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## Effects of 18-months metformin versus placebo in combination with three insulin regimens on RNA and DNA oxidation in individuals with type 2 diabetes: A post-hoc analysis of a randomized clinical trial

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## ABSTRACT

Formation of reactive oxygen species has been linked to the development of diabetes complications. Treatment with metformin has been associated with a lower risk of developing diabetes complications, including when used in combination with insulin. Metformin inhibits Complex 1 in isolated mitochondria and thereby decreases the formation of reactive oxygen species. Thus, we *post-hoc* investigated the effect of metformin in combination with different insulin regimens on RNA and DNA oxidation in individuals with type 2 diabetes. Four hundred and fifteen individuals with type 2 diabetes were randomized (1:1) to blinded treatment with metformin (1,000 mg twice daily) versus placebo and to (1:1:1) open-label biphasic insulin, basal-bolus insulin, or basal insulin therapy in a 2 × 3 factorial design. RNA and DNA oxidation were determined at baseline and after 18 months measured as urinary excretions of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), respectively. Urinary excretion of 8-oxoGuo changed by +7.1% (95% CI: 0.5% to 14.0%,  $P = 0.03$ ) following metformin versus placebo, whereas changes in 8-oxodG were comparable between intervention groups. Biphasic insulin decreased urinary excretion of 8-oxoGuo (within-group: −9.6% (95% CI: −14.4% to −4.4%)) more than basal-bolus insulin (within-group: 5.2% (95% CI: −0.5% to 11.2%)),  $P = 0.0002$

**Abbreviations:** 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; IQR, interquartile range; ROS, reactive oxygen species; SD, standard deviation.

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between groups, and basal insulin (within-group: 3.7% (95% CI: –2.0% to 9.7%)),  $P = 0.0007$  between groups. Urinary excretion of 8-oxodG decreased more in the biphasic insulin group (within-group: –9.9% (95% CI: –14.4% to –5.2%)) than basal-bolus insulin (within group effect: –1.2% (95% CI: –6.1% to 3.9%)),  $P = 0.01$  between groups, whereas no difference was observed compared with basal insulin. In conclusion, eighteen months of metformin treatment in addition to different insulin regimens increased RNA oxidation, but not DNA oxidation. Biphasic insulin decreased both RNA and DNA oxidation compared with other insulin regimens.

## 1. Introduction

Individuals with type 2 diabetes have increased risk of micro- and macrovascular complications [1], neurodegenerative diseases [2], and several forms of cancers [3]. The biological mechanisms that mediate these associations are not fully understood and have attracted much attention in order to potentially prevent development and progression of these complications through, e.g., a targeted intervention. Increased formation of reactive oxygen species during energy metabolism in the mitochondria has been proposed as a mechanism for the development of diabetes complications [4].

Metformin is recommended as first-line medical treatment to manage hyperglycemia in individuals diagnosed with type 2 diabetes [1]. In addition, several guidelines recommend metformin as an adjunct to insulin therapy primarily due to less weight gain, reduction of HbA<sub>1c</sub>, and less insulin requirement compared with insulin treatment alone [5]. Metformin has been known for a century and as medication from the 1950's [6]; however, the target organ and mode of actions remain debated. For long, the liver was considered the target organ responsible for the antihyperglycemic effects through a modulation of gluconeogenesis and glycogenolysis [7]. However, recently the gut and its derived hormones have been identified as important in mediating the antidiabetic effects of metformin [6]. An emerging amount of new receptors activated by metformin are continuously identified [6], thereby indicating pleiotropic effects of metformin. This suggest that treatment with metformin may have additional effects on health status beside improving glycemic control. Some studies, but not all, indicate that metformin may reduce the risk of cardiovascular disease, neurodegenerative diseases, and several forms of cancer, which may be mediated through a reduction of oxidative stress [2,3,8–10].

Inhibition of Complex I in the mitochondria is one of the established molecular mechanism of metformin [11,12]. Several *in vitro* studies have confirmed this mechanism; however, the clinical relevance of the supraphysiological concentrations used (i.e., millimolar instead of micromolar) has been questioned [13]. Inhibition of Complex I decreases the formation rate of reactive oxygen species (ROS) [14–16], and *in vitro* work has shown that metformin inhibits mitochondrial ROS production through the inhibition of Complex I [17]. In a clinical setting, a direct measurement of ROS formation is inapplicable; thus, oxidized macromolecules using, e.g., nucleic acids are often used as an indirect measurement [18]. Historically, DNA oxidation has attracted much attention due to the potential pre-mutagenic GC to TA transversion caused by oxidation of guanosine which is associated with cancer development [19]. During the last decade, RNA oxidation has attracted focus in individuals with type 2 diabetes, as individuals with diabetes-related complications present a higher formation rate of RNA oxidation [20] and increased formation rates are associated with increased mortality [21–23]. *In vitro* studies reveal that oxidation of RNA disturbs protein synthesis as well as cellular signaling [24,25], which suggest that RNA oxidation may be part of the pathogenesis and not just a result hereof. We hypothesized that metformin in combination with different insulin regimens decreases RNA and DNA oxidation in individuals with type 2 diabetes. A potential reduction of RNA and DNA oxidation may explain the proposed association with lower risks of cardiovascular disease and cancers following metformin treatment [3,9,10].

