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:	*BMJ Open*
Manuscript ID	bmjopen-2016-014728
Article Type:	Protocol
Date Submitted by the Author:	14-Oct-2016
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 Vilsbøll, 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

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

Emil List Larsen[1,2,4], **Vanja Cejvanovic**[1,2,3,6] **Laura Kofoed Kjær**[1,2,3,6], **Tina Vilsbøll**[4], **Filip Krag Knop**[4], **Jørgen Rungby**[4,5], **Henrik Enghusen Poulsen**[1,2,3*]

[1]Laboratory of Clinical Pharmacology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
[2]Department of Clinical Pharmacology, Bispebjerg and Frederiksberg Hospital, University of Copenhagen, Copenhagen, Denmark
[3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[4]Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Hellerup, Denmark
[5]Department of Biomedicine, Aarhus University, Aarhus, Denmark

[6]These authors contributed equally to this work

***Corresponding author**
Professor, Henrik Enghusen Poulsen, MD
Laboratory of Clinical Pharmacology, Q7642
Rigshospitalet, University of Copenhagen
Ole Maaløes Vej 26, Entrance 76
DK-2200 Copenhagen N
Denmark
E-mail: hepo@rh.dk
Phone: +45 3545 7671
Fax: +45 3545 2745

Words: 2535
Figures: 1
Tables: 0
References: 37
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 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

Abbreviations: Type 2 diabetes, T2D; Ribonucleic acid, RNA; Deoxyribonucleic acid, DNA; 8-oxo-7,8-dihydroguanosine, 8-oxoGuo; 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG; malondialdehyde, MDA; sodium/glucose cotransporter 2, SGLT-2; ultra-performance liquid chromatography tandem mass-spectrometry, UPLC-MS/MS; high-performance liquid chromatography, HPLC; 8-iso prostaglandin f2α, 8-iso-PGF2α; Suspected Unexpected Serious Adverse Reactions, SUSARs.

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-dihydriguanosiene (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 inconsistence 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.

Hypothesis and aims

We hypothesise that empagliflozin lowers oxidative nucleic acid modifications, measurable by urinary excretion of 8-oxoGuo and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). Further, we evaluate if there is a compartmentalisation of oxidative modifications in the organism and within the cell by also differentiate the effect of empagliflozin on lipid peroxidation by plasma levels of malondialdehyde (MDA).

In addition to our primary hypothesis, this study investigates whether iron, transferrin, transferrin-saturation, and ferritin are correlated to the levels of oxidative modifications (8-oxoGuo, 8-oxodG, and MDA) in patients with T2D.

Study objectives

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

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

Further, this study determines the concentration of iron, transferrin, transferrin-saturation, and ferritin before and after the intervention.

METHODS AND ANALYSIS

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

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

Trial design and setting

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

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

Eligibility of participants

Inclusion criteria

Male diagnosed with T2D

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

Exclusion criteria

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

Intervention

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

Trial medicine comply Good Manufacturing Practice regulations.[36] Participants receive text messages reminders of medicine intake during the intervention period.
Participants will be evaluated compliant to medicine intake at adherence level of at least 65%.

Outcomes

Primary outcome measurements

The primary outcome of the trial will be the difference between 24h urinary excretion of 8-oxoGuo and 8-oxodG before and after the intervention, which will be compared between the two groups.

Secondary outcome measurements

The secondary outcome will be the difference between MDA in plasma before and after the intervention, which will be compared between the two groups. Further, this study correlates the concentration of iron, transferrin, transferrin-saturation, and ferritin with the urinary excretion of 8-oxoGuo and 8-oxodG.

Participant timeline

Participant timeline is illustrated in Figure 1. Potential participants will be informed about the trial in relation to their outpatient consultation at the Hospital. If they are interested in participating, a screening visit will be conducted after informed consent. At the screening visit eligibility will be investigated. Blood pressure, pulse, height, and weight will be measured.
Participants will be fasting at the randomisation and end-of-study visit. At the randomisation visit, trial medicine will be dispensed to participants, 24h urine sample and a venous blood sample will be collected. After a 14-days treatment schedule, the end-of-study visit will take place. Participants will deliver the 24h urine sample and excess trial medicine, and a venous blood sample will be collected. All adverse events will be registered.
The 24h urine sample will be collected from the morning the day before the visit until the morning the day of the visit. The participant will empty his bladder before the collection of 24h urine will begin.
The first participant's screening visit is expected to be October the 25th 2016. The last participant's end-of-study visit is expected to be March the 31th 2017.

