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The effect of empagliflozin on oxidative nucleic acid modifications in patients with type 2 diabetes: protocol for a randomised, double-blinded, placebo-controlled trial

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Complete List of Authors:	Larsen, Emil List; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Cejvanovic, Vanja; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Kjær, Laura; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Vilbøll, Tina; Gentofte Hospital, University of Copenhagen, Center for Diabetes Research, Department of Internal Medicine Knop, Filip; Gentofte Hospital, University of Copenhagen, Diabetes Research Division, Department of Internal Medicine Rungby, Jørgen; Gentofte Hospital, University of Copenhagen, Center for Diabetes Research, Department of Internal Medicine; Aarhus University, Department of Biomedicine Poulsen, Henrik; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology; University of Copenhagen, Faculty of Health and Medical Sciences
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4 **1 The effect of empagliflozin on oxidative nucleic acid modifications in patients**
5 **2 with type 2 diabetes: protocol for a randomised, double-blinded, placebo-**
6 **3 controlled trial**

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10 **5 Emil List Larsen^{1,2,4}, Vanja Cejvanovic^{1,2,3,6} Laura Kofoed Kjær^{1,2,3,6}, Tina Vilsbøll⁴,**
11 **6 Filip Krag Knop⁴, Jørgen Rungby^{4,5}, Henrik Enghusen Poulsen^{1,2,3*}**
12

13 ¹Laboratory of Clinical Pharmacology, Rigshospitalet, University of Copenhagen,
14 Copenhagen, Denmark

15 ²Department of Clinical Pharmacology, Bispebjerg and Frederiksberg Hospital, University of
16 Copenhagen, Copenhagen, Denmark

17 ³Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of
18 Copenhagen, Copenhagen, Denmark

19 ⁴Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Hellerup,
20 Denmark

21 ⁵Department of Biomedicine, Aarhus University, Aarhus, Denmark

22 ⁶These authors contributed equally to this work
23
24

25
26
27 ***Corresponding author**

28 Professor, Henrik Enghusen Poulsen, MD
29 Laboratory of Clinical Pharmacology, Q7642
30 Rigshospitalet, University of Copenhagen
31 Ole Maaløes Vej 26, Entrance 76
32 DK-2200 Copenhagen N
33 Denmark

34 E-mail: hypo@rh.dk

35 Phone: +45 3545 7671

36 Fax: +45 3545 2745
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ABSTRACT

Introduction: Cardiovascular disease is the leading cause of morbidity and mortality in patients with type 2 diabetes (T2D). Although glycaemic control reduces microvascular complications, the effect of intensive treatment strategies or individual drugs on macrovascular cardiovascular diseases is still debated.

Ribonucleic acid (RNA) oxidation is positively associated with mortality in patients with T2D. Inspired by animal studies showing effect of a sodium-glucose cotransporter-2 (SGLT-2) inhibitor (empagliflozin) on oxidative stress and a recent trial evaluating empagliflozin, that demonstrated improved cardiovascular outcomes in patients with T2D at high risk of cardiovascular events, we hypothesise that empagliflozin lowers oxidative stress.

Methods and analysis: In this randomised, double-blinded, and placebo-controlled study, 34 adult males with T2D will be randomised (1:1) to empagliflozin or placebo once-daily for 14 days as add-on to ongoing therapy. The primary endpoints will be changes in 24-hour urinary excretion of 8-oxo-7,8-dihydriguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) determined before and after intervention (by ultra-performance liquid chromatography tandem mass-spectrometry). Additionally, fasting levels of malondialdehyde (MDA) will be determined in plasma before and after intervention (by high-performance liquid chromatography).

Further, the plasma levels of iron, transferrin, transferrin-saturation, and ferritin are determined to correlate the iron metabolism to the markers of oxidative modifications.

Ethics and dissemination: The study protocol has been approved by The Regional Committee on Biomedical Research Ethics (approval number H-16017433) and the Danish Data Protection Agency, and will be carried out under the surveillance and guidance of the GCP unit at Bispebjerg Frederiksberg Hospital, University of Copenhagen in compliance with the ICH-GCP guidelines and in accordance with the Helsinki Declaration.

The results of this study will be presented at national and international conferences, and submitted to a peer-reviewed international journal with authorship in accordance to ICMJE Recommendations state.

Trial registration: Study name: EMPOX; clinicaltrials.gov: NCT02890745
Protocol version 5.1 - August, 2016.

Article summary

Strengths and limitations of this study

► This study will investigate the effect of empagliflozin on urinary excretion of 8-oxoGuo, since 8-oxoGuo is a prognostic biomarker for mortality in patients with T2D

► The trial design will be randomised, double-blinded, and placebo-controlled

► The methods for analysis of the outcomes are precise and reliable

► The limitation of this study is, that the study outcome is not mortality in a long-term follow up, but a biomarker prognostic for mortality in patients with T2D

Key words: Diabetes Mellitus, Type 2; Empagliflozin; Oxidative modifications; Oxidative Nucleic Acid Modifications; 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxo-7,8-dihydroguanosine

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80 **Abbreviations:** Type 2 diabetes, T2D; Ribonucleic acid, RNA; Deoxyribonucleic acid, DNA; 8-
81 oxo-7,8-dihydroguanosine, 8-oxoGuo; 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG;
82 malondialdehyde, MDA; sodium/glucose cotransporter 2, SGLT-2; ultra-performance liquid
83 chromatography tandem mass-spectrometry, UPLC-MS/MS; high-performance liquid
84 chromatography, HPLC; 8-iso prostaglandin f2 α , 8-iso-PGF2 α ; Suspected Unexpected Serious
85 Adverse Reactions, SUSARs.

For peer review only

INTRODUCTION

Diabetes is an increasing health issue affecting around 422 million people in the world, a doubling in prevalence since 1980.[1] More than 90% have type 2 diabetes (T2D). Patients with T2D have increased risk of developing cardiovascular diseases.[2]

The treatment target and strategy of T2D are individualised based on disease complications and co-morbidity, with glycaemic control as the major focus.[3] Although glycaemic control reduces the risk of microvascular complications,[4] the effect of intensive treatment strategies or individual drugs on macrovascular cardiovascular diseases is still debated.[5–11]

T2D is in general characterised as a metabolic disease with insulin resistance and defect insulin production.[12] Additionally, increased oxidative nucleic acid modifications have been observed.[13–15] Of particular importance, ribonucleic acid (RNA) oxidation measured by urinary excretion of 8-oxo-7,8-dihydriguanosine (8-oxoGuo) has been shown to be associated with mortality in both newly diagnosed patients with T2D[14] and in patients with a 6-years disease duration of T2D.[15] However, still no drug treatments have been demonstrated to reduce urinary excretion of 8-oxoGuo.[16–19]

