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Cardiovascular disease in diabetes Focus on pathophysiology, risk markers and GLP-1 RAs

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Cardiovascular disease in diabetes
Focus on pathophysiology, risk markers and GLP-1 RAs

A dissertation submitted to the University of Copenhagen
Faculty of Health and Medical Sciences for consideration
for the degree of Doctor of Medical Science

By

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Preface

This thesis is based upon studies carried out during my time at Steno Diabetes Center. I wish to thank all the participants involved; your voluntary contributions made the studies possible.

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To everyone else in "520", to all the co-authors and collaborators not mentioned: Thank you! Especially, thank you to Rasmus Ripa for a great collaboration on Liraflame.

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Vedbæk, January 2023.

Emilie Hein Zobel

Indhold

List of publications

This thesis is based on the following original and published/accepted papers:

1) Higher Parathyroid Hormone Level Is Associated With Increased Arterial Stiffness in Type 1 Diabetes.
   Zobel EH, Theilade S, von Scholten BJ, Persson F, Tarnow L, Lajer M, Hansen TW, Rossing P.
   Diabetes Care. 2017, 40(3):e32-e33.

2) Myocardial flow reserve assessed by cardiac 82Rb positron emission tomography/computed tomography is associated with albumin excretion in patients with Type 1 diabetes.
   Zobel EH, Winther SA, Hasbak P, von Scholten BJ, Holmvang L, Kjaer A, Rossing P, Hansen TW.
   European Heart Journal Cardiovascular Imaging. 2018, 20(7):796-803.

3) Cardiac Autonomic Function is Associated With Myocardial Flow Reserve in Type 1 Diabetes.
   Zobel EH, Hasbak P, Winther SA, Stevns C, Fleischer J, von Scholten BJ, Holmvang L, Kjaer A, Rossing P, Hansen TW.
   Diabetes. 2019, 68(6):1277-1286.

4) Relation of cardiac adipose tissue to coronary calcification and myocardial microvascular function in type 1 and type 2 diabetes.
   Zobel EH, Christensen RH, Winther SA, Hasbak P, Hansen CS, von Scholten BJ, Holmvang L, Kjaer A, Rossing P, Hansen TW.
   Cardiovascular Diabetology. 2020, 19(1):16.

5) Toe-brachial index as a predictor of cardiovascular disease and all-cause mortality in people with type 2 diabetes and microalbuminuria.
   Zobel EH, von Scholten BJ, Reinhard H, Persson F, Hansen TW, Parving HH, Jacobsen PK, Rossing P.
   Diabetologia. 2017, 60(10):1883-1891.

6) Symmetric and asymmetric dimethylarginine as risk markers of cardiovascular disease, all-cause mortality and deterioration in kidney function in persons with type 2 diabetes and microalbuminuria
   Zobel EH, von Scholten BJ, Reinhard H, Persson F, Teerlink T, Hansen TW, Parving HH, Jacobsen PK, Rossing P.

Cardiovascular Diabetology. 2017, 16(1):88.

7) Cuff inflations do not affect night-time blood pressure: comparison of 24-hour ambulatory blood pressure measured by a cuff and a tonometric device in type 2 diabetes.
Petersen EH, Theilade S, Hansen TW, Lindhardt MK, Rossing P.
Blood Pressure Monitoring. 2015, 20(6):369-72.

8) Pleiotropic effects of liraglutide treatment on renal risk factors in type 2 diabetes: Individual effects of treatment.
Zobel EH, von Scholten BJ, Lindhardt M, Persson F, Hansen TW, Rossing P.
Journal of Diabetes and Its Complications. 2017, 31(1):162-8.

9) Pleiotropic effects of liraglutide in patients with type 2 diabetes and moderate renal impairment: Individual effects of treatment.
Zobel EH, von Scholten BJ, Goldman B, Persson F, Hansen TW, Rossing P.
Diabetes, Obesity and Metabolism. 2019, 21(5):1261-1265

10) Effect of Liraglutide on Arterial Inflammation Assessed as [(18)F]FDG Uptake in Patients With Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Trial.
Ripa RS*, Zobel EH*, von Scholten BJ, Jensen JK, Binderup T, Diaz LJ, Curovic VR, Hansen TW, Rossing P, Kjaer A.
Circulation Cardiovascular Imaging. 2021:CIRCIMAGING120012174.
**Authors contributed equally*.