Sample size

An a priori power calculation has been performed with type I error risk of 5% and type II error risk of 20%.
Values of the primary outcomes are about 30 nmol per 24h. We wish to be able to detect an effect size of 20%. The standard deviation is 6 nmol per 24h.
The power calculation estimated 17 participants in each group using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Assignment of intervention

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

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

The physicians responsible for the treatment of patients with T2D in the outpatient clinic will inform potential participants about the trial and will offer them an information meeting. At the information meeting EL, LK, and/or VC will inform potential participants about the trial, and the potential participants will receive written information as well. The screening visit will be performed by EL, LK, and/or VC after written informed consent is obtained. EL, JR, TV, and FK will be responsible for the enrolment of participants and assignment of intervention. The end-of-study visit will be performed by EL, LK, and/or VC. HP is sponsor-investigator of the study. JR is principle investigator of the study.
Only HP, EL, LK, and VC will have access to the final dataset until after analysis of the data. After analysis and code break of the trial all investigators have full access to the dataset.
Participants, research coordinator, investigators, and laboratorian technicians will be blinded until data is analysed.
Code break of a participant's trial medicine will occur only under exceptional circumstances when knowledge of treatment is absolutely necessary. Only the principal Investigator and an employee who is otherwise uninvolved in the study will be able to perform a code break. Since they will not be part of the data analysis, a potential code break will not be of critical importance.

Data collection, analysis and management

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

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

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

The study will collect demographic and baseline information from the participant's physicians.

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

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

The treatment period will be short, and empagliflozin is in general well tolerated. Therefore only few adverse reactions are to be expected. Participants will be informed of possible adverse reactions and told to contact an investigator if he experiences a possible adverse reaction. Adverse events will be registered at the end-of-study visit. All adverse events will be reported to the Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics at the end of trial.
Fatal or life-threatening Suspected Unexpected Serious Adverse Reactions (SUSARs) will be reported to Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics within 7 days. Other SUSARs will be reported to Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics within 15 days. The participants will be insurance by the Capital Region of Denmark, Gentofte Hospital. Principal investigator will have the final decision on whether an adverse event requires additional treatment, exclusion from the trial, or termination the entire trial.

Statistical analysis

All analyses will be conducted per-protocol. Any drop-out of the trial will be described in the results.
Baseline demographic and clinical characteristics will be compared using a Student's t-test. If the two groups are not equal after randomisation in aspect of demographic or clinical parameters, then a logistic regression will be used to correlate the result for these parameters.
The difference in primary and secondary outcomes before and after intervention will be compared between the groups using Student's t-test.
Data will be evaluated for normal distribution using graphical illustration and the Shapiro-Wilk test, and further validated using a non-parametric test. If data are not normally distributed, data will be log-transformed before analyses or subjected to a non-parametric test for analysis.
A two-sided *P*-value <0.05 will be considered statistically significant.

The strengths and limitations of this study

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

ETHICS AND DISSEMINATION

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

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

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

Contributors

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

Competing interests

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

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

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

EL has received a 12 months scholarship from Boehringer Ingelheim.

All other authors declare no conflict of interest.

Funding

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

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

Data sharing statement

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

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

REFERENCES

1 World Health Organization. Global Report on Diabetes. Published Online First: 2016.http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf

2 Shah AD, Langenberg C, Rapsomaniki E, et al. Type 2 diabetes and incidence of cardiovascular diseases: A cohort study in 1.9 million people. *Lancet Diabetes Endocrinol* 2015;**3**:105–14. doi:10.1016/S2213-8587(14)70219-0

3 Inzucchi SE, Bergenstal RM, Buse JB, et al. Management of hyperglycaemia in type 2 diabetes, 2015: a patient-centred approach. Update to a Position Statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetologia* 2015;**58**:429–42. doi:10.1007/s00125-014-3460-0

4 Patel A, MacMahon S, Chalmers J, et al. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. (ADVANCE Trial). *N Engl J Med* 2008;**358**:2560–72. doi:10.1056/NEJMoa0802987

5 Turnbull FM, Abraira C, Anderson RJ, et al. Intensive glucose control and macrovascular outcomes in type 2 diabetes. *Diabetologia* 2009;**52**:2288–98. doi:10.1007/s00125-009-1470-0

6 Currie CJ, Peters JR, Tynan A, et al. Survival as a function of HbA1c in people with type 2 diabetes: a retrospective cohort study. *Lancet* 2010;**375**:481–9. doi:10.1016/S0140-6736(09)61969-3

7 Scirica BM, Bhatt DL, Braunwald E, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med* 2013;**369**:1317–26. doi:10.1056/NEJMoa1307684

8 White WB, Cannon CP, Heller SR, et al. Alogliptin after Acute Coronary Syndrome in Patients with Type 2 Diabetes. *N Engl J Med* 2013;**369**:1327–35. doi:10.1056/NEJMoa1305889

9 Green JB, Bethel MA, Armstrong PW, et al. Effect of Sitagliptin on Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med* 2015;**373**:150608133014007. doi:10.1056/NEJMoa1501352

10 Pfeffer MA, Claggett B, Diaz R, et al. Lixisenatide in Patients with Type 2 Diabetes and Acute Coronary Syndrome. *N Engl J Med* 2015;**373**:2247–57. doi:10.1056/NEJMoa1509225

11 Gæde P, Oellgaard J, Carstensen B, et al. Years of life gained by multifactorial intervention in patients with type 2 diabetes mellitus and microalbuminuria : 21 years follow-up on the Steno-2 randomised trial. *Diabetologia* Published Online First: 2016. doi:10.1007/s00125-016-4065-6