Empagliflozin is a selective sodium/glucose cotransporter 2 (SGLT-2)-inhibitor, that increases urinary excretion of glucose, and thereby improves glycaemia in patients with T2D.[20] Recently, the cardiovascular outcome trial EMPA-REG-OUTCOME study demonstrated that empagliflozin added to standard care in doses of 10 or 25 mg once-daily compared to placebo treatment reduces all-cause mortality and death from cardiovascular causes in patients with T2D and high-risk of cardiovascular disease.[21] The effect of empagliflozin occurred fast, only three months after treatment initiation.[22] The mode of action behind these effects of empagliflozin remains debated. Additional effects to empagliflozin treatment such as lowering blood pressure and increased diuresis have been suggested, but not confirmed.[23,24]

Empagliflozin reduces multiple markers of oxidative modifications in rat models of type 1 diabetes.[25,26] In addition, it has been reported that empagliflozin lowers 8-iso prostaglandin f₂α (8-iso-PGF₂α) (a marker of lipid peroxidation) in patients with T2D. However, the methodology, by which 8-iso-PGF₂α was measured, was not reported.[27]

Several biomarkers of oxidative modifications are proven potential clinical relevant for different diseases.[28] Studies have demonstrated inconsistency between markers of oxidative modifications in different cellular compartments.[14,15,29] Hereby, this study differentiates the effect of empagliflozin on three different cellular compartments.

Iron contributes to increased levels of oxidative modifications, and iron is hypothesised to be associated with development of diabetes.[30] This study evaluates the effect of empagliflozin on the concentration of iron, transferrin, transferrin-saturation, and ferritin in order to correlate the iron metabolism to the markers of oxidative modifications.

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4 128 **Hypothesis and aims**

5 129 We hypothesise that empagliflozin lowers oxidative nucleic acid modifications, measurable
6 130 by urinary excretion of 8-oxoGuo and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).
7 131 Further, we evaluate if there is a compartmentalisation of oxidative modifications in the
8 132 organism and within the cell by also differentiate the effect of empagliflozin on lipid
9 133 peroxidation by plasma levels of malondialdehyde (MDA).
10 134 In addition to our primary hypothesis, this study investigates whether iron, transferrin,
11 135 transferrin-saturation, and ferritin are correlated to the levels of oxidative modifications (8-
12 136 oxoGuo, 8-oxodG, and MDA) in patients with T2D.
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14 138 **Study objectives**

15 139 The primary objectives are 24h urinary excretion of 8-oxoGuo and 8-oxodG before and after
16 140 treatment with empagliflozin compared to placebo as a measurement of whole body RNA-
17 141 and DNA oxidation, respectively.[31]
18 142

19 143 The secondary objectives encompass the concentration of MDA in plasma before and after
20 144 treatment with empagliflozin compared to placebo treatment as a measurement of lipid
21 145 peroxidation.[32]

22 146 Further, this study determines the concentration of iron, transferrin, transferrin-saturation,
23 147 and ferritin before and after the intervention.
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148 **METHODS AND ANALYSIS**

149 The protocol is developed in accordance with the SPIRIT (Standard Protocol Items:
150 Recommendations for Interventional Trials) recommendation.[33,34]

151 The trial is registered at <http://clinicaltrials.gov> (NCT02890745). Important changes in the
152 protocol will be registered at the website, and addressed to the Danish Medicines Authority
153 and The Regional Committee on Biomedical Research Ethics.

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155 **Trial design and setting**

156 The trial will be randomised, placebo-controlled, and double-blinded with two parallel
157 groups.

158 Participants will be recruited from the diabetes outpatient clinic, Department of Medicine,
159 Gentofte Hospital, University of Copenhagen, Hellerup, Denmark in relation to outpatient
160 consultations. The recruitment continues until 34 participants successfully have completed
161 the trial.

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163 **Eligibility of participants**

164 *Inclusion criteria*

165 Male diagnosed with T2D

- 166 ▶ Age: 18-75 years
- 167 ▶ HbA1c: 6.5-9.0%
- 168 ▶ Capable of understanding oral- and written information

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170 *Exclusion criteria*

- 171 ▶ Estimated glomerular filtration rate (eGFR) < 60 mL/hour/1.73 m²
- 172 ▶ Currently receiving insulin treatment
- 173 ▶ Coronary artery bypass grafting, percutaneous coronary intervention, acute coronary
174 syndrome, stroke, lung embolism, deep vein thrombosis, or transitory cerebral ischemia
175 within 6 months
- 176 ▶ Genital infection within 14 days
- 177 ▶ Plasma alanine aminotransferase ≥3 times upper normal limit
- 178 ▶ Treatment with SGLT-2 inhibitor within 2 months
- 179 ▶ Hyperglycaemic symptoms
- 180 ▶ Psychiatric disorder
- 181 ▶ Intolerance to empagliflozin or other agents relevant to study
- 182 ▶ Non-compliant

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184 **Intervention**

185 Participants will be randomised to receive once-daily empagliflozin 25 mg or placebo (in the
186 morning) for two weeks as add-on to ongoing therapy. Empagliflozin and placebo will be
187 manufactured and labelled by Boehringer Ingelheim. The dosage of empagliflozin is standard
188 medical treatment of T2D.[35] Possible administration of sulfonylurea drugs to participants
189 will be paused one week before the start visit and during the intervention to avoid
190 hypoglycaemia during the trial. Otherwise, there will be no restriction to concomitant
191 treatment. Participants are encouraged not to change their lifestyle during the trial.

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4 192 Trial medicine comply Good Manufacturing Practice regulations.[36] Participants receive text
5 193 messages reminders of medicine intake during the intervention period.
6 194 Participants will be evaluated compliant to medicine intake at adherence level of at least
7 195 65%.
8 196

10 197 **Outcomes**

11 198 *Primary outcome measurements*

12 199 The primary outcome of the trial will be the difference between 24h urinary excretion of 8-
13 200 oxoGuo and 8-oxodG before and after the intervention, which will be compared between
14 201 the two groups.
15 202

17 203 *Secondary outcome measurements*

18 204 The secondary outcome will be the difference between MDA in plasma before and after the
19 205 intervention, which will be compared between the two groups.
20 206 Further, this study correlates the concentration of iron, transferrin, transferrin-saturation,
21 207 and ferritin with the urinary excretion of 8-oxoGuo and 8-oxodG.
22 208

24 209 **Participant timeline**

25 210 Participant timeline is illustrated in Figure 1. Potential participants will be informed about
26 211 the trial in relation to their outpatient consultation at the Hospital. If they are interested in
27 212 participating, a screening visit will be conducted after informed consent. At the screening
28 213 visit eligibility will be investigated. Blood pressure, pulse, height, and weight will be
29 214 measured.

30 215 Participants will be fasting at the randomisation and end-of-study visit. At the randomisation
31 216 visit, trial medicine will be dispensed to participants, 24h urine sample and a venous blood
32 217 sample will be collected. After a 14-days treatment schedule, the end-of-study visit will take
33 218 place. Participants will deliver the 24h urine sample and excess trial medicine, and a venous
34 219 blood sample will be collected. All adverse events will be registered.