No clinical studies have investigated the effect of metformin

treatment on RNA oxidation in a randomized setting. A few studies have explored effects on DNA oxidation with overall inconclusive results [26,27]. Surprisingly, a recent cross-sectional study in individuals with type 2 diabetes suggested increased RNA and DNA oxidation, measured as urinary excretion rates of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), in metformin-treated individuals [28]. However, a conservative adjustment for multiple testing suggested that this finding was not statistically significant. The study additionally revealed that individuals treated with insulin presented lower excretion rates of 8-oxodG [28]. The cross-sectional study design does not allow interpretation regarding causality, because of high risk of confounding [29]. Thus, we find it of great importance to explore the proposed effects of treatment with metformin and insulin regimens, respectively on RNA and DNA oxidation in a randomized setting that has a lower risk of confounding and therefore allows a stronger interpretation on causality [29].

## 2. Material and methods

The current study was an investigator-initiated, multicenter, randomized 2 × 3 factorial trial with eight Danish diabetes centers in the Copenhagen area participating in the study. The study was designed to evaluate the effects of metformin treatment in combination with three insulin analogues regimens on carotid intima-media thickness (primary outcome). These results have been reported elsewhere together with a detailed description of study design, setting, and participants [30–32]. In brief, eligible participants were diagnosed with type 2 diabetes, aged >30 years with a BMI >25 kg/m<sup>2</sup>, HbA<sub>1c</sub> ≥ 7.5% [≥58 mmol/mol], and receiving anti-diabetic medicine (oral antidiabetic drugs for at least 1 year and/or insulin therapy for at least 3 months). The antidiabetic treatment was stopped 12 hour prior to initiation in the study. Participants were randomized in a 2 × 3 factorial design to 18 months of treatment with metformin (1,000 mg two times daily) versus placebo (1:1) in a blinded setting combined with randomization (1:1:1) to either biphasic insulin (biphasic insulin aspart) (one to three times daily), basal bolus insulin (insulin aspart three times daily combined with insulin detemir once daily), or basal insulin (insulin detemir, once daily) in an open-label setting. The insulin regimen was dosed individually with a treatment goal of HbA<sub>1c</sub> of 7.0% [53 mmol/mol]. Metformin and placebo were produced and delivered by Merck Company. During the study, the therapeutic target for blood pressure was 130/80 mmHg in individuals without microalbuminuria and 125/80 mmHg in individuals with microalbuminuria. The therapeutic targets for total cholesterol and low-density lipid cholesterol were 4.5 and 2.5 mmol/L, respectively [30]. The randomization was performed using a central, computer-based randomization service and stratified by age (>65 years), prior insulin therapy, and inclusion center (Steno Diabetes Center or not Steno Diabetes Center) at the Copenhagen Trial Unit. The trial was conducted in accordance with The Declaration of Helsinki and guidelines for Good Clinical Practice with informed consent from each participant. The trial was approved by the Regional Committee on Biomedical Research Ethics (H-D-2007-112), the Danish Medicines Agency, and the Danish Data protection Agency. This sub-study was additionally approved by the Regional Committee on Biomedical Research Ethics (H-17002764) and the Danish Data Protection Agency. The trial is registered at [clinicaltrials.gov](https://clinicaltrials.gov) (NCT00657943).

### 2.1. Effects of metformin on carotid intima-media thickness, HbA<sub>1c</sub>, body weight, and dose of insulin after 18 months

As previously reported [31], the current trial demonstrated no beneficial effect of metformin treatment on carotid intima-media thickness compared with placebo treatment. However, metformin treatment reduced HbA<sub>1c</sub> compared with placebo treatment. Similarly, weight gain was smaller during metformin treatment compared with placebo treatment. The insulin dose increased in both treatment groups; however, the increase was smallest among metformin treated participants. The number of adverse events were similar between treatment groups; however, more metformin treated individuals reported non-severe hypoglycemia [31].

### 2.2. Effects of insulin regimens on carotid intima-media thickness, HbA<sub>1c</sub>, body weight, and dose of insulin after 18 months

As previously reported [32], the current trial demonstrated no differences in changes of carotid intima-media thickness following the different insulin regimens. HbA<sub>1c</sub> was reduced more in the biphasic insulin treated individuals compared with both basal-bolus insulin and basal insulin treated individuals. Weight gain was most pronounced in the biphasic insulin and basal-bolus insulin treated groups compared with the basal insulin group. At the end of the trial, insulin dose was lowest in the biphasic insulin treated group and highest in the basal insulin group. The number of adverse events were similar between treatment groups; however, more biphasic insulin treated individuals reported non-severe hypoglycemia [32].