12 Defronzo RA. From the triumvirate to the ominous octet: A new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 2009;**58**:773–95. doi:10.2337/db09-9028

13 Broedbaek K, Weimann A, Stovgaard ES, et al. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker in type 2 diabetes. *Free Radic Biol Med* 2011;**51**:1473–9. doi:10.1016/j.freeradbiomed.2011.07.007

14 Broedbaek K, Siersma V, Henriksen T, et al. Urinary markers of nucleic acid oxidation and long-term mortality of newly diagnosed type 2 diabetic patients. *Diabetes Care* 2011;**34**:2594–6. doi:10.2337/dc11-1620

15 Broedbaek K, Siersma V, Henriksen T, et al. Association Between Urinary Markers of

Nucleic Acid Oxidation and Mortality in Type 2 Diabetes: A population-based cohort study. *Diabetes Care* 2013;**36**:669–76. doi:10.2337/dc12-0998

16 Glintborg B, Weimann A, Kensler TW, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: Unaltered oxidative biomarkers. *Free Radic Biol Med* 2006;**41**:1010–4. doi:10.1016/j.freeradbiomed.2006.06.015

17 Broedbaek K, Henriksen T, Weimann A, et al. Long-term effects of irbesartan treatment and smoking on nucleic acid oxidation in patients with type 2 diabetes and microalbuminuria: An irbesartan in patients with type 2 diabetes and microalbuminuria (IRMA 2) substudy. *Diabetes Care* 2011;**34**:1192–8. doi:10.2337/dc10-2214

18 Poulsen HE, Specht E, Broedbaek K, et al. RNA modifications by oxidation: A novel disease mechanism? *Free Radic Biol Med* 2012;**52**:1353–61. doi:10.1016/j.freeradbiomed.2012.01.009

19 Rasmussen ST, Andersen JT, Nielsen TK, et al. Simvastatin and oxidative stress in humans: A randomized, double-blinded, placebo-controlled clinical trial. *Redox Biol* 2016;**9**:32–8. doi:10.1016/j.redox.2016.05.007

20 Ferrannini E, Solini A. SGLT2 inhibition in diabetes mellitus: rationale and clinical prospects. *Nat Rev Endocrinol* 2012;**8**:495–502. doi:10.1038/nrendo.2011.243

21 Zinman B, Wanner C, Lachin JM, et al. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *N Engl J Med* 2015;**373**:2117–28. doi:10.1056/NEJMoa1504720

22 Abdul-Ghani M, Del Prato S, Chilton R, et al. SGLT2 Inhibitors and Cardiovascular Risk: Lessons Learned From the EMPA-REG OUTCOME Study. *Diabetes Care* 2016;**39**:717–25. doi:10.2337/dc16-0041

23 Tikkanen I, Chilton R, Johansen OE. Potential role of sodium glucose cotransporter 2 inhibitors in the treatment of hypertension. *Curr Opin Nephrol Hypertens* 2016;**25**:81–6. doi:10.1097/MNH.0000000000000199

24 Sattar N, McLaren J, Kristensen SL, et al. SGLT2 Inhibition and cardiovascular events: why did EMPA-REG Outcomes surprise and what were the likely mechanisms? *Diabetologia* Published Online First: 2016. doi:10.1007/s00125-016-3956-x

25 Oelze M, Kröller-Schön S, Welschof P, et al. The Sodium-Glucose Co-Transporter 2 Inhibitor Empagliflozin Improves Diabetes-Induced Vascular Dysfunction in the Streptozotocin Diabetes Rat Model by Interfering with Oxidative Stress and Glucotoxicity. *PLoS One* 2014;**9**:1–13. doi:10.1371/journal.pone.0112394

26 Cheng STW, Chen L, Li SYT, et al. The Effects of Empagliflozin, an SGLT2 Inhibitor, on Pancreatic β-Cell Mass and Glucose Homeostasis in Type 1 Diabetes. *PLoS One* 2016;**11**:1–12. doi:10.1371/journal.pone.0147391

27 Nishimura R, Tanaka Y, Koiwai K, et al. Effect of empagliflozin monotherapy on postprandial glucose and 24-hour glucose variability in Japanese patients with type 2 diabetes mellitus: a randomized, double-blind, placebo-controlled, 4-week study. *Cardiovasc Diabetol* 2015;**14**:11. doi:10.1186/s12933-014-0169-9

28 Frijhoff J, Winyard PG, Zarkovic N, et al. Clinical Relevance of Biomarkers of Oxidative Stress. *Antioxid Redox Signal* 2015;**23**:1144–70. doi:10.1089/ars.2015.6317

29 Broedbaek K, Poulsen HE, Weimann A, et al. Urinary excretion of biomarkers of oxidatively damaged DNA and RNA in hereditary hemochromatosis. *Free Radic Biol*

Med 2009;**47**:1230–3. doi:10.1016/j.freeradbiomed.2009.08.004

30 Stroh M, Swerdlow RH, Zhu H. Common defects of mitochondria and iron in neurodegeneration and diabetes (MIND): A paradigm worth exploring. *Biochem Pharmacol* 2014;**88**:573–83. doi:10.1016/j.bcp.2013.11.022

31 Poulsen HE, Nadal LL, Broedbaek K, et al. Detection and interpretation of 8-oxodG and 8-oxoGua in urine, plasma and cerebrospinal fluid. *Biochim Biophys Acta - Gen Subj* 2014;**1840**:801–8. doi:10.1016/j.bbagen.2013.06.009

32 Lykkesfeldt J. Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: Comparison with ultraviolet-visible spectrophotometry. *Clin Chem* 2001;**47**:1725–7.