11) Effect of Liraglutide on Vascular Inflammation Evaluated by [64Cu]DOTATATE.
Zobel EH, Ripa RS, von Scholten BJ, Curovic VR, Diaz LJ, Hansen TW, Rossing P, Kjaer A.
Diagnostics. 2021;11(8):1431.

12) Ceramides and phospholipids are downregulated with liraglutide treatment: results from the LiraFlame randomized controlled trial.
Zobel EH, Wretlind A, Ripa RS, Rotbain Curovic V, von Scholten BJ, Suvitaival T, Hansen TW, Kjaer A, Legido-Quigley C, Rossing P.
BMJ Open Diabetes Research & Care. 2021, 9(1).

13) The importance of addressing multiple risk markers in type 2 diabetes: results from the LEADER and SUSTAIN 6 trials.

Zobel EH, von Scholten BJ, Hansen TW, Persson F, Rasmussen S, Wolthers B, et Rossing P.

Diabetes, Obesity and Metabolism. 2021, accepted for publication.

Abbreviations

ADMA	Asymmetric dimethylarginine
[^{64}Cu]DOTATATE	[1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid]-D-Phe1, Tyr3-octreotate
[^{18}F]FDG	^{18}F-Fluorodeoxyglucose
GFR	Glomerular filtration rate
GLP-1 RA	Glucagon-like peptide-1 receptor agonist
IDI	Integrated discrimination improvement
LEADER	Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results
LIRA-RENAL	Efficacy and Safety of Liraglutide Versus Placebo as Add-on to Glucose-Lowering Therapy in Patients With Type 2 Diabetes and Moderate Renal Impairment
MIBG	Metaiodobenzylguanidine
MRA	Mineralocorticoid receptor antagonist
PET	Positron emission tomography
ROC	Reciever Operating Characteristic
REWIND	Researching Cardiovascular Events With a Weekly Incretin in Diabetes trial
SDMA	Symmetric dimethylarginine
SUSTAIN-6	Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes

Introduction and aims

Cardiovascular disease is the leading cause of morbidity and mortality in diabetes(1). Although, the risk of cardiovascular disease and mortality have declined substantially over the last decade, the risk of fatal cardiovascular disease remains about eight times higher in persons with type 1 diabetes and two times higher in persons with type 2 diabetes compared to the general population(1).

Type 1 diabetes has an earlier onset than type 2 diabetes, and there are other important differences in the underlying pathophysiology, still, most of the risk factors for cardiovascular disease overlap in type 1 and type 2 diabetes, including hyperglycemia, obesity, hypertension, dyslipidemia, and kidney disease(2). Obesity and the metabolic syndrome which is dominating in type 2 diabetes, have also become more prevalent in type 1 diabetes in relation to epidemiological shifts in the population(2).

Despite the encouraging improvement, the excess mortality due to cardiovascular disease in diabetes underscores the need for better and earlier diagnostic using imaging, clinical markers or biomarkers, and a need for new treatment options. The overall aim of this thesis was therefore to obtain novel knowledge on the pathophysiology underlying cardiovascular disease in diabetes, on risk markers for cardiovascular disease in diabetes, and on understanding the clinical effects and mode-of-action of glucagon-like peptide-1 receptor agonists (GLP-1 RAs), a new treatment option with the ability to reduce the risk of cardiovascular disease in diabetes.

I) Understanding the pathophysiology of cardiovascular complications in diabetes

Part I Despite the higher risk of cardiovascular disease in type 1 diabetes, the underlying pathophysiology is not well understood. We explored bone markers and myocardial microvascular dysfunction as potential new pathophysiological aspects of cardiovascular disease in type 1 diabetes.

Presence of cardiovascular disease and osteoporosis are often coexisting, and epidemiological and biological evidence has questioned whether these are just independent age-related coexistent diseases(3). To explore the hypothesis that stiffening of the large arteries may in part be mediated through abnormalities in bone metabolism, we investigated in study A the associations between arterial stiffness and bone mass density as well as a panel of clinical bone markers and markers of mineral metabolism in a cross-sectional study in type 1 diabetes. Due to the high prevalence of vascular calcification and reduced bone mass density it is ideal to investigate a possible link between bone demineralisation and arterial stiffness in a diabetic population.