### 2.3. Analyses

Details regarding pre-specified primary and secondary outcomes are available elsewhere [30–32]. Urine samples were collected at baseline and at the end of the study. Participants that dropped out of the study prior to termination of the study period were invited to a prior ‘final visit’, when possible. RNA and DNA oxidation were investigated by urinary excretion rates of 8-oxoGuo and 8-oxodG as a *post-hoc* analysis [30]. Changes were investigated following metformin treatment compared with placebo treatment in combination with insulin therapy. As defined in the protocol for other outcomes, effects of individual insulin regimens were analyzed in a separate analysis if no interaction between the effect of metformin treatment and insulin treatment were present [30]. The urine samples were stored at –80 °C at Steno Diabetes Center Copenhagen, transferred to the Laboratory of Clinical Pharmacology, Rigshospitalet – Bispebjerg Frederiksberg Hospital stored in dry-ice, and stored at –20 °C until batch analysis. Before analysis, the frozen urine samples were thawed, mixed, heated (to 37 °C for 5 min), and centrifuged at 10,000 g for 5 min. Detailed description of the analysis and quality control is available elsewhere [33]. In brief, a reverse phase chromatographic separation was applied using Acquity UPLC 1-class system (Waters, Milford, MA, USA) with an Acquity UPLC BEH Shield RP18 Column (Waters) and a VanGuard precolumn (Waters). Detection was performed by a Xevo TQ-S triple quadrupole mass spectrometer (Waters) with negative ionization electrospray method and multi-reaction monitor mode. Urine creatinine was determined by Jaffe’s Method as described elsewhere [34].

Other laboratory parameters were determined at the local Department of Biochemistry at each hospital using routine and standardized methods. Similarly, was HbA<sub>1c</sub> determined locally using International Federation of Clinical Chemistry (IFCC) standardized methods.

Details regarding measurement of carotid intima-media thickness are reported elsewhere [31,32,35]. In brief, the same two technicians measured carotid intima-media thickness at baseline and at the end of the study by ultrasound in the common carotid artery 10 mm proximal to the carotid bulb using General Healthcare logic 9 [31,32].

### 2.4. Statistical analysis

R version 3.6.1 [36] was used for all statistical tests and graphical illustrations. Distribution of data was assessed graphically. Descriptive data is provided as mean ± standard deviation (SD) or median with interquartile range (IQR) if Gaussian distribution criteria were not met. A linear mixed-effect model (package ‘lme3’, function ‘lmer’) allowing baseline differences was applied for the analysis. A random subject effect was included, and fixed effects constituted stratifying variables (age, prior insulin therapy, and inclusion center) as well as design variables (sex, prior cardiovascular disease, statin treatment, and positive glutamic acid decarboxylase antibodies) as defined prior to study initiation [30]. Data were log-transformed, if appropriate, to meet model assumptions. Associations between carotid intima-media thickness and RNA oxidation were investigated graphically and using Pearson’s correlation. The sample size was calculated based on the primary outcome, carotid intima-media thickness, and reported elsewhere [30]. A two-sided *P*-value <0.05 without adjustment for multiplicity was considered statistically significant.

