidance from JO and HEP. LKK, JO and HEP planned the statistical analyses and contributed to interpretation and discussion of results. TH performed the urine analyses and interpreted the analyses. All authors contributed to the concept, design, interpretation and revision of the manuscript. All authors were responsible for the final decision to submit for publication.

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