From bone markers to study B where we explored the link between renal and heart disease in a cross-sectional study in type 1 diabetes. Diabetic kidney disease is a complication that is strongly linked to a higher risk of cardiovascular disease in diabetes(4). We used quantitative cardiac positron emission tomography (PET) to quantify myocardial microvascular function. Cardiac PET estimate myocardial blood flow at rest and during pharmacologically induced hyperaemia, and the ratio between the two is termed the myocardial flow reserve. The myocardial flow reserve is a measure that integrates the haemodynamic effects of epicardial stenoses, diffuse atherosclerotic disease, and myocardial microvascular dysfunction, and a low myocardial flow reserve is a risk factor for cardiac mortality(5). We hypothesised that presence of albuminuria is linked to a lower myocardial flow reserve. We further pursued the hypothesis that cardiac autonomic dysfunction including loss of sympathetic integrity is linked to impaired myocardial blood flow regulation in diabetes. We evaluated the association between myocardial flow reserve and cardiac autonomic function assessed using heart rate variability indices, cardiovascular autonomic reflex tests, and cardiac ^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) imaging. In a cross-sectional study similar to study B, our group has previously assessed myocardial flow reserve in persons with type 2 diabetes and healthy controls using the same equipment and similar protocols(6). We pooled the two studies to explore the hypothesis that amount of cardiac adipose tissue was negatively associated with myocardial flow reserve and positively associated with coronary artery calcium score in type 1 diabetes, type 2 diabetes and in healthy controls.

II) Identifying high-risk individuals with type 2 diabetes

Part II. There is a growing need to identify the individuals with type 2 diabetes at the highest risk of developing cardiovascular disease. This is on a background of an increasing number of persons with type 2 diabetes worldwide resulting in need to prioritize treatment and care, including the new and more expensive anti-diabetic treatments with the potential to reduce the risk of cardio-renal complications in type 2 diabetes. The aim of the second part of this thesis was to explore both new and established risk measures for cardiovascular disease in type 2 diabetes.

Measurement of toe-brachial index and ankle-brachial index are diagnostic tests for peripheral arterial disease, but these markers also reflect systemic atherosclerosis. Few studies have evaluated the added prognostic value of toe-brachial index in type 2 diabetes(7, 8), and the value of ankle-brachial index in diabetes is debated as ankle-pressures may be elevated because of medial arterial calcification(9). We hypothesized that lower toe-brachial index and ankle-brachial index are independent risk factors of cardiovascular disease and mortality in type 2 diabetes and explored this in a prospective study with 6-years of follow up (study C). Assessment of toe-brachial index and ankle-brachial index is non-invasive,

inexpensive and easily performed in most clinical settings, and therefore measures of atherosclerosis applicable for clinical practice. In the same cohort, we also explored asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) as markers of a different aspect of the pathophysiology underlying vascular disease in diabetes. ADMA is an analogue of L-arginine and is a naturally occurring product of metabolism, found in the human circulation(10). ADMA directly inhibits the production of nitric oxide, and this biological function has fueled an interest in ADMA and its structural isomer SDMA as risk factors. Higher ADMA is considered an independent risk factor of cardiovascular disease and mortality in different populations(11) but results in type 2 diabetes are conflicting(12, 13). Studies of SDMA as a risk factor in type 2 diabetes are few(12, 14). We hypothesized that higher ADMA and SDMA are independent risk factors for cardiovascular disease and mortality in type 2 diabetes.

In contrast to toe-brachial index, SDMA and ADMA, blood pressure is a well-established risk factor for cardiovascular disease in diabetes(15), and correct blood pressure measurement is a cornerstone in identification of persons with type 2 diabetes at the highest risk of cardiovascular disease(16). The level of 24-hour ambulatory blood pressure is a better predictor of cardiovascular complications and mortality as compared to blood pressure measured in the office(17). Moreover, the interest in measurements of blood pressure during the nighttime is growing, as data suggesting prognostic superiority compared to daytime blood pressure are emerging(18). Traditionally, 24-hour blood pressure devices are upper arm-type self-inflating cuff monitors. Some persons find the frequent arm-compressions by the cuff uncomfortable which may affect compliance, and especially, the nighttime blood pressure might be false elevated because of impaired sleep-quality. In study D, we investigated the impact of cuff inflations on subjective and objective quality of sleep and level of nighttime blood pressure in persons with type 2 diabetes, by comparing 24-hour and nighttime blood pressure measured by a cuff-less wrist device and a standard self-inflating arm cuff-device. We hypothesized that cuff-inflations would impair sleep quality and reduce the nocturnal systolic blood pressure decline.