33 Chan A-W, Tetzlaff JM, Altman DG, et al. Research and Reporting Methods Annals of Internal Medicine SPIRIT 2013 Statement : Defining Standard Protocol Items for Clinical Trials. 2015;**1**. doi:10.7326/0003-4819-158-3-201302050-00583

34 Chan A-W, Tetzlaff JM, Gøtzsche PC, et al. SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ* 2013;**346**:e7586. doi:10.1136/bmj.e7586

35 Jardiance®. http://pro.medicin.dk/Medicin/Praeparater/7463 (accessed 10 Jun2016).

36 European-Commision. Good Manufacturing Practice. Medicinal Products for Human and Vetrinary use. Annex 13. Investigational Medicinal Prodcts 2010: ;**4**.

37 ICH Harmonised Tripartite Guideline. Guideline for Good Clinical Practice E6(R1). *ICH Harmon Tripart Guidel* Published Online First: 1996. doi:10.1056/NEJMp1012246

FIGURE LEGENDS

Figure 1. Participant timeline.

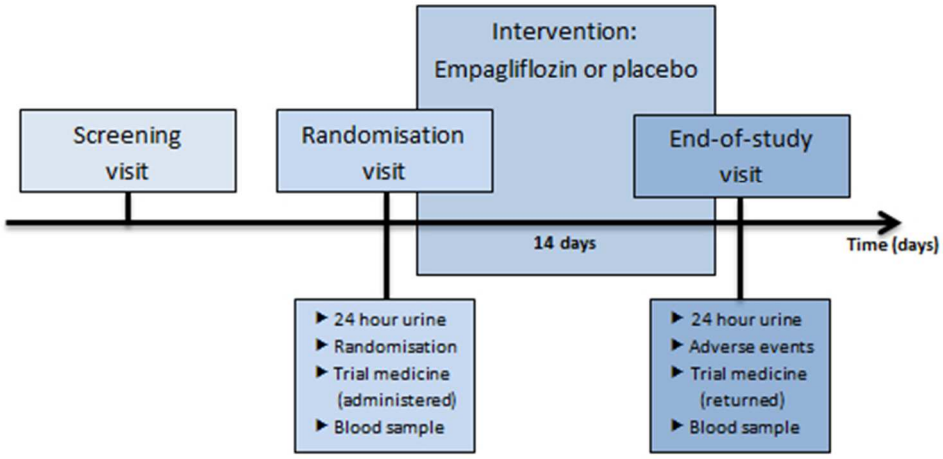

Figure 1. Participant timeline.
Figure 1

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

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
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
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
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
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 8
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 8
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Page 8
Methods: Data collection, management, and analysis			
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
	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

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
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
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 9
	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
Methods: Monitoring			
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
	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
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
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
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 10
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

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
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Page 10
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
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 11
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
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
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
	31b	Authorship eligibility guidelines and any intended use of professional writers	Page 2
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No plans, not applicable
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix 1
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

*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" license.

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:	*BMJ Open*
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 Vilsbøll, 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

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

Emil List Larsen[1,2,4], **Vanja Cejvanovic**[1,2,3,6] **Laura Kofoed Kjær**[1,2,3,6], **Tina Vilsbøll**[4], **Filip Krag Knop**[4], **Jørgen Rungby**[4,5], **Henrik Enghusen Poulsen**[1,2,3*]

[1]Laboratory of Clinical Pharmacology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
[2]Department of Clinical Pharmacology, Bispebjerg and Frederiksberg Hospital, University of Copenhagen, Copenhagen, Denmark
[3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[4]Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Hellerup, Denmark
[5]Department of Biomedicine, Aarhus University, Aarhus, Denmark

[6]These authors contributed equally to this work

***Corresponding author**
Professor, Henrik Enghusen Poulsen, MD
Laboratory of Clinical Pharmacology, Q7642
Rigshospitalet, University of Copenhagen
Ole Maaløes Vej 26, Entrance 76
DK-2200 Copenhagen N
Denmark
E-mail: hepo@rh.dk
Phone: +45 3545 7671
Fax: +45 3545 2745

Words: 2797
Figures: 1
Tables: 0
References: 40
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

Abbreviations: 8-iso prostaglandin f2α, 8-iso-PGF2α; 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG; 8-oxo-7,8-dihydroguanosine, 8-oxoGuo; Deoxyribonucleic acid, DNA; high-performance liquid chromatography, HPLC; malondialdehyde, MDA; Ribonucleic acid, RNA; sodium/glucose cotransporter 2, SGLT-2; Suspected Unexpected Serious Adverse Reactions, SUSARs; Type 2 diabetes, T2D; ultra-performance liquid chromatography tandem mass-spectrometry, UPLC-MS/MS;.