## 3. Results

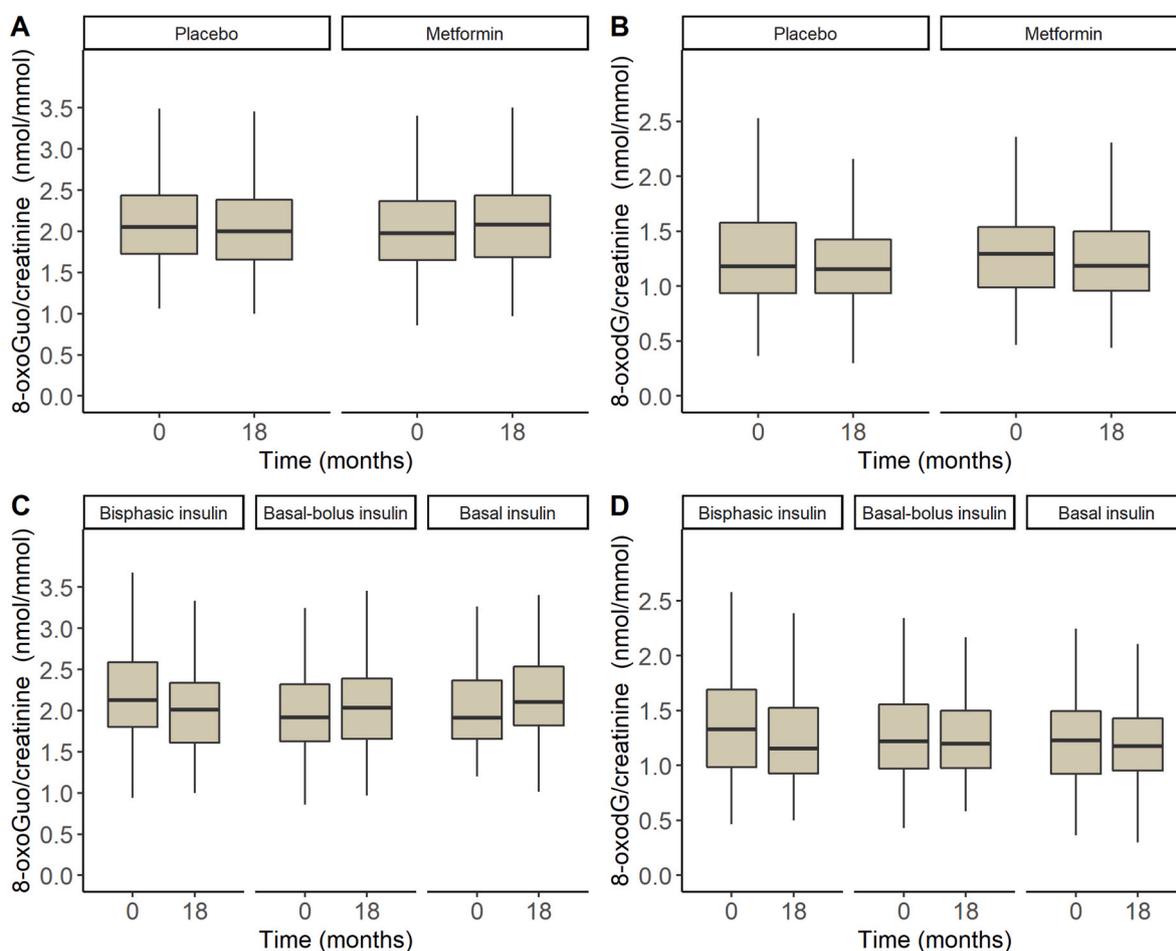
### 3.1. Trial participants

As previously reported [31,32], a total number of 464 potential participants were accessed for eligibility between April 2008 and December 2012. Forty-nine potential participants did not meet the criteria for participating or withdrew prior to randomization. Thus, a total number of 415 was randomized to trial intervention. Eighty participants did not complete the 18 months treatment period and three participants were excluded due to loss of randomization code, leaving a total of 332 participants completing the study [31,32]. At baseline, urine samples were missing for 15 participants. At the end of the study, urine samples were missing for 62 participants with comparable distribution between intervention groups.

The baseline characteristics were similar between intervention groups, as reported elsewhere [31,32]. In summary, the trial population were (median (IQR)) aged 62.5 years (55.0 years–66.4 years) predominantly males (*n* = 281 (68.2%)) with a BMI of 31.8 kg/m<sup>2</sup> (29.2 kg/m<sup>2</sup> to 34.9 kg/m<sup>2</sup>) and HbA<sub>1c</sub> of 8.3% (7.8%–9.3%) [67 mmol/mol (62 mmol/mol to 78 mmol/mol)] at baseline. The entire study population had a median total cholesterol of 4.0 mmol/L (3.5 mmol/L to 4.7 mmol/L) and a median LDL cholesterol of 2.1 mmol/L (1.6 mmol/L to 2.6 mmol/L) at baseline. Cholesterol concentrations of each treatment group as well as changes during the study period are reported elsewhere [31,32]. The baseline urinary excretion rates of 8-oxoGuo/creatinine was 2.03 nmol/mmol (1.70 nmol/mmol to 2.44 nmol/mmol) and of 8-oxodG/creatinine 1.27 nmol/mmol (0.96 nmol/mmol to 1.57 nmol/mmol) in the entire trial population. Baseline excretion rates of each intervention group are available in Fig. 1. Prior to inclusion in the study, the study participants were treated with metformin (*n* = 343 (83.3%)), insulin (*n* = 185 (69.2%)), sulphonylurea (*n* = 116 (28.2%)), and other antihyperglycemic drugs (*n* = 59 (14.3%)). At baseline, 308 (74.8%) participants received renin-angiotensin system inhibitors, 133 (56.6%) participants received other antihypertensive agents, and 358 (86.9%) participants were treated with statins. The prior antihyperglycemic as well as concomitant antihypertensive and lipid lowering treatment were similar between treatment groups and presented elsewhere [31,32].