III) Understanding the clinical effects and mode-of-action of GLP-1 RAs

Part III Several large cardiovascular outcome trials have demonstrated that treatment with some glucagon-like peptide-1 receptor agonists (GLP-1 RAs) reduce the risk of major adverse cardiovascular events in type 2 diabetes(19-23). GLP-1 RAs with proven cardiovascular benefits are recommended as part of the glycemic management among persons with type 2 diabetes with established atherosclerotic cardiovascular disease(24). The mechanism behind the cardiovascular protection observed with GLP-1 RAs in outcome studies are largely unknown, and the aim of the third part of this thesis was to improve the understanding of the clinical effects and mode-of-actions of GLP-1 RAs.

GLP-1 RAs have beneficial effects on several cardio-renal risk factors including body weight, glycemic control, LDL-cholesterol, systolic blood pressure, albuminuria and renal function(25-28). Little is known about the individual treatment response to GLP-1 RAs, and we investigated whether response in one risk factor was associated with response in other risk factors (cross-dependency). These associations were first explored in a post-hoc analysis of a small intervention study(29), and then pursued in a post-hoc analysis of the Efficacy and Safety of Liraglutide Versus Placebo as Add-on to Glucose-Lowering Therapy in Patients With Type 2 Diabetes and Moderate Renal Impairment (LIRA-RENAL) study, a multicenter randomized clinical trial(30). Both studies used the GLP-1 RA liraglutide. We hypothesized that some individuals would be good responders with a response in several risk factors, whereas others would not respond on any of the risk factors.

In terms of understanding the mode-of-action of GLP-1 RAs, the magnitude of the cardio-protection observed in the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial using liraglutide(19) have raised the possibility that mechanisms other than those observed in the trial – improvement in glycemic control, reduction in body weight, small decrease in systolic blood pressure, slightly slower estimated GFR decline and reduction in albuminuria – may be at play(31). Based on mostly preclinical findings we hypothesized that treatment with liraglutide reduces arterial inflammation(32) and we investigated this hypothesis in a randomized clinical trial (study G). We assessed arterial inflammation using ^{18}F-fluorodeoxyglucose ([^{18}F]FDG) PET/CT and [1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid]-D-Phe1, Tyr3-octreotate ([^{64}Cu]DOTATATE) PET/CT. GLP-1 RA treatment is associated with favorable changes in the lipid profile with modest, but consistent reductions in LDL-cholesterol, total-cholesterol and triglyceride level(33). Studies have suggested that other lipid species are important for the development of atherosclerosis, and therefore, in the same trial, we evaluated liraglutides effect on a broad range on different lipids with the aim to identify possible downstream effects of liraglutide on lipid species important for the development of atherosclerosis. Thus, we hypothesized that treatment with liraglutide have beneficial effects on atherogenic lipids.

With GLP-1 RAs it is possible to improve multiple cardio-renal risk factors, using one drug. The importance of targeting multiple risk factors is evident from the small randomized Steno 2 study, where multifactorial intervention targeting several modifiable risk factors resulted in 50% reduced risk of cardiovascular disease as compared to treatment with standard of care(34). We explored the importance of multiple risk factor response for cardiovascular and renal outcomes in type 2 diabetes in a post-hoc analyses of the GLP-1 RA cardiovascular outcome trials, LEADER(19) and the Trial to Evaluate Cardiovascular and Other Long-term

Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6)(20). We hypothesized that multiple risk factor response is important for outcomes in type 2 diabetes.