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-dihydriguanosiene (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 inconsistence 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.

Hypothesis and aims

We hypothesise that empagliflozin lowers oxidative nucleic acid modifications, measurable by urinary excretion of 8-oxoGuo and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). Further, we evaluate if there is a compartmentalisation of oxidative modifications in the organism and within the cell by also differentiate the effect of empagliflozin on lipid peroxidation by plasma levels of malondialdehyde (MDA).

In addition to our primary hypothesis, this study investigates whether iron, transferrin, transferrin-saturation, and ferritin are correlated to the levels of oxidative modifications (8-oxoGuo, 8-oxodG, and MDA) in patients with T2D.

Study objectives

The primary objectives are 24h urinary excretion of 8-oxoGuo and 8-oxodG before and after treatment with empagliflozin compared to placebo as a measurement of whole body RNA- and DNA oxidation, respectively.[30]

The secondary objectives encompass the concentration of MDA in plasma before and after treatment with empagliflozin compared to placebo treatment as a measurement of lipid peroxidation.[31]

Further, this study determines the concentration of iron, transferrin, transferrin-saturation, and ferritin before and after the intervention to confirm and further explore the association between the iron metabolism and oxidative modifications.

METHODS AND ANALYSIS

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

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

Trial design and setting

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

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

Eligibility of participants

Inclusion criteria

Male diagnosed with T2D

- Age: 18-75 years
- HbA1c: 6.5-9.0%
- Capable of understanding oral- and written information
- **Caucasian**

Exclusion criteria

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

Intervention

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

treatment. Participants are encouraged not to change their lifestyle (i.e. diet, exercise, and smoking status) during the trial.
Trial medicine comply Good Manufacturing Practice regulations.[35] Participants receive text messages reminders of medicine intake during the intervention period.
Participants will be evaluated compliant to medicine intake at adherence level of at least 65%.

Outcomes

Primary outcome measurements

The primary outcome of the trial will be the difference between 24h urinary excretion of 8-oxoGuo and 8-oxodG before and after the intervention, which will be compared between the two groups.

Secondary outcome measurements

The secondary outcome will be the difference between MDA in plasma before and after the intervention, which will be compared between the two groups. Further, this study correlates the concentration of iron, transferrin, transferrin-saturation, and ferritin with the urinary excretion of 8-oxoGuo and 8-oxodG.

Participant timeline

Participant timeline is illustrated in Figure 1. Potential participants will be informed about the trial in relation to their outpatient consultation at the Hospital. If they are interested in participating, a screening visit will be conducted after informed consent. At the screening visit eligibility will be investigated. Blood pressure, pulse, height, and weight will be measured.
Participants will be fasting at the randomisation and end-of-study visit. At the randomisation visit, trial medicine will be dispensed to participants, 24h urine sample and a venous blood sample will be collected. After a 14-days treatment schedule, the end-of-study visit will take place. Participants will deliver the 24h urine sample and excess trial medicine, and a venous blood sample will be collected. All adverse events will be registered.
The 24h urine sample will be collected from the morning the day before the visit until the morning the day of the visit. The participant will empty his bladder before the collection of 24h urine will begin.
The first participant's screening visit is expected to be October the 25th 2016. The last participant's end-of-study visit is expected to be May the 31th 2017.

Sample size

An a priori power calculation has been performed with type I error risk of 5% and type II error risk of 20%. Values of the primary outcomes are about 30 nmol with standard deviation of 6 nmol per 24h based on baseline values from the PENTRIOX trial[36]. Smoking increases urinary excretion of 8-oxodG by 50% (95% CI 31-69%)[37]. We wish to be able to detect an effect size of 20%, since we do not believe smaller changes are clinical relevant. The type 1 and type 2 errors are set at conventional values.
The power calculation based on these assumptions estimated 17 participants in each group using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Assignment of intervention

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

The physicians responsible for the treatment of patients with T2D in the outpatient clinic will inform potential participants about the trial and will offer them an information meeting. At the information meeting EL, LK, and/or VC will inform potential participants about the trial, and the potential participants will receive written information as well. The screening visit will be performed by EL, LK, and/or VC after written informed consent is obtained. EL, JR, TV, and FK will be responsible for the enrolment of participants and assignment of intervention. The end-of-study visit will be performed by EL, LK, and/or VC. HP is sponsor-investigator of the study. JR is principle investigator of the study.
Only HP, EL, LK, and VC will have access to the final dataset until after analysis of the data. After analysis and code break of the trial all investigators have full access to the dataset.
Participants, research coordinator, investigators, and laboratorian technicians will be blinded until data is analysed.
Code break of a participant's trial medicine will occur only under exceptional circumstances when knowledge of treatment is absolutely necessary. Only the principal Investigator and an employee who is otherwise uninvolved in the study will be able to perform a code break. Since they will not be part of the data analysis, a potential code break will not be of critical importance.