35 220 The 24h urine sample will be collected from the morning the day before the visit until the
36 221 morning the day of the visit. The participant will empty his bladder before the collection of
37 222 24h urine will begin.

38 223 The first participant's screening visit is expected to be October the 25th 2016. The last
39 224 participant's end-of-study visit is expected to be March the 31th 2017.
40 225

44 226 **Sample size**

45 227 An a priori power calculation has been performed with type I error risk of 5% and type II
46 228 error risk of 20%.

47 229 Values of the primary outcomes are about 30 nmol per 24h. We wish to be able to detect an
48 230 effect size of 20%. The standard deviation is 6 nmol per 24h.

49 231 The power calculation estimated 17 participants in each group using SAS version 9.4 (SAS
50 232 Institute, Inc., Cary, NC, USA).
51 233

54 234 **Assignment of intervention**

55 235 The randomisation will be carried out through a simple randomisation with a 1:1 allocation
56 236 ratio from a computer-generated list of random numbers.
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4 237 The list of random numbers will be made by an employee who otherwise will be uninvolved
5 238 in the study. The allocation sequence will be concealed from the investigators and
6 239 healthcare staff enrolling and assessing participants.

8 240 The physicians responsible for the treatment of patients with T2D in the outpatient clinic will
9 241 inform potential participants about the trial and will offer them an information meeting. At
10 242 the information meeting EL, LK, and/or VC will inform potential participants about the trial,
11 243 and the potential participants will receive written information as well. The screening visit will
12 244 be performed by EL, LK, and/or VC after written informed consent is obtained. EL, JR, TV, and
13 245 FK will be responsible for the enrolment of participants and assignment of intervention. The
14 246 end-of-study visit will be performed by EL, LK, and/or VC. HP is sponsor-investigator of the
15 247 study. JR is principle investigator of the study.

18 248 Only HP, EL, LK, and VC will have access to the final dataset until after analysis of the data.
19 249 After analysis and code break of the trial all investigators have full access to the dataset.

20 250 Participants, research coordinator, investigators, and laboratorian technicians will be blinded
21 251 until data is analysed.

22 252 Code break of a participant's trial medicine will occur only under exceptional circumstances
23 253 when knowledge of treatment is absolutely necessary. Only the principal Investigator and an
24 254 employee who is otherwise uninvolved in the study will be able to perform a code break.
25 255 Since they will not be part of the data analysis, a potential code break will not be of critical
26 256 importance.

28 257

30 258 **Data collection, analysis and management**

31 259 Urine and plasma samples will be collected at the randomisation visit and the end-of-study
32 260 visit. In order to improve adherence of the 24h urine sample, participants will receive three
33 261 text messages reminders of the sample collection during the sample collection period. If
34 262 participants record loss of a urine void, 150 mL will be added to the recorded diuresis.
35 263 Participants will be evaluated compliant to 24h urinary collection at adherence level of at
36 264 least 75%.

38 265

39 266 Urine samples will be stored at -20°C until data analysis. The samples will be analysed after
40 267 last participant's end-of-study visit. Analysis of 8-oxoGuo and 8-oxodG will be performed by
41 268 a method based on ultra-performance liquid chromatography tandem mass-spectrometry
42 269 (UPLC-MS/MS) as previously described.[19]

44 270

45 271 Plasma samples will be stored at -80°C until data analysis. The samples will be analysed after
46 272 last participant's end-of-study visit. Analysis of the plasma will be performed using a high-
47 273 performance liquid chromatography (HPLC) method as previously described.[32]

48 274

50 275 The study will collect demographic and baseline information from the participant's
51 276 physicians.

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53 278 All data will be written in Case Report Form (CRF). CRFs will only be available for
54 279 investigators and research coordinator, stored at trial site locked away according to ICH-GCP
55 280 guidelines.[37] The data will be entered into a locked database by double data entry after
56 281 last participant's end-of-study visit.

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282 The trial will be monitored by the GCP unit, Bispebjerg Hospital, University of Copenhagen,
283 Copenhagen, Denmark. The GCP unit and the Danish Medicines Authority will be able to
284 conduct an audit of the trial independent of sponsor-investigator and investigators.
285

286 The treatment period will be short, and empagliflozin is in general well tolerated. Therefore
287 only few adverse reactions are to be expected. Participants will be informed of possible
288 adverse reactions and told to contact an investigator if he experiences a possible adverse
289 reaction. Adverse events will be registered at the end-of-study visit. All adverse events will
290 be reported to the Danish Medicines Authority and the Danish National Committee on
291 Biomedical Research Ethics at the end of trial.

292 Fatal or life-threatening Suspected Unexpected Serious Adverse Reactions (SUSARs) will be
293 reported to Danish Medicines Authority and the Danish National Committee on Biomedical
294 Research Ethics within 7 days. Other SUSARs will be reported to Danish Medicines Authority
295 and the Danish National Committee on Biomedical Research Ethics within 15 days.
296 The participants will be insured by the Capital Region of Denmark, Gentofte Hospital.
297 Principal investigator will have the final decision on whether an adverse event requires
298 additional treatment, exclusion from the trial, or termination the entire trial.
299

300 **Statistical analysis**

301 All analyses will be conducted per-protocol. Any drop-out of the trial will be described in the
302 results.

303 Baseline demographic and clinical characteristics will be compared using a Student's t-test. If
304 the two groups are not equal after randomisation in aspect of demographic or clinical
305 parameters, then a logistic regression will be used to correlate the result for these
306 parameters.

307 The difference in primary and secondary outcomes before and after intervention will be
308 compared between the groups using Student's t-test.

309 Data will be evaluated for normal distribution using graphical illustration and the Shapiro-
310 Wilk test, and further validated using a non-parametric test. If data are not normally
311 distributed, data will be log-transformed before analyses or subjected to a non-parametric
312 test for analysis.

313 A two-sided *P*-value <0.05 will be considered statistically significant.
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315 **The strengths and limitations of this study**

316 The strength of this study is the randomised, placebo-controlled, double-blinded trial design.
317 The biomarker 8-oxoGuo is prognostic for mortality in patients with T2D, and the methods
318 for analysis of both primary and secondary outcomes are precise and reliable.
319 The limitation of this study is that the study outcome is not mortality with a long-term follow
320 up, but a biomarker prognostic for mortality in patients with T2D.

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4 321 **ETHICS AND DISSEMINATION**

5 322 The study has received approval from The Regional Committee on Biomedical Research
6 323 Ethics (approval number H-16017433), the Danish Medicines Authority, and the Danish Data
7 324 Protection Agency. The study is performed with informed consent from participants in
8 325 agreement to the declaration of Helsinki.

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11 326 Additional plasma samples will be stored at -80°C for use in future research, locked away at
12 327 the Department of Pharmacology, University Hospital Rigshospitalet.