Participants, designs and methods

Table 1. Characteristics of the studies including designs and participants

Study	Name of study	Study design	Number of participants	Key inclusion criteria	Settings	Inclusion time	Paper(s)
A	Kano	Cross-sectional	347	T1DM	SDCC	2009-11	1
B	Rubin	Cross-sectional	60 (30[60*])	T1DM, normo- or macroalbuminuria (healthy controls, [T2DM*])	SDCC	2016-18 (2013-14)	2,3,4
C	BNPcure	Prospective, follow-up	200	T2DM and micro-albuminuria	SDCC	2006-08	5, 6
D	Rem-brandt	Longitudinal, cross-over	53	T2DM and hypertension	SDCC	2014	7
E	Liratime	Post hoc analysis	31	T2DM, hyperten-sion and preserved renal function	SDCC	2012-14	8
F	LIRA-RENAL	Post hoc analysis	279	T2DM and moderate renal impairment	Internatio-nal multi-centre	2012-13	9
G	Lira-Flame	Randomized clinical trial	102	T2DM, ≥ 50 years	SDCC	2017-18	10, 11, 12
H	LEADER	Post hoc analysis	8638	T2DM, symptoms or history of CVD	Internatio-nal multi-centre	2010-12	13
I	SUSTAIN 6	Post hoc analysis	3040	T2DM, symptoms or history of CVD	Internatio-nal multi-centre	2013	13

*Type 1 and type 2 diabetes were defined according to WHO criteria at the time of inclusion in the studies. SDCC: Steno Diabetes Center Copenhagen. *A retrospective analysis included study B and a previous study of 30 healthy controls and 60 persons with T2DM. T1DM: Type 1 diabetes mellitus. T2DM: type 2 diabetes mellitus. LEADER, The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome*

Results. LIRA-RENAL, Efficacy and Safety of Liraglutide Versus Placebo as Add-on to Glucose-Lowering Therapy in Patients With Type 2 Diabetes and Moderate Renal Impairment. SUSTAIN 6, The Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes.

All participants provided informed consent in writing prior to participation. The studies were approved by relevant ethical committees and conducted in accordance to the declaration of Helsinki.

Methods

The publications include description of clinical measurements and of the used standard laboratory and biomarker assays(35-47).

Arterial stiffness

Arterial stiffness was assessed as pulse wave velocity measured between the carotid and femoral arteries (cfPWV). Recordings were obtained with the SphygmoCor (AtCor Medical, Sydney, Australia). Three cfPWV measurements were performed after 15 minutes supine rest and averaged.

Bone mineral density

Bone mass density (g/cm^2) was measured in the femoral neck by DXA (Hologic Discovery, Apex 3.3).

Toe-brachial index and ankle-brachial index

Systolic blood pressure was measured on both legs in the first toe and in the ankle using a strain gauge technique. The lowest pressure measured was divided with the average brachial blood pressure, measured in the dominant arm. Detailed description in(39).

24-hour blood pressure measurement

Measurements were performed with a cuff-less wrist device (BPro, HealthStats, Singapore) and a standard self-inflating arm cuff-device (Takeda, TM2430, Japan). Recordings were made every 15 minutes with the tonometric device and every 15 minutes between 7 am and 11 pm and every 30 minutes between 11 pm and 7 am with the cuff-device. Detailed description in(41).

Cardiac ^{123}I-MIBG scintigraphy to assess cardiac autonomic function

A Philips SKYLight Gamma Camera with JETstream software (Philips Medical Systems, Best, the Netherlands) obtained planar anterior-posterior images 15 minutes (early) and 240 minutes (late) after ^{123}I-

MIBG injection. Images were processed using the Extended Brilliance Workspace NM Application Suite version 4.5.3.40140 (Philips Medical Systems), and the myocardial washout rate from early to late images was calculated. Detailed description in(37).

Heart rate analyses to assess cardiac autonomic function

The Vagus device (Medicus Engineering, Aarhus, Denmark) was used to measure the resting heart rate variability in time and frequency domains and to measure heart rate variability during cardiac autonomic reflex tests including 1) response to standing, 2) response to deep breathing and 3) response to the Valsalva maneuver. Detailed description in(37).

Cardiac adipose tissue

Cardiac adipose tissue was measured from non-contrast CT images using the cardiac software, Syngo.via Frontier—Cardiac risk assessment (Siemens, AG; Healthcare Sector, Germany). Myocardial and adipose tissue borders were traced automatically and adipose tissue volume was quantified based on the specific density of fat, as described in(38).

Coronary artery calcium score

CT based coronary artery calcium score was calculated using Agatston method(48).