### 3.2. Relationship between RNA oxidation and carotid intima-media thickness

Baseline excretion rates of 8-oxoGuo/creatinine were not correlated with baseline mean (*r* = –0.04 (95% CI: –0.13 to 0.06), *P* = 0.47) or baseline maximal carotid intima-media thickness (*r* = –0.04 (95% CI:



**Fig. 1.** The urinary excretion rates of 8-oxo-7,8-dihydroguanosine (8-oxoGuo)/creatinine in the metformin intervention groups (A) and each insulin intervention group (C) as well as the urinary excretion rates of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)/creatinine in the metformin intervention groups (B) and each insulin intervention group (D) are visualized as boxplots at baseline and the end of study.

–0.14 to 0.06),  $P = 0.44$ ). Nor was there any correlation between changes in excretion rates of 8-oxoGuo/creatinine and changes of mean ( $r = -0.01$  (95% CI: –0.11 to 0.10),  $P = 0.91$ ) or changes maximal carotid intima-media thickness ( $r = -0.01$  (95% CI: –0.12 to 0.10)  $P = 0.87$ ), Fig. 2.

### 3.3. Metformin treatment

No interaction between the effect of metformin and the effect of insulin treatment on 8-oxoGuo/creatinine ( $P = 0.66$ ) and 8-oxodG/creatinine ( $P = 0.52$ ) were evident. Thus, the effects of metformin treatment and insulin treatment on 8-oxoGuo/creatinine and 8-oxodG/creatinine were investigated separately.

The participants treated with metformin changed 8-oxoGuo/creatinine by +7.1% (95% CI: 0.5% to 14.4%,  $P = 0.03$ ) compared with placebo treatment. No significant within-group changes were evident (Fig. 1A, Table 1). The change in urinary excretion of 8-oxodG/creatinine was similar between treatment groups ( $P = 0.76$ ). As such, both intervention groups decreased urinary excretion of 8-oxodG/creatinine (metformin-treated group: –6.0% (95% CI: –9.9% to –2.0%); placebo-treated group: –4.4% (95% CI: –8.3% to –0.3%)) (Fig. 1B, Table 1).

### 3.4. Insulin treatment

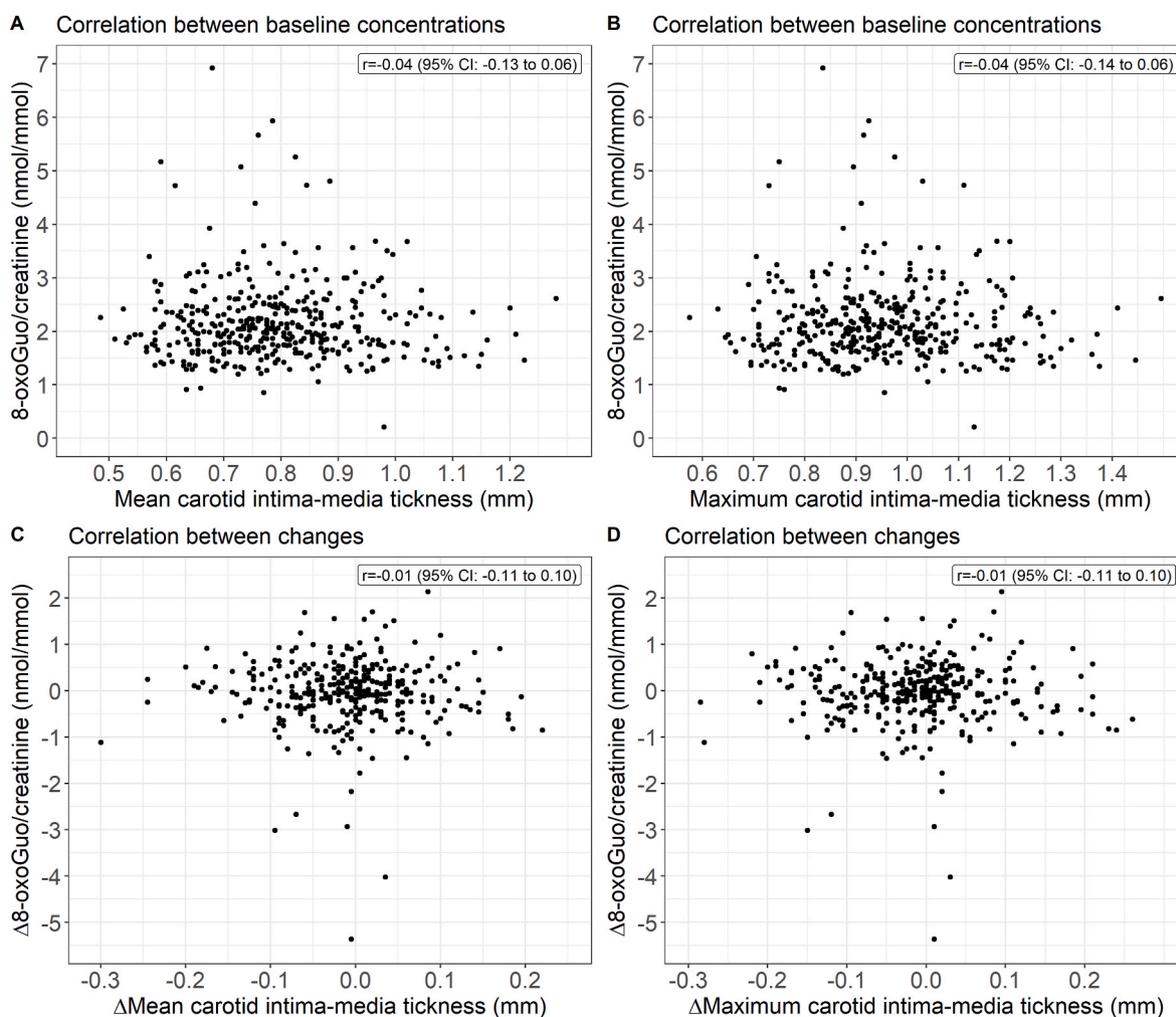
Urinary excretion of 8-oxoGuo/creatinine decreased more in the biphasic insulin group (within-group: –9.6% (95% CI: –14.4% to