Data collection, analysis and management

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

Urine samples will be stored at -20°C until data analysis. The samples will be analysed after last participant's end-of-study visit by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The frozen urine samples will be heated to 37°C and centrifuged at 10.000 g for 5 minutes prior to addition of Lithiumacetate and internal standard. The chromatographic separation will be performed by an Acquity UPLC I-class system with an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 x 100 mm) (Waters, Milford, MA, USA) with mobile phases A: 5 mM ammoniumacetate and B: Acetonitrile. The mass spectrometric detection will be performed by a Xevo TQ-S triple quadrulope mass spectrometer (Waters, Milford, MA, USA) with Intellistart function (MassLynx 4.1).[18] Further details of the method and quality control are described elsewhere [18] Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the preferred method to determine urinary excretion of 8-oxoGuo and 8-oxodG due to the high specificity[38].

Plasma samples will be stored at -80°C until data analysis. The samples will be analysed after last participant's end-of-study visit by high-performance liquid chromatography (HPLC). Phosphotungstic acid will be added to the samples before centrifuged for 3 minutes at 16.000 g. Then butylated hydroxytoluene will be added to avoid further peroxidation. TBA reagent and acetic acid will be added and the mixture will be heated to 95°C for 60 minutes to form $MDA(TBA)_2$ adduct. $MDA(TBA)_2$ will be determined by HPLC with fluroscence detection (excitation, 515 nm; emmision.[31] Further details of the method and quality control are described elsewhere.[31]
The HPLC method has a high specificity, thus preferred for determining MDA[39].

The study will collect demographic and baseline information from the participant's physicians.

All data will be written in Case Report Form (CRF). CRFs will only be available for investigators and research coordinator, stored at trial site locked away according to ICH-GCP guidelines.[40] The data will be entered into a locked database by double data entry after last participant's end-of-study visit.
The trial will be monitored by the GCP unit, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark. The GCP unit and the Danish Medicines Authority will be able to conduct an audit of the trial independent of sponsor-investigator and investigators.

The treatment period will be short, and empagliflozin is in general well tolerated. Therefore only few adverse reactions are to be expected. Participants will be informed of possible adverse reactions and told to contact an investigator if he experiences a possible adverse reaction. Adverse events will be registered at the end-of-study visit. All adverse events will be reported to the Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics at the end of trial.
Fatal or life-threatening Suspected Unexpected Serious Adverse Reactions (SUSARs) will be reported to Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics within 7 days. Other SUSARs will be reported to Danish Medicines Authority and the Danish National Committee on Biomedical Research Ethics within 15 days. The participants will be insurance by the Capital Region of Denmark, Gentofte Hospital. Principal investigator will have the final decision on whether an adverse event requires additional treatment, exclusion from the trial, or termination the entire trial.

Statistical analysis

All analyses will be conducted per-protocol. Any drop-out of the trial will be described in the results.
Baseline demographic and clinical characteristics will be compared using a Student's t-test. If the two groups are not equal after randomisation in aspect of demographic or clinical parameters, then a logistic regression will be used to correlate the result for these parameters.
The difference in primary and secondary outcomes before and after intervention will be compared between the groups using Student's t-test.

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

A two-sided *P*-value <0.05 will be considered statistically significant.

The strengths and limitations of this study

The study explores if empagliflozin influences oxidation of nucleic acids as a prerequisite before conducting a controlled trial with oxidative stress and mortality as endpoints. The strength of this study is the randomised, placebo-controlled, double-blinded trial design. The biomarker 8-oxoGuo is prognostic for mortality in patients with T2D**,** and the methods for analysis of both primary and secondary outcomes are precise and reliable. The limitation of this study is that the study outcome is not mortality with a long-term follow up, but a biomarker prognostic for mortality in patients with T2D.

ETHICS AND DISSEMINATION

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

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

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

Contributors

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

Competing interests

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

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

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

EL has received a 12 months scholarship from Boehringer Ingelheim.

All other authors declare no conflict of interest.

Funding

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

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

Data sharing statement

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

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

REFERENCES

1 World Health Organization. Global Report on Diabetes. Published Online First: 2016.http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf

2 Shah AD, Langenberg C, Rapsomaniki E, et al. Type 2 diabetes and incidence of cardiovascular diseases: A cohort study in 1.9 million people. *Lancet Diabetes Endocrinol* 2015;**3**:105–14. doi:10.1016/S2213-8587(14)70219-0

3 Inzucchi SE, Bergenstal RM, Buse JB, et al. Management of hyperglycaemia in type 2 diabetes, 2015: a patient-centred approach. Update to a Position Statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetologia* 2015;**58**:429–42. doi:10.1007/s00125-014-3460-0

4 Patel A, MacMahon S, Chalmers J, et al. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. (ADVANCE Trial). *N Engl J Med* 2008;**358**:2560–72. doi:10.1056/NEJMoa0802987

5 Turnbull FM, Abraira C, Anderson RJ, et al. Intensive glucose control and macrovascular outcomes in type 2 diabetes. *Diabetologia* 2009;**52**:2288–98. doi:10.1007/s00125-009-1470-0