Cardiac PET/CT imaging to asses myocardial blood flow

We used a combined PET/CT scanner in 3D mode (Siemens Biograph mCT 128, Siemens, Munich, Germany). After injection of the ^{82}Rb tracer (CardioGen-82, Bracco Diagnostics, Monroe Township, NJ, USA) images were obtained during rest and during maximal hyperemia, induced by adenosine infusion. Myocardial blood-flow at rest and stress was calculated using the Siemens Syngo MBF 2.3 (Siemens Medical Solutions, Malvern, PA, USA). Detailed description in(36).

PET/CT imaging to asses arterial inflammation

We used a combined PET/CT-scanner (Siemens Biograph mCT64, Siemens, Berlin, Germany) and two different tracers with separate imaging protocols previously described in detail (44, 45). In brief, two hours after ^{18}F-FDG injection, PET was obtained in 3D mode from head to groin for 5 minutes per field of view. A low dose CT scan and a diagnostic CT scan of the neck arteries was used for attenuation correction and to localize the aorta and the carotid arteries, respectively. Similarly, one hour after [^{64}CU]-DOTATATE injection, PET was obtained in 3D mode for 10 minutes centered at the carotid bifurcation. A low dose CT scan was

used for attenuation correction and to localize the carotid arteries. Quantification of the PET images was performed using OsiriX MD 11.0 (Pixmeo, Bernex, Switzerland).

Lipidomics

Lipids were extracted from plasma samples and analyzed by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS)(46).

In two publications supportive endpoints included ultrasound assessed carotid intima media thickness(40, 44), while reactive hyperemia index evaluated with EndoPat™ (Itamar Medical, Israel), and lastly glycocalyx integrity evaluated with the GlycoCheck® device (Maasticht, The Netherlands) was supportive endpoints in (44), with a detailed description in the supplementary in (44).

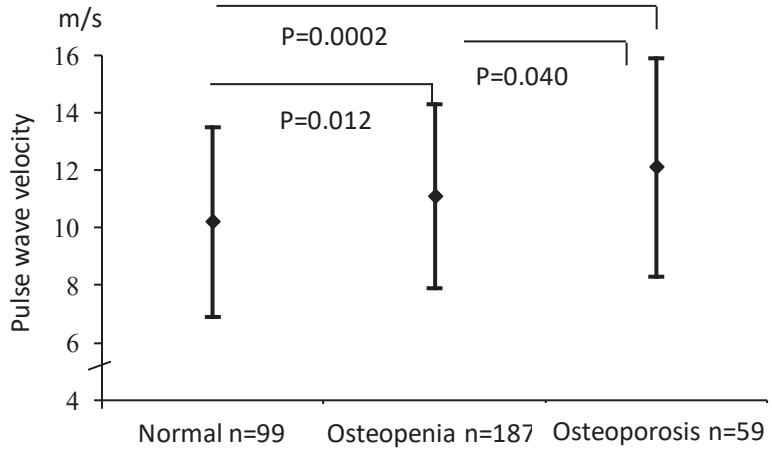
I) Understanding the pathophysiology of cardiovascular complications in diabetes

In this part of the thesis, we explored bone markers and myocardial microvascular dysfunction as potential new pathophysiological aspects of cardiovascular disease in type 1 diabetes.

Results

In a cross-sectional study in 347 persons with type 1 diabetes (Paper 1, study A, Table 1), we explored possible associations between arterial stiffness and bone mass density as well as a panel of clinical bone markers and markers of mineral metabolism. We demonstrated associations between carotid-femoral pulse wave velocity and bone mineral density (Figure 1), several clinical bone markers and mineral metabolism factors. These associations lost significance after comprehensive adjustment, except for the relationship between higher parathyroid hormone (PTH) and increased carotid-femoral pulse wave velocity (p=0.014)(35).