13 328 The results of the study will be presented at national and international conferences, and
14 329 submitted at a peer-review international journal.
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60**330 Contributors**

331 This study is based on an idea of HP. HP, EL, VC, LK, TV, FK, and JR contributed to the trial
332 design. EL wrote the first protocol draft. HP, VC, LK, TV, FK, and JR contributed to
333 development of study protocol and approved the final draft.

334

335 Competing interests

336 JR has received lecture fees from AstraZeneca, Boehringer Ingelheim Pharmaceuticals, Eli
337 Lilly and Company, Merck Sharp & Dohme, Novo Nordisk, Sanofi, and is a member of the
338 Advisory boards of Novo Nordisk, Merck Sharp & Dohme and Sanofi.

339 TV has received lecture fees from AstraZeneca, Boehringer Ingelheim Pharmaceuticals,
340 Bristol-Myers Squibb, Eli Lilly and Company, Merck Sharp & Dohme, Novo Nordisk, Novartis,
341 Sanofi, and Zealand Pharma, and is member of the Advisory Boards of Amgen, Novo Nordisk,
342 Merck Sharp & Dohme and Bristol-Myers Squibb/AstraZeneca.

343 FK has received lecture fees from, is a member of the Advisory Boards of, has consulted for,
344 and/or received research support from AstraZeneca, Boehringer Ingelheim Pharmaceuticals,
345 Eli Lilly, Gilead Sciences, Merck Sharp & Dohme, Novo Nordisk, Ono Pharmaceuticals, Sanofi
346 and Zealand Pharma.

347 EL has received a 12 months scholarship from Boehringer Ingelheim.
348 All other authors declare no conflict of interest.

349

350 Funding

351 This study is sponsor-investigator-initiated and investigator-driven and financed by a
352 unrestricted grant from Boehringer Ingelheim. Empagliflozin and placebo are manufactured
353 and provided by Boehringer Ingelheim.

354 VC has received the Faculty PhD Scholarship from the Faculty of Health and Medical
355 Sciences, University of Copenhagen.

356

357 Data sharing statement

358 HP, EL, LK, and VC will have access to the final dataset. After analysis and code break of the
359 trial all investigators have full access to the dataset.

360 The results of the study will be presented at national and international conferences, and
361 submitted at a peer-review international journal.

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473 **FIGURE LEGENDS**

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475 **Figure 1.** Participant timeline.

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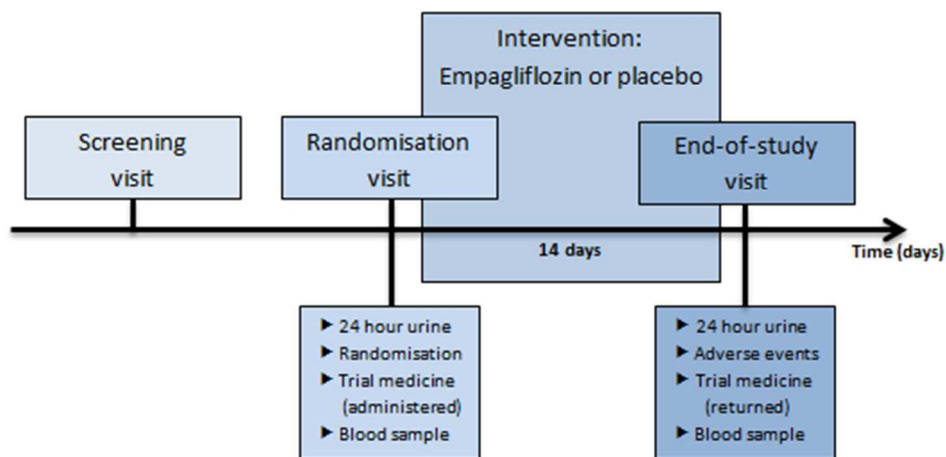


Figure 1. Participant timeline.
Figure 1

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 2
	2b	All items from the World Health Organization Trial Registration Data Set	Not applicable
Protocol version	3	Date and version identifier	Page 2
Funding	4	Sources and types of financial, material, and other support	Page 11
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 11
	5b	Name and contact information for the trial sponsor	Page 1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Page 8
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Page 8+9

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Page 4
	6b	Explanation for choice of comparators	Page 4+5
Objectives	7	Specific objectives or hypotheses	Page 5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Page 6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Page 6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 6-7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	Page 9
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Page 7 + 8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Page 6
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Page 7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Page 7 + Figure 1

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3	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Page 7
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6	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
7				
8	Methods: Assignment of interventions (for controlled trials)			
9				
10	Allocation:			
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12	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Page 7-8
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18	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Page 7-8
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22	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 8
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25	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 8
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28		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Page 8
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32	Methods: Data collection, management, and analysis			
33				
34	Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Page 8
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39		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Page 8
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3	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 8
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7	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Page 9
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10		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 9
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12		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Page 9
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16	Methods: Monitoring			
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18	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	Page 9
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23		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	Page 9, no interim
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26	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 8+9
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29	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Page 9
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33	Ethics and dissemination			
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35	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 10
36				
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38	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	Page 6
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3	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8
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6		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Page 10
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9	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 8
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12	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 11
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15	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 8
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18	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 9
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21	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 10
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26		31b	Authorship eligibility guidelines and any intended use of professional writers	Page 2
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28		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No plans, not applicable
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31	Appendices			
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33	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix 1
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36	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Page 10
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39 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.
 40 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons
 41 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.
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BMJ Open

The effect of empagliflozin on oxidative nucleic acid modifications in patients with type 2 diabetes: protocol for a randomised, double-blinded, placebo-controlled trial

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2016-014728.R1
Article Type:	Protocol
Date Submitted by the Author:	24-Feb-2017
Complete List of Authors:	Larsen, Emil List; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Cejvanovic, Vanja; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Kjær, Laura; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology Vilbøll, Tina; Gentofte Hospital, University of Copenhagen, Center for Diabetes Research, Department of Internal Medicine Knop, Filip; Gentofte Hospital, University of Copenhagen, Diabetes Research Division, Department of Internal Medicine Rungby, Jørgen; Gentofte Hospital, University of Copenhagen, Center for Diabetes Research, Department of Internal Medicine; Aarhus University, Department of Biomedicine Poulsen, Henrik; Rigshospitalet - Bispebjerg Frederiksberg Hospital, Laboratory & Department of Clinical Pharmacology; University of Copenhagen, Faculty of Health and Medical Sciences
Primary Subject Heading:	Diabetes and endocrinology
Secondary Subject Heading:	Pharmacology and therapeutics
Keywords:	Diabetes Mellitus, Type 2, Empagliflozin, Oxidative modifications, Oxidative Nucleic Acid Modifications, 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxo-7,8-dihydroguanosine

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Manuscripts

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4 **1 The effect of empagliflozin on oxidative nucleic acid modifications in patients**
5 **2 with type 2 diabetes: protocol for a randomised, double-blinded, placebo-**
6 **3 controlled trial**