–4.4%)) than both basal-bolus insulin (within-group: 5.2% (95% CI: –0.5% to 11.2%)),  $P = 0.0002$  between groups, and basal insulin (within-group: 3.7% (95% CI: –2.0% to 9.7%))  $P = 0.0007$ . Additionally, urinary excretion of 8-oxodG/creatinine decreased more in the biphasic insulin group (within-group: –9.9% (95% CI: –14.4% to –5.2%)) than basal-bolus insulin (within group: –1.2% (95% CI: –6.1% to 3.9%))  $P = 0.01$  between groups). No difference was observed in urinary excretion of 8-oxodG/creatinine between the biphasic insulin group and the basal insulin group ( $P = 0.11$  between groups) (Fig. 1C–D, Table 2).

As previously reported [32], HbA<sub>1c</sub> decreased more in the biphasic insulin group (–1.0% (95% CI: –1.16% to –0.83%) [–11 mmol/mol (–13 mmol/mol to –9 mmol/mol)]) than both basal-bolus (–0.45% (95% CI: –0.61% to –0.28%) [–5 mmol/mol (–7 mmol/mol to –3 mmol/mol)]),  $p < 0.001$  between groups, and basal insulin (–0.26% (95% CI: –0.43% to –0.09%) [–3 mmol/mol (–5 mmol/mol to –1 mmol/mol)]),  $P < 0.001$  between groups, following 18 months of treatment [32].

## 4. Discussion

In this factorial, randomized clinical trial, we demonstrated a minor increase in RNA oxidation following 18-months metformin treatment compared with placebo treatment in combination with insulin therapy in individuals with type 2 diabetes. Additionally, the biphasic insulin regimen decreased both RNA and DNA oxidation compared with the other insulin regimens.



**Fig. 2.** Correlation between baseline urinary excretion rates of 8-oxoGuo and baseline ultrasound measured mean carotid intima-media thickness (A) as well as maximum carotid intima-media thickness (B). And correlation between changes in urinary excretion rates of 8-oxodG and changes in baseline ultrasound measured mean carotid intima-media thickness (C) as well as maximum carotid intima-media thickness (D).

**Table 1**

Estimates and 95% confidence intervals (CI) of the percentage change in urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo)/creatinine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)/creatinine derived from the linear mixed effect models of individuals treated with placebo or metformin. The first two rows with results provide within-group effects, whereas the last row provide between-group effects.

	8-oxoGuo/creatinine		8-oxodG/creatinine	
	Estimate (95% CI)	P	Estimate (95% CI)	P
Placebo (within group)	-4.1% (-8.7% to 0.6%)	0.09	-4.4% (-8.3% to -0.3%)	0.03
Metformin (within group)	3.0% (-1.6% to 7.6%)	0.20	-6.0% (-9.9% to -2.0%)	0.004
Metformin vs. placebo	7.1% (0.5% to 14.0%)	0.03	-1.7% (-7.4% to 4.3%)	0.76

The finding that metformin treatment increases RNA oxidation in individuals with type 2 diabetes supports the growing evidence that questions whether metformin inhibit Complex 1 in the mitochondria *in vivo* [13]. An inhibition of Complex 1 decreases ROS formation in isolated mitochondria [14–16] and given the close cellular location of RNA to the mitochondria [18], this would decrease the formation rate of RNA oxidation.

This is the first clinical trial to explore effects of metformin treatment

**Table 2**

Estimates and 95% confidence interval (CI) of the percentage change in urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo)/creatinine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)/creatinine derived from the linear mixed effect models of individuals treated with biphasic insulin (biphasic insulin aspart), basal-bolus insulin (aspart + detemir), or basal insulin (detemir insulin). The first three rows with results provide within-group effects, whereas the last three rows provide between-group effects.