Figure 1. Carotid-femoral pulse wave velocity and osteoporosis status

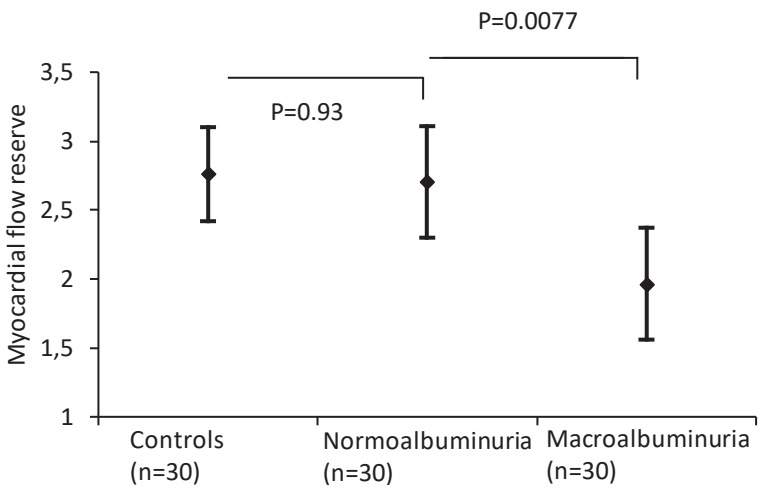


Carotid-femoral pulse wave velocity mean ± standard deviation in relation to osteoporosis status. P values represent differences between groups.

In another cross-sectional study we explored the association between myocardial microvascular dysfunction and renal function. The study included 60 persons with type 1 diabetes stratified by albumin excretion (30 with normoalbuminuria and 30 with macroalbuminuria) and 30 sex and age matched healthy controls (paper 2, study B, Table 1). We observed that 1) the myocardial flow reserve was comparable in healthy controls and persons with type 1 diabetes and normoalbuminuria and 2) persons with type 1 diabetes and history of macroalbuminuria had lower (impaired) myocardial flow reserve than persons with type 1 diabetes and normoalbuminuria (Figure 2). Coronary artery calcium score was higher in persons with type 1 diabetes than in the healthy controls, but comparable between persons with type 1 diabetes and

normo- or macroalbuminuria. When the coronary artery calcium score was dichotomized, coronary artery calcium score >300 was more frequent among persons with type 1 diabetes and macroalbuminuria than among persons with type 1 diabetes and normoalbuminuria(36).

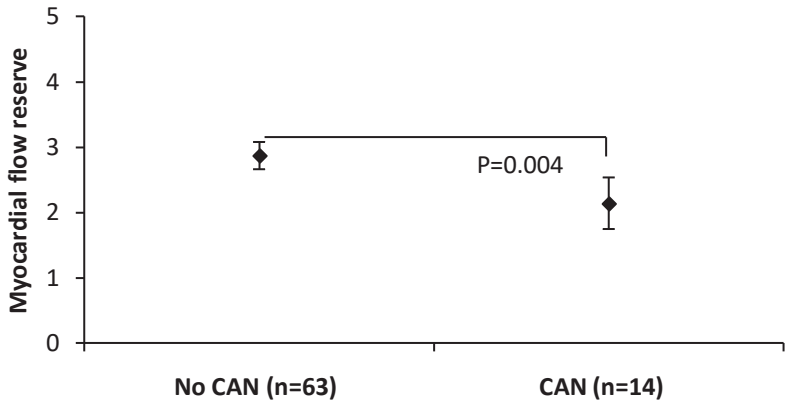
Figure 2. Myocardial flow reserve in type 1 diabetes and healthy controls.



Myocardial flow reserve estimated marginal means with 95% Confidence Intervals. Adjusted for age, sex, 24-hour systolic blood pressure, eGFR and smoking according to groups. Adapted from Zobel et al., European Heart Journal - Cardiovascular Imaging, 2018(36).

In the same cross-sectional study in type 1 diabetes (paper 3, study B, Table 1), we evaluated the association between cardiac autonomic function and the myocardial flow reserve. Cardiac autonomic function was evaluated indirectly with simple bedside tests using heart rate variability indices and cardiovascular reflex tests and directly using MIBG scintigraphy, that asses integrity of the adrenergic cardiac innervation of the heart. Late heart-to-mediastinum ratio (from the MIBG scintigraphy) and the 30-to-15 ratio (a cardiovascular autonomic reflex test) were positively associated with myocardial flow reserve, and these associations persisted after comprehensive adjustments ($p \leq 0.04$). Prevalence of cardiac autonomic neuropathy was 18% based on the cardiovascular reflex tests, and myocardial flow reserve was lower in this group than among those without cardiac autonomic neuropathy (Figure 3). Cardiac autonomic neuropathy was more frequent among persons with type 1 diabetes and macroalbuminuria than among persons with type 1 diabetes and normoalbuminuria. We further demonstrated an impaired cardiac autonomic function in type 1 diabetes compared to the healthy controls when evaluated using both the cardiovascular reflex tests and the MIBG scintigraphy(37).