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5 **Emil List Larsen^{1,2,4}, Vanja Cejvanovic^{1,2,3,6} Laura Kofoed Kjær^{1,2,3,6}, Tina Vilsbøll⁴,**
6 **Filip Krag Knop⁴, Jørgen Rungby^{4,5}, Henrik Enghusen Poulsen^{1,2,3*}**
7

8 ¹Laboratory of Clinical Pharmacology, Rigshospitalet, University of Copenhagen,
9 Copenhagen, Denmark

10 ²Department of Clinical Pharmacology, Bispebjerg and Frederiksberg Hospital, University of
11 Copenhagen, Copenhagen, Denmark

12 ³Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of
13 Copenhagen, Copenhagen, Denmark

14 ⁴Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Hellerup,
15 Denmark

16 ⁵Department of Biomedicine, Aarhus University, Aarhus, Denmark

17
18 ⁶These authors contributed equally to this work
19

20 ***Corresponding author**

21 Professor, Henrik Enghusen Poulsen, MD
22 Laboratory of Clinical Pharmacology, Q7642
23 Rigshospitalet, University of Copenhagen
24 Ole Maaløes Vej 26, Entrance 76
25 DK-2200 Copenhagen N
26 Denmark

27 E-mail: hypo@rh.dk

28 Phone: +45 3545 7671

29 Fax: +45 3545 2745
30

31 **Words:** 2797

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33 **Tables:** 0

34 **References:** 40

35 **Supplementary files:** 0

ABSTRACT

Introduction: Cardiovascular disease is the leading cause of morbidity and mortality in patients with type 2 diabetes (T2D). Although glycaemic control reduces microvascular complications, the effect of intensive treatment strategies or individual drugs on macrovascular cardiovascular diseases is still debated.

Ribonucleic acid (RNA) oxidation is associated with increased mortality in patients with T2D. Inspired by animal studies showing effect of a sodium-glucose cotransporter-2 (SGLT-2) inhibitor (empagliflozin) on oxidative stress and a recent trial evaluating empagliflozin, that demonstrated improved cardiovascular outcomes in patients with T2D at high risk of cardiovascular events, we hypothesise that empagliflozin lowers oxidative stress.

Methods and analysis: In this randomised, double-blinded, and placebo-controlled study, 34 adult males with T2D will be randomised (1:1) to empagliflozin or placebo once-daily for 14 days as add-on to ongoing therapy. The primary endpoints will be changes in 24-hour urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) determined before and after intervention (by ultra-performance liquid chromatography tandem mass-spectrometry). Additionally, fasting levels of malondialdehyde (MDA) will be determined in plasma before and after intervention (by high-performance liquid chromatography).

Further, the plasma levels of iron, transferrin, transferrin-saturation, and ferritin are determined to correlate the iron metabolism to the markers of oxidative modifications.

Ethics and dissemination: The study protocol has been approved by The Regional Committee on Biomedical Research Ethics (approval number H-16017433) and the Danish Data Protection Agency, and will be carried out under the surveillance and guidance of the GCP unit at Bispebjerg Frederiksberg Hospital, University of Copenhagen in compliance with the ICH-GCP guidelines and in accordance with the Helsinki Declaration.

The results of this study will be presented at national and international conferences, and submitted to a peer-reviewed international journal with authorship in accordance to ICMJE Recommendations state.

Trial registration: Study name: EMPOX; clinicaltrials.gov: NCT02890745
Protocol version 5.1 - August, 2016.

Article summary

Strengths and limitations of this study

► This study will investigate the effect of empagliflozin on urinary excretion of 8-oxoGuo, since 8-oxoGuo is a prognostic biomarker for mortality in patients with T2D

► The trial design will be randomised, double-blinded, and placebo-controlled

► The methods for analysis of the outcomes are precise and reliable

► The limitation of this study is, that the study outcome is not mortality in a long-term follow up, but a biomarker prognostic for mortality in patients with T2D

Key words: Diabetes Mellitus, Type 2; Empagliflozin; Oxidative modifications; Oxidative Nucleic Acid Modifications; 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxo-7,8-dihydroguanosine

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4 80 **Abbreviations:** 8-iso prostaglandin f2 α , 8-iso-PGF2 α ; 8-oxo-7,8-dihydro-2'-deoxyguanosine,
5 81 8-oxodG; 8-oxo-7,8-dihydroguanosine, 8-oxoGuo; Deoxyribonucleic acid, DNA; high-
6 82 performance liquid chromatography, HPLC; malondialdehyde, MDA; Ribonucleic acid, RNA;
7 83 sodium/glucose cotransporter 2, SGLT-2; Suspected Unexpected Serious Adverse Reactions,
8 84 SUSARs; Type 2 diabetes, T2D; ultra-performance liquid chromatography tandem mass-
9 85 spectrometry, UPLC-MS/MS;.

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INTRODUCTION

Diabetes is an increasing health issue affecting around 422 million people in the world, a doubling in prevalence since 1980.[1] More than 90% have type 2 diabetes (T2D). Patients with T2D have increased risk of developing cardiovascular diseases.[2]

The treatment target and strategy of T2D are individualised based on disease complications and co-morbidity, with glycaemic control as the major focus.[3] Although glycaemic control reduces the risk of microvascular complications,[4] the effect of intensive treatment strategies or individual drugs on macrovascular cardiovascular diseases is still debated.[5–10]

T2D is in general characterised as a metabolic disease with insulin resistance and defect insulin production.[11] Additionally, increased oxidative nucleic acid modifications have been observed.[12–14] Of particular importance, ribonucleic acid (RNA) oxidation measured by urinary excretion of 8-oxo-7,8-dihydriguanosine (8-oxoGuo) has been shown to be associated with increased mortality in both newly diagnosed patients with T2D[13] and in patients with a 6-years disease duration of T2D.[14] However, still no drug treatments have been demonstrated to reduce urinary excretion of 8-oxoGuo.[15–18]

Empagliflozin is a selective sodium/glucose cotransporter 2 (SGLT-2)-inhibitor, that increases urinary excretion of glucose, and thereby improves glycaemia in patients with T2D.[19] Recently, the cardiovascular outcome trial EMPA-REG-OUTCOME study demonstrated that empagliflozin added to standard care in doses of 10 or 25 mg once-daily compared to placebo treatment reduces all-cause mortality and death from cardiovascular causes in patients with T2D and high-risk of cardiovascular disease.[20] The effect of empagliflozin occurred fast, only three months after treatment initiation.[21] The mode of action behind these effects of empagliflozin remains debated. Additional effects to empagliflozin treatment such as lowering blood pressure and increased diuresis have been suggested, but not confirmed.[22,23]

Empagliflozin reduces multiple markers of oxidative modifications in rat models of type 1 diabetes.[24,25] In addition, it has been reported that empagliflozin lowers 8-iso prostaglandin f₂α (8-iso-PGF₂α) (a marker of lipid peroxidation) in patients with T2D. However, the methodology, by which 8-iso-PGF₂α was measured, was not reported.[26]

Several biomarkers of oxidative modifications are proven potential clinical relevant for different diseases.[27] Studies have demonstrated inconsistency between markers of oxidative modifications in different cellular compartments.[13,14,28] Hereby, this study differentiates the effect of empagliflozin on three different cellular compartments.