6 Currie CJ, Peters JR, Tynan A, et al. Survival as a function of HbA1c in people with type 2 diabetes: a retrospective cohort study. *Lancet* 2010;**375**:481–9. doi:10.1016/S0140-6736(09)61969-3

7 Scirica BM, Bhatt DL, Braunwald E, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med* 2013;**369**:1317–26. doi:10.1056/NEJMoa1307684

8 White WB, Cannon CP, Heller SR, et al. Alogliptin after Acute Coronary Syndrome in Patients with Type 2 Diabetes. *N Engl J Med* 2013;**369**:1327–35. doi:10.1056/NEJMoa1305889

9 Green JB, Bethel MA, Armstrong PW, et al. Effect of Sitagliptin on Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med* 2015;**373**:150608133014007. doi:10.1056/NEJMoa1501352

10 Pfeffer MA, Claggett B, Diaz R, et al. Lixisenatide in Patients with Type 2 Diabetes and Acute Coronary Syndrome. *N Engl J Med* 2015;**373**:2247–57. doi:10.1056/NEJMoa1509225

11 Defronzo RA. From the triumvirate to the ominous octet: A new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 2009;**58**:773–95. doi:10.2337/db09-9028

12 Broedbaek K, Weimann A, Stovgaard ES, et al. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker in type 2 diabetes. *Free Radic Biol Med* 2011;**51**:1473–9. doi:10.1016/j.freeradbiomed.2011.07.007

13 Broedbaek K, Siersma V, Henriksen T, et al. Urinary Markers of Nucleic Acid Oxidation and Long-Term Mortality of Newly Diagnosed Type 2 Diabetic Patients. *Diabetes Care* 2011;**34**:2594–6. doi:10.2337/dc11-1620

14 Broedbaek K, Siersma V, Henriksen T, et al. Association Between Urinary Markers of Nucleic Acid Oxidation and Mortality in Type 2 Diabetes: A population-based cohort study. *Diabetes Care* 2013;**36**:669–76. doi:10.2337/dc12-0998

15 Glintborg B, Weimann A, Kensler TW, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: Unaltered oxidative biomarkers. *Free Radic Biol Med*

2006;**41**:1010–4. doi:10.1016/j.freeradbiomed.2006.06.015
16 Broedbaek K, Henriksen T, Weimann A, et al. Long-term effects of irbesartan treatment and smoking on nucleic acid oxidation in patients with type 2 diabetes and microalbuminuria: An irbesartan in patients with type 2 diabetes and microalbuminuria (IRMA 2) substudy. *Diabetes Care* 2011;**34**:1192–8. doi:10.2337/dc10-2214
17 Poulsen HE, Specht E, Broedbaek K, et al. RNA modifications by oxidation: A novel disease mechanism? *Free Radic Biol Med* 2012;**52**:1353–61. doi:10.1016/j.freeradbiomed.2012.01.009
18 Rasmussen ST, Andersen JT, Nielsen TK, et al. Simvastatin and oxidative stress in humans: A randomized, double-blinded, placebo-controlled clinical trial. *Redox Biol* 2016;**9**:32–8. doi:10.1016/j.redox.2016.05.007
19 Ferrannini E, Solini A. SGLT2 inhibition in diabetes mellitus: rationale and clinical prospects. *Nat Rev Endocrinol* 2012;**8**:495–502. doi:10.1038/nrendo.2011.243
20 Zinman B, Wanner C, Lachin JM, et al. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *N Engl J Med* 2015;**373**:2117–28. doi:10.1056/NEJMoa1504720
21 Abdul-Ghani M, Del Prato S, Chilton R, et al. SGLT2 Inhibitors and Cardiovascular Risk: Lessons Learned From the EMPA-REG OUTCOME Study. *Diabetes Care* 2016;**39**:717–25. doi:10.2337/dc16-0041
22 Tikkanen I, Chilton R, Johansen OE. Potential role of sodium glucose cotransporter 2 inhibitors in the treatment of hypertension. *Curr Opin Nephrol Hypertens* 2016;**25**:81–6. doi:10.1097/MNH.0000000000000199
23 Sattar N, McLaren J, Kristensen SL, et al. SGLT2 Inhibition and cardiovascular events: why did EMPA-REG Outcomes surprise and what were the likely mechanisms? *Diabetologia* Published Online First: 2016. doi:10.1007/s00125-016-3956-x
24 Oelze M, Kröller-Schön S, Welschof P, et al. The Sodium-Glucose Co-Transporter 2 Inhibitor Empagliflozin Improves Diabetes-Induced Vascular Dysfunction in the Streptozotocin Diabetes Rat Model by Interfering with Oxidative Stress and Glucotoxicity. *PLoS One* 2014;**9**:1–13. doi:10.1371/journal.pone.0112394
25 Cheng STW, Chen L, Li SYT, et al. The Effects of Empagliflozin, an SGLT2 Inhibitor, on Pancreatic β-Cell Mass and Glucose Homeostasis in Type 1 Diabetes. *PLoS One* 2016;**11**:1–12. doi:10.1371/journal.pone.0147391
26 Nishimura R, Tanaka Y, Koiwai K, et al. Effect of empagliflozin monotherapy on postprandial glucose and 24-hour glucose variability in Japanese patients with type 2 diabetes mellitus: a randomized, double-blind, placebo-controlled, 4-week study. *Cardiovasc Diabetol* 2015;**14**:11. doi:10.1186/s12933-014-0169-9
27 Frijhoff J, Winyard PG, Zarkovic N, et al. Clinical Relevance of Biomarkers of Oxidative Stress. *Antioxid Redox Signal* 2015;**23**:1144–70. doi:10.1089/ars.2015.6317
28 Broedbaek K, Poulsen HE, Weimann A, et al. Urinary excretion of biomarkers of oxidatively damaged DNA and RNA in hereditary hemochromatosis. *Free Radic Biol Med* 2009;**47**:1230–3. doi:10.1016/j.freeradbiomed.2009.08.004
29 Stroh M, Swerdlow RH, Zhu H. Common defects of mitochondria and iron in neurodegeneration and diabetes (MIND): A paradigm worth exploring. *Biochem Pharmacol* 2014;**88**:573–83. doi:10.1016/j.bcp.2013.11.022