	8-oxoGuo/creatinine		8-oxodG/creatinine	
	Estimate (95% CI)	P	Estimate (95% CI)	P
Biphasic insulin (within group)	-9.6% (-14.4% to -4.4%)	0.0004	-9.9% (-14.4% to -5.2%)	0.0001
Basal-bolus insulin (within group)	5.2% (-0.5% to 11.2%)	0.07	-1.2% (-6.1% to 3.9%)	0.63
Basal insulin (within group)	3.7% (-2.0% to 9.7%)	0.21	-4.3% (-9.2% to 0.8%)	0.09
Basal-bolus vs. biphasic insulin	16.3% (7.5% to 25.8%)	0.0002	9.6% (2.0% to 17.8%)	0.01
Basal vs. biphasic insulin	14.6% (5.9% to 24.0%)	0.0007	6.2% (-1.3% to 14.2%)	0.11
Basal vs. Basal-bolus insulin	-1.4% (-8.9% to 6.7%)	0.72	-3.2% (-9.9% to 4.1%)	0.39

on RNA oxidation in a randomized and placebo-controlled setting. However, a previous cross-sectional study suggested increased excretion rates of RNA oxidative products in individuals treated with metformin [28]. This is in accordance with our present findings. Oxidation of messenger RNA disrupts the translation process which lead to mutated or truncated proteins [24]. Furthermore, oxidation of micro-RNAs result in changed cellular signaling [25]. Thus, increased RNA oxidation could be harmful, especially when considering the association between RNA oxidation and neurodegenerative as well as cardiovascular diseases found in observational studies [23,37,38]. The baseline concentration of urinary excretion of 8-oxoGuo in the trial population was comparable with the lowest quartile of a previous cohort study in individuals with type 2 diabetes that had the lowest hazard ratio of all-cause mortality [39]. Therefore, we may not expect major changes in the excretion rate of 8-oxoGuo. To our knowledge, the potential clinical significance of a 7% increase in urinary excretion of 8-oxoGuo is not established. In comparison, the difference between the lowest and highest quartile of urinary excretion of 8-oxoGuo in individuals with type 2 diabetes is approximately 100%. However, the safety profile of metformin has been thoroughly investigated during the last decades of treatment, and the drug has been recommended as first-line treatment in individuals with type 2 diabetes for more than the last decade [1,8–10].

There was no difference in changes of urinary excretion of 8-oxodG between the metformin group versus the placebo group. However, in both groups DNA oxidation decreased from start to the end of study. This effect may be mediated through the concurrent insulin therapy and/or improved glycemic control, but is also observed in other randomized trials and could be an unspecific effect from life style changes mediated through trial participation [18]. Two previous studies have investigated the effect of metformin on 8-oxodG formation [26,27]. One study revealed decreased plasma concentrations of 8-oxodG following metformin treatment, but only in a subgroup of women with polycystic ovary syndrome [26]. Another study revealed that dapagliflozin decreased urinary excretion of 8-oxodG compared with metformin treatment in patients with type 2 diabetes [27]. Altogether, this is consistent with our finding of no improved beneficial effects of metformin compared with placebo, both in combination with insulin therapy, on DNA oxidation.

Biphasic insulin decreased RNA and DNA oxidation to a greater extent than both basal-bolus insulin and basal insulin. A previous cross-sectional study from our laboratory showed that individuals with type 2 diabetes treated with insulin presented lower excretion rates of 8-oxodG [28]. No distinction was made between insulin types [28], which underscores the novelty of our present findings and point at potentially important differences in pharmacodynamic profiles of different insulin regimens. The mechanisms are unknown, but notably, biphasic insulin treatment improved glycemic control more than basal-bolus and basal insulin treatment and required a lower insulin dosage [32]. Biphasic insulin and basal-bolus insulin target postprandial hyperglycemia unlike basal insulin. Targeting postprandial hyperglycemia has been linked with decreased oxidative stress [40], which may in combination with better glycemic control explain the beneficial effects of biphasic insulin on oxidative stress compared to basal insulin. Cellular hyperglycemia induces ROS formation [4]. However, until now, no direct linkage between systemic oxidized nucleic acids measured *in vivo* and glycemic control has been established [22]. Collectively, the combined effects of decreased HbA<sub>1c</sub> (as previously shown [32]) and decreased RNA oxidation following biphasic insulin treatment compared with other insulin regimens, suggest that biphasic insulin may have favorable effects compared with basal-bolus and basal insulin in terms of surrogate markers related to diabetes complications in individuals with type 2 diabetes.

Carotid intima-media thickness is a predictor of vascular events [41]. Similarly, urinary excretion of 8-oxoGuo associates with cardiovascular disease in individuals with type 2 diabetes [23,37]. Thus, we expected that the two may be related. However, the current study revealed no

relationship between the two surrogate markers of cardiovascular disease, which does not support common pathogenetic roles.