Iron contributes to increased levels of oxidative modifications, and iron is hypothesised to be associated with development of diabetes.[29] This study evaluates the correlation of iron, transferrin, transferrin-saturation, and ferritin with the markers of oxidative modifications.

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4 127 **Hypothesis and aims**

5 128 We hypothesise that empagliflozin lowers oxidative nucleic acid modifications, measurable
6 129 by urinary excretion of 8-oxoGuo and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).
7 130 Further, we evaluate if there is a compartmentalisation of oxidative modifications in the
8 131 organism and within the cell by also differentiate the effect of empagliflozin on lipid
9 132 peroxidation by plasma levels of malondialdehyde (MDA).
10 133 In addition to our primary hypothesis, this study investigates whether iron, transferrin,
11 134 transferrin-saturation, and ferritin are correlated to the levels of oxidative modifications (8-
12 135 oxoGuo, 8-oxodG, and MDA) in patients with T2D.
13 136

14 137 **Study objectives**

15 138 The primary objectives are 24h urinary excretion of 8-oxoGuo and 8-oxodG before and after
16 139 treatment with empagliflozin compared to placebo as a measurement of whole body RNA-
17 140 and DNA oxidation, respectively.[30]
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19 142 The secondary objectives encompass the concentration of MDA in plasma before and after
20 143 treatment with empagliflozin compared to placebo treatment as a measurement of lipid
21 144 peroxidation.[31]

22 145 Further, this study determines the concentration of iron, transferrin, transferrin-saturation,
23 146 and ferritin before and after the intervention to confirm and further explore the association
24 147 between the iron metabolism and oxidative modifications.
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148 **METHODS AND ANALYSIS**

149 The protocol is developed in accordance with the SPIRIT (Standard Protocol Items:
150 Recommendations for Interventional Trials) recommendation.[32,33]

151 The trial is registered at <http://clinicaltrials.gov> (NCT02890745). Important changes in the
152 protocol will be registered at the website, and addressed to the Danish Medicines Authority
153 and The Regional Committee on Biomedical Research Ethics.

154

155 **Trial design and setting**

156 The trial will be randomised, placebo-controlled, and double-blinded with two parallel
157 groups.

158 Participants will be recruited from the diabetes outpatient clinic, Department of Medicine,
159 Gentofte Hospital, University of Copenhagen, Hellerup, Denmark in relation to outpatient
160 consultations. The recruitment continues until 34 participants successfully have completed
161 the trial.

162

163 **Eligibility of participants**

164 *Inclusion criteria*

165 Male diagnosed with T2D

- 166 ▶ Age: 18-75 years
- 167 ▶ HbA1c: 6.5-9.0%
- 168 ▶ Capable of understanding oral- and written information
- 169 ▶ **Caucasian**

170

171 *Exclusion criteria*

- 172 ▶ Estimated glomerular filtration rate (eGFR) < 60 mL/hour/1.73 m²
- 173 ▶ Currently receiving insulin treatment
- 174 ▶ Coronary artery bypass grafting, percutaneous coronary intervention, acute coronary
175 syndrome, stroke, lung embolism, deep vein thrombosis, or transitory cerebral ischemia
176 within 6 months
- 177 ▶ Genital infection within 14 days
- 178 ▶ Plasma alanine aminotransferase ≥3 times upper normal limit
- 179 ▶ Treatment with SGLT-2 inhibitor within 2 months
- 180 ▶ Hyperglycaemic symptoms
- 181 ▶ Psychiatric disorder
- 182 ▶ Intolerance to empagliflozin or other agents relevant to study
- 183 ▶ Non-compliant

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185 **Intervention**

186 Participants will be randomised to receive once-daily empagliflozin 25 mg or placebo (in the
187 morning) for two weeks as add-on to ongoing therapy. Empagliflozin and placebo will be
188 manufactured and labelled by Boehringer Ingelheim. The dosage of empagliflozin is standard
189 medical treatment of T2D.[34] Possible administration of sulfonylurea drugs to participants
190 will be paused one week before the start visit and during the intervention to avoid
191 hypoglycaemia during the trial. Otherwise, there will be no restriction to concomitant

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4 192 treatment. Participants are encouraged not to change their lifestyle (i.e. diet, exercise, and
5 193 smoking status) during the trial.
6 194 Trial medicine comply Good Manufacturing Practice regulations.[35] Participants receive text
7 195 messages reminders of medicine intake during the intervention period.
8 196 Participants will be evaluated compliant to medicine intake at adherence level of at least
9 197 65%.
10 198

11 199 **Outcomes**

12 200 *Primary outcome measurements*

13 201 The primary outcome of the trial will be the difference between 24h urinary excretion of 8-
14 202 oxoGuo and 8-oxodG before and after the intervention, which will be compared between
15 203 the two groups.
16 204

17 205 *Secondary outcome measurements*

18 206 The secondary outcome will be the difference between MDA in plasma before and after the
19 207 intervention, which will be compared between the two groups.
20 208 Further, this study correlates the concentration of iron, transferrin, transferrin-saturation,
21 209 and ferritin with the urinary excretion of 8-oxoGuo and 8-oxodG.
22 210

23 211 **Participant timeline**

24 212 Participant timeline is illustrated in Figure 1. Potential participants will be informed about
25 213 the trial in relation to their outpatient consultation at the Hospital. If they are interested in
26 214 participating, a screening visit will be conducted after informed consent. At the screening
27 215 visit eligibility will be investigated. Blood pressure, pulse, height, and weight will be
28 216 measured.

29 217 Participants will be fasting at the randomisation and end-of-study visit. At the randomisation
30 218 visit, trial medicine will be dispensed to participants, 24h urine sample and a venous blood
31 219 sample will be collected. After a 14-days treatment schedule, the end-of-study visit will take
32 220 place. Participants will deliver the 24h urine sample and excess trial medicine, and a venous
33 221 blood sample will be collected. All adverse events will be registered.

34 222 The 24h urine sample will be collected from the morning the day before the visit until the
35 223 morning the day of the visit. The participant will empty his bladder before the collection of
36 224 24h urine will begin.

37 225 The first participant's screening visit is expected to be October the 25th 2016. The last
38 226 participant's end-of-study visit is expected to be May the 31th 2017.
39 227

40 228 **Sample size**

41 229 An a priori power calculation has been performed with type I error risk of 5% and type II
42 230 error risk of 20%. Values of the primary outcomes are about 30 nmol with standard
43 231 deviation of 6 nmol per 24h based on baseline values from the PENTRIOX trial[36].
44 232 Smoking increases urinary excretion of 8-oxodG by 50% (95% CI 31-69%)[37]. We wish to be
45 233 able to detect an effect size of 20%, since we do not believe smaller changes are clinical
46 234 relevant. The type 1 and type 2 errors are set at conventional values.