Figure 3. Myocardial flow reserve according to cardiac autonomic neuropathy (CAN) status.



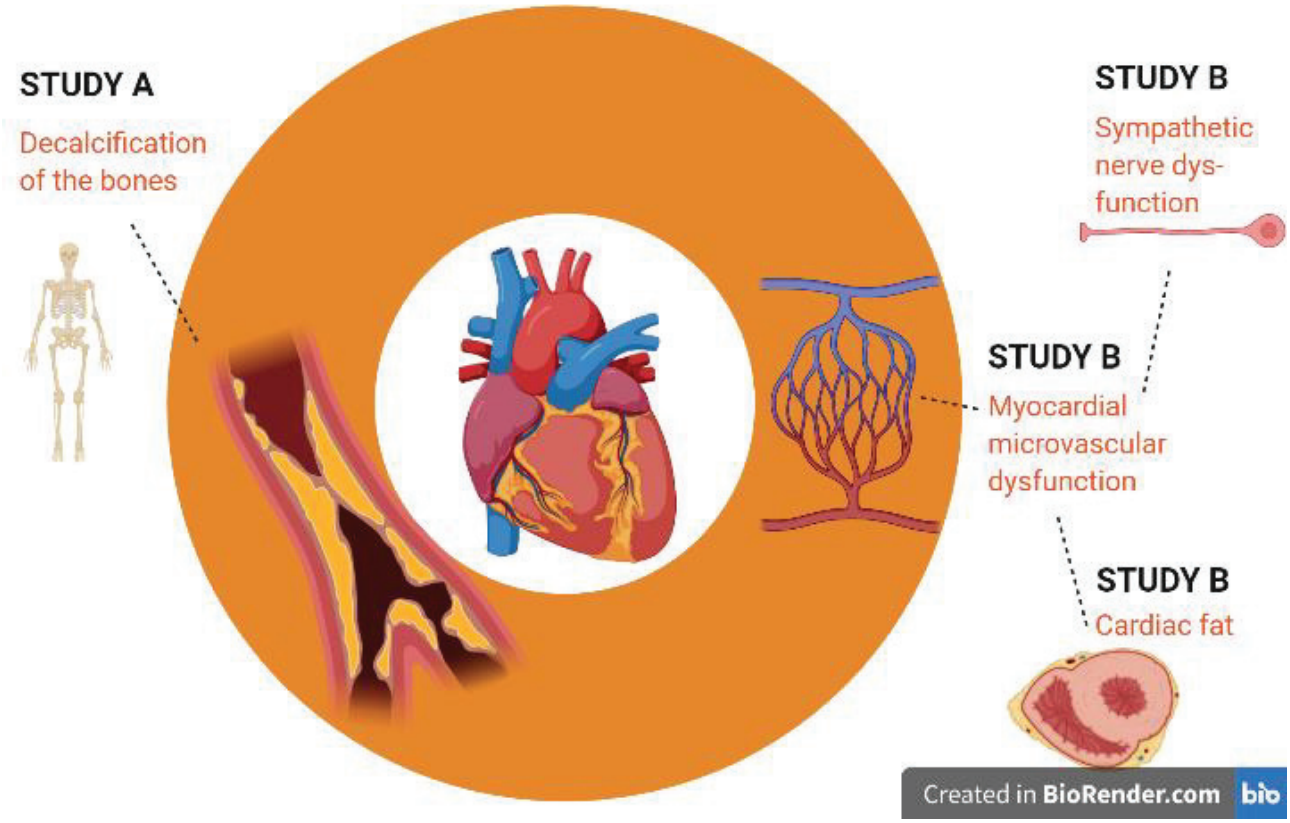
Adapted from Zobel et al., Diabetes, 2019(37).

Our group has previously assessed myocardial flow reserve in persons with type 2 diabetes and healthy controls using the same equipment and similar protocols. In an analysis including these 30 healthy controls, the 60 persons with type 2 diabetes and the 60 persons with type 1 diabetes from study B, we explored the association between cardiac fat and myocardial flow reserve (paper 4, study B, Table 1). Mean (standard deviation) cardiac adipose tissue level was comparable in the healthy controls and the persons with type 1 diabetes [99 (61) mL and 106 (78) mL] respectively, but higher in the persons with type 2 diabetes [228 