The strength of the current study is the randomized, placebo-controlled design. The relatively long study duration allows interpretation of longer-term effects, and the relatively large sample size allows detection of smaller, but potential clinically relevant changes. The ultra-performance liquid chromatography tandem mass spectrometry used for measuring 8-oxoGuo and 8-oxodG is the reference standard method due to the high specificity [18]. The study is limited by the fact that measurement of both 8-oxoGuo and 8-oxodG were not pre-specified, and as such, analyses are post-hoc and should be seen as hypothesis-generating only. Nevertheless, analysis of the urine samples is an objective measurement performed blinded, and, thus, not subject to bias; and the hypothesis and statistical analysis followed the a priori design of the study and adhered to well-founded theory. Spot urine samples instead of 24-hour urine samples were collected. Thus, creatinine adjustment to correct urinary flow is made. Given that the intervention is not assumed to affect urine creatinine, this does not seem to weaken the study markedly.

No distinction between organ of origin is made, when measuring 8-oxoGuo and 8-oxodG in the urine. As such, changes in smaller organs may not be detected. Furthermore, the contribution from the nucleotide pool is uncertain. The amount of guanine incorporated in RNA is estimated to be 75 times greater than the amount of guanine found in the nucleotide pool [42]. Thus, it would require a considerable oxidation in the nucleotide pool, if the nucleotide pool contribute most to 8-oxoGuo and 8-oxodG excreted in the urine. However, the nucleotide pool may be more prone to oxidation [43].

In conclusion, 18 months of treatment with metformin increased RNA oxidation to a minor extent, whereas DNA oxidation did not change compared with the placebo group. The findings from the current study provide information regarding the mechanism of metformin, but does not support the hypothesis that decreased RNA and DNA oxidation explains the proposed protective effect of metformin treatment against cardiovascular disease, neurodegenerative diseases, and cancer [2,8]. Since our results do not support metformin treatment decreases the formation of ROS *in vivo*, there may be no clinically relevant upstream inhibition of Complex 1 by metformin. At last, the study provides novel insights into effects of different insulin regimens on markers of oxidative stress. The decreased RNA and DNA oxidation in relation to biphasic insulin treatment add-on to existing evidence of clinically relevant beneficial effects compared with basal-bolus and basal insulin.

#### Author contribution

E.L.L. researched data based on previous analysis protocol for the current trial, interpreted the findings, and wrote the first manuscript draft. L.K.K. and H.E.P. initiated the current sub-study and interpreted results. L.L.-C., S.M., T.P.A. developed the study protocol, conducted the clinical study, and initiated this sub-study. T.W.B., L.B., C.G., C.H., T.K., S.S.L., E.R.M., H.P., S.B.S., L.T, B.T., and H.V. developed the trial protocol and conducted the clinical trial. All authors reviewed the manuscript and approved the final version of the manuscript. E.L.L. is guarantor of the work and takes full responsibility hereof.

#### Declaration of competing interest

The trial was funded by an unrestricted research grant from Novo Nordisk A/S. Novo Nordisk A/S did not decide trial design and was not involved in clinical conduction, data analyses, interpretation, or writing of manuscript. Novo Nordisk A/S was allowed to comment on the protocol.

E.L.L. and H.E.P.: received research funding for an unrelated investigator-initiated study by Boehringer Ingelheim; L.L.-C. owns shares in Novo Nordisk A/S; S.M.: *Advisory boards*: AstraZeneca; Boehringer Ingelheim; Eli Lilly; Merck Sharp & Dohme; Novo Nordisk; Sanofi,

Bayer. *Lecture fees*: AstraZeneca; Boehringer Ingelheim; Merck Sharp & Dohme; Novo Nordisk; Sanofi. *Research grant recipient*: Novo Nordisk, Boehringer-Ingelheim; T.P.A. owns shares in Novo Nordisk A/S. S.S.L. owns shares in Novo Nordisk A/S and shares in dynamically traded investment funds, which may own stocks from pharmaceutical companies. S.S.L. has reported former employment at Steno Diabetes Center, which is a diabetes hospital and academic institution previously owned by Novo Nordisk. S.S.L. is now employed at Boehringer Ingelheim International GmbH, Ingelheim, Germany. S.S.L.'s contribution was his alone and does not necessarily reflect the official position of Boehringer Ingelheim. T.W.B. owns shares in Novo Nordisk A/A. T.W.B. has reported former employment at Steno Diabetes Center, which is a diabetes hospital and academic institution previously owned by Novo Nordisk. T.W.B. is now employed at Novo Nordisk. T.W.B.'s contribution was hers alone and does not reflect the official position at Novo Nordisk. L.B. has received fees from and/or attended advisory for Novo Nordisk A/S, Merck Sharp & Dohme, Bayer, Sanofi, and Boehringer Ingelheim. All other authors declare no study relevant conflict of interest.

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