47 235 The power calculation based on these assumptions estimated 17 participants in each group
48 236 using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).
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5 238 **Assignment of intervention**

6 239 The randomisation will be carried out through a simple randomisation with a 1:1 allocation
7 240 ratio from a computer-generated list of random numbers.

8 241 The list of random numbers will be made by an employee who otherwise will be uninvolved
9 242 in the study. The allocation sequence will be concealed from the investigators and
10 243 healthcare staff enrolling and assessing participants.

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13 244 The physicians responsible for the treatment of patients with T2D in the outpatient clinic will
14 245 inform potential participants about the trial and will offer them an information meeting. At
15 246 the information meeting EL, LK, and/or VC will inform potential participants about the trial,
16 247 and the potential participants will receive written information as well. The screening visit will
17 248 be performed by EL, LK, and/or VC after written informed consent is obtained. EL, JR, TV, and
18 249 FK will be responsible for the enrolment of participants and assignment of intervention. The
19 250 end-of-study visit will be performed by EL, LK, and/or VC. HP is sponsor-investigator of the
20 251 study. JR is principle investigator of the study.

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22 252 Only HP, EL, LK, and VC will have access to the final dataset until after analysis of the data.
23 253 After analysis and code break of the trial all investigators have full access to the dataset.

24 254 Participants, research coordinator, investigators, and laboratorian technicians will be blinded
25 255 until data is analysed.

26
27 256 Code break of a participant's trial medicine will occur only under exceptional circumstances
28 257 when knowledge of treatment is absolutely necessary. Only the principal Investigator and an
29 258 employee who is otherwise uninvolved in the study will be able to perform a code break.
30 259 Since they will not be part of the data analysis, a potential code break will not be of critical
31 260 importance.

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34 262 **Data collection, analysis and management**

35 263 Urine and plasma samples will be collected at the randomisation visit and the end-of-study
36 264 visit. In order to improve adherence of the 24h urine sample, participants will receive three
37 265 text messages reminders of the sample collection during the sample collection period. If
38 266 participants record loss of a urine void, 150 mL will be added to the recorded diuresis.
39 267 Participants will be evaluated compliant to 24h urinary collection at adherence level of at
40 268 least 75%.

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44 270 Urine samples will be stored at -20°C until data analysis. The samples will be analysed after
45 271 last participant's end-of-study visit by ultra-performance liquid chromatography tandem
46 272 mass spectrometry (UPLC-MS/MS). The frozen urine samples will be heated to 37°C and
47 273 centrifuged at 10.000 g for 5 minutes prior to addition of Lithiumacetate and internal
48 274 standard. The chromatographic separation will be performed by an Acquity UPLC I-class
49 275 system with an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 x 100 mm) (Waters,
50 276 Milford, MA, USA) with mobile phases A: 5 mM ammoniumacetate and B: Acetonitrile. The
51 277 mass spectrometric detection will be performed by a Xevo TQ-S triple quadrupole mass
52 278 spectrometer (Waters, Milford, MA, USA) with Intellistart function (MassLynx 4.1).[18]
53 279 Further details of the method and quality control are described elsewhere [18]
54 280 Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the preferred method to
55 281 determine urinary excretion of 8-oxoGuo and 8-oxodG due to the high specificity[38].
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5 283 Plasma samples will be stored at -80°C until data analysis. The samples will be analysed after
6 284 last participant's end-of-study visit by high-performance liquid chromatography (HPLC).
7 285 Phosphotungstic acid will be added to the samples before centrifuged for 3 minutes at
8 286 16.000 g. Then butylated hydroxytoluene will be added to avoid further peroxidation. TBA
9 287 reagent and acetic acid will be added and the mixture will be heated to 95°C for 60 minutes
10 288 to form MDA(TBA)₂ adduct. MDA(TBA)₂ will be determined by HPLC with fluorescence
11 289 detection (excitation, 515 nm; emission.[31] Further details of the method and quality
12 290 control are described elsewhere.[31]
13 291 The HPLC method has a high specificity, thus preferred for determining MDA[39].
14 292

15 293 The study will collect demographic and baseline information from the participant's
16 294 physicians.
17 295

18 296 All data will be written in Case Report Form (CRF). CRFs will only be available for
19 297 investigators and research coordinator, stored at trial site locked away according to ICH-GCP
20 298 guidelines.[40] The data will be entered into a locked database by double data entry after
21 299 last participant's end-of-study visit.

22 300 The trial will be monitored by the GCP unit, Bispebjerg Hospital, University of Copenhagen,
23 301 Copenhagen, Denmark. The GCP unit and the Danish Medicines Authority will be able to
24 302 conduct an audit of the trial independent of sponsor-investigator and investigators.
25 303

26 304 The treatment period will be short, and empagliflozin is in general well tolerated. Therefore
27 305 only few adverse reactions are to be expected. Participants will be informed of possible
28 306 adverse reactions and told to contact an investigator if he experiences a possible adverse
29 307 reaction. Adverse events will be registered at the end-of-study visit. All adverse events will
30 308 be reported to the Danish Medicines Authority and the Danish National Committee on
31 309 Biomedical Research Ethics at the end of trial.

32 310 Fatal or life-threatening Suspected Unexpected Serious Adverse Reactions (SUSARs) will be
33 311 reported to Danish Medicines Authority and the Danish National Committee on Biomedical
34 312 Research Ethics within 7 days. Other SUSARs will be reported to Danish Medicines Authority
35 313 and the Danish National Committee on Biomedical Research Ethics within 15 days.
36 314 The participants will be insured by the Capital Region of Denmark, Gentofte Hospital.
37 315 Principal investigator will have the final decision on whether an adverse event requires
38 316 additional treatment, exclusion from the trial, or termination the entire trial.
39 317

318 **Statistical analysis**

40 319 All analyses will be conducted per-protocol. Any drop-out of the trial will be described in the
41 320 results.

42 321 Baseline demographic and clinical characteristics will be compared using a Student's t-test. If
43 322 the two groups are not equal after randomisation in aspect of demographic or clinical
44 323 parameters, then a logistic regression will be used to correlate the result for these
45 324 parameters.

46 325 The difference in primary and secondary outcomes before and after intervention will be
47 326 compared between the groups using Student's t-test.