30 Poulsen HE, Nadal LL, Broedbaek K, et al. Detection and interpretation of 8-oxodG and 8-oxoGua in urine, plasma and cerebrospinal fluid. *Biochim Biophys Acta - Gen Subj* 2014;**1840**:801–8. doi:10.1016/j.bbagen.2013.06.009

31 Lykkesfeldt J. Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: Comparison with ultraviolet-visible spectrophotometry. *Clin Chem* 2001;**47**:1725–7.

32 Chan A-W, Tetzlaff JM, Altman DG, et al. Research and Reporting Methods Annals of Internal Medicine SPIRIT 2013 Statement : Defining Standard Protocol Items for Clinical Trials. 2015;**1**. doi:10.7326/0003-4819-158-3-201302050-00583

33 Chan A-W, Tetzlaff JM, Gøtzsche PC, et al. SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ* 2013;**346**:e7586. doi:10.1136/bmj.e7586

34 Jardiance®. http://pro.medicin.dk/Medicin/Praeparater/7463 (accessed 10 Jun2016).

35 European-Commision. Good Manufacturing Practice. Medicinal Products for Human and Vetrinary use. Annex 13. Investigational Medicinal Prodcts 2010: ;**4**.

36 Larsen EL, Cejvanovic V, Kjær LK, et al. Clarithromycin, trimethoprim, and penicillin and oxidative nucleic acid modifications in humans: randomised, controlled trials. *Br J Clin Pharmacol* Published Online First: 2017. doi:10.1111/bcp.13261

37 Loft S, Vistisen K, Ewertz M, et al. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* 1992;**13**:2241–7.

38 Weimann A, Broedbaek K, Henriksen T, et al. Assays for urinary biomarkers of oxidatively damaged nucleic acids. *Free Radic Res* 2012;**46**:531–40. doi:10.3109/10715762.2011.647693

39 Lykkesfeldt J. Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. *Clin Chim Acta* 2007;**380**:50–8. doi:10.1016/j.cca.2007.01.028

40 ICH Harmonised Tripartite Guideline. Guideline for Good Clinical Practice E6(R1). *ICH Harmon Tripart Guidel* Published Online First: 1996. doi:10.1056/NEJMp1012246

FIGURE LEGENDS

Figure 1. Participant timeline.

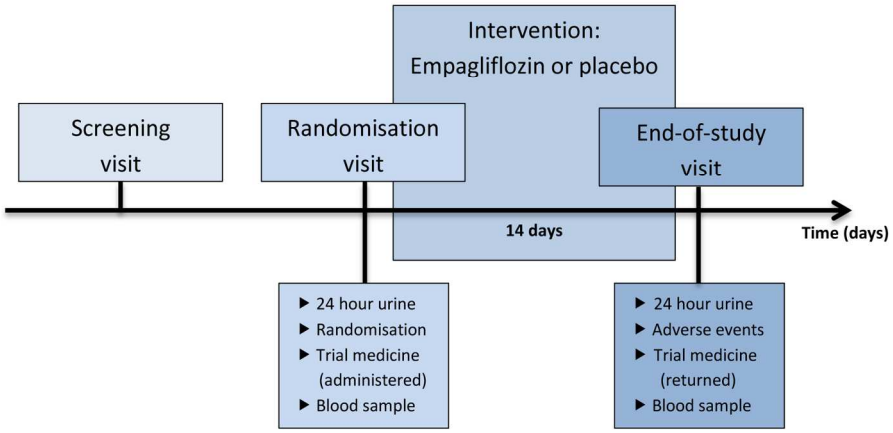

Figure 1. Participant timeline.
Figure 1
156x105mm (300 x 300 DPI)

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

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
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
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
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
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 8
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 8
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Page 8
Methods: Data collection, management, and analysis			
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
	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

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
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
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 9
	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
Methods: Monitoring			
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
	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
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
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
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 10
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

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
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Page 10
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
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 11
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
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
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
	31b	Authorship eligibility guidelines and any intended use of professional writers	Page 2
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No plans, not applicable
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix 1
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

*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.