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4 327 Data will be evaluated for normal distribution using graphical illustration and the Shapiro-
5 328 Wilk test, and further validated using a non-parametric test. If data are not normally
6 329 distributed, data will be log-transformed before analyses or subjected to a non-parametric
7 330 test for analysis.
8
9 331 A two-sided *P*-value <0.05 will be considered statistically significant.
10 332

11 333 **The strengths and limitations of this study**

12 334 The study explores if empagliflozin influences oxidation of nucleic acids as a prerequisite
13 335 before conducting a controlled trial with oxidative stress and mortality as endpoints.
14 336 The strength of this study is the randomised, placebo-controlled, double-blinded trial design.
15 337 The biomarker 8-oxoGuo is prognostic for mortality in patients with T2D, and the methods
16 338 for analysis of both primary and secondary outcomes are precise and reliable.
17 339 The limitation of this study is that the study outcome is not mortality with a long-term follow
18 340 up, but a biomarker prognostic for mortality in patients with T2D.
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4 341 **ETHICS AND DISSEMINATION**

5 342 The study has received approval from The Regional Committee on Biomedical Research
6 343 Ethics (approval number H-16017433), the Danish Medicines Authority, and the Danish Data
7 344 Protection Agency. The study is performed with informed consent from participants in
8 345 agreement to the declaration of Helsinki.

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11 346 Additional plasma samples will be stored at -80°C for use in future research, locked away at
12 347 the Department of Pharmacology, University Hospital Rigshospitalet.

13 348 The results of the study will be presented at national and international conferences, and
14 349 submitted at a peer-review international journal.
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4 350 **Contributors**

5 351 This study is based on an idea by HP. HP, EL, VC, LK, TV, FK, and JR contributed to the trial
6 352 design. EL wrote the first protocol draft. HP, VC, LK, TV, FK, and JR contributed to
7 353 development of study protocol and approved the final draft.
8
9 354

10 355 **Competing interests**

11 356 JR has received lecture fees from AstraZeneca, Boehringer Ingelheim Pharmaceuticals, Eli
12 357 Lilly and Company, Merck Sharp & Dohme, Novo Nordisk, Sanofi, and is a member of the
13 358 Advisory boards of Novo Nordisk, Merck Sharp & Dohme and Sanofi.

14 359 TV has received lecture fees from AstraZeneca, Boehringer Ingelheim Pharmaceuticals,
15 360 Bristol-Myers Squibb, Eli Lilly and Company, Merck Sharp & Dohme, Novo Nordisk, Novartis,
16 361 Sanofi, and Zealand Pharma, and is member of the Advisory Boards of Amgen, Novo Nordisk,
17 362 Merck Sharp & Dohme and Bristol-Myers Squibb/AstraZeneca.

18 363 FK has received lecture fees from, is a member of the Advisory Boards of, has consulted for,
19 364 and/or received research support from AstraZeneca, Boehringer Ingelheim Pharmaceuticals,
20 365 Eli Lilly, Gilead Sciences, Merck Sharp & Dohme, Novo Nordisk, Ono Pharmaceuticals, Sanofi
21 366 and Zealand Pharma.

22 367 EL has received a 12 months scholarship from Boehringer Ingelheim.
23 368 All other authors declare no conflict of interest.
24 369

25 370 **Funding**

26 371 This study is sponsor-investigator-initiated and investigator-driven and financed by a
27 372 unrestricted grant from Boehringer Ingelheim. Empagliflozin and placebo are manufactured
28 373 and provided by Boehringer Ingelheim.

29 374 VC has received the Faculty PhD Scholarship from the Faculty of Health and Medical
30 375 Sciences, University of Copenhagen.
31 376

32 377 **Data sharing statement**

33 378 HP, EL, LK, and VC will have access to the final dataset. After analysis and code break of the
34 379 trial all investigators have full access to the dataset.

35 380 The results of the study will be presented at national and international conferences, and
36 381 submitted at a peer-review international journal.
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30 497 40 ICH Harmonised Tripartite Guideline. Guideline for Good Clinical Practice E6(R1). *ICH*
31 498 *Harmon Tripart Guidel* Published Online First: 1996. doi:10.1056/NEJMp1012246
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4 500 **FIGURE LEGENDS**

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6 502 **Figure 1.** Participant timeline.
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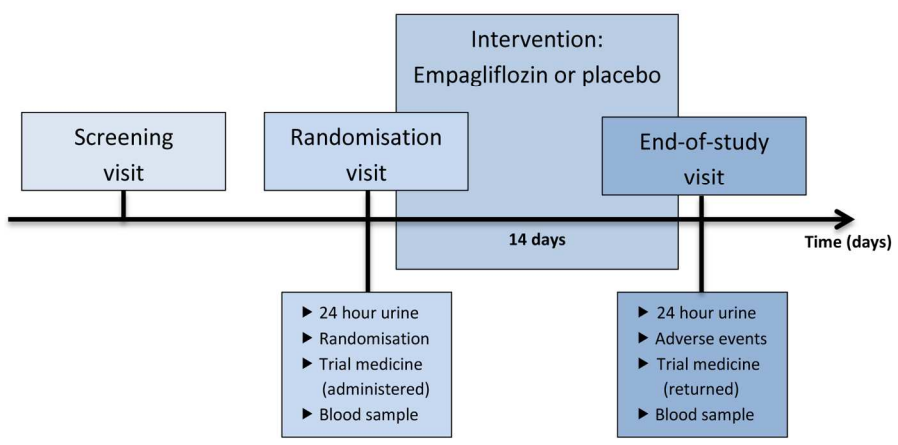


Figure 1. Participant timeline.
Figure 1
156x105mm (300 x 300 DPI)

view only



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 2
	2b	All items from the World Health Organization Trial Registration Data Set	Not applicable
Protocol version	3	Date and version identifier	Page 2
Funding	4	Sources and types of financial, material, and other support	Page 11
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 11
	5b	Name and contact information for the trial sponsor	Page 1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Page 8
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Page 8+9

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49**Introduction**

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Page 4
	6b	Explanation for choice of comparators	Page 4+5
Objectives	7	Specific objectives or hypotheses	Page 5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Page 6

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Page 6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 6-7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	Page 9
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Page 7 + 8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Page 6
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Page 7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Page 7 + Figure 1

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3	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Page 7
4				
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6	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
7				

8 **Methods: Assignment of interventions (for controlled trials)**

9 Allocation:

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12	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Page 7-8
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18	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Page 7-8
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22	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 8
23				
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25	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 8
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28		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Page 8
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32 **Methods: Data collection, management, and analysis**

33				
34	Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Page 8
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39		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Page 8
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3	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 8
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7	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Page 9
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10		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 9
11				
12		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Page 9
13				
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16	Methods: Monitoring			
17				
18	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	Page 9
19				
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23		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	Page 9, no interim
24				
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26	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 8+9
27				
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29	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Page 9
30				
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33	Ethics and dissemination			
34				
35	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 10
36				
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38	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	Page 6
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3	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8
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6		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Page 10
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9	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 8
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12	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 11
13				
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15	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 8
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18	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 9
19				
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21	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 10
22				
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26		31b	Authorship eligibility guidelines and any intended use of professional writers	Page 2
27				
28		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No plans, not applicable
29				
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31	Appendices			
32				
33	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix 1
34				
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36	Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Page 10
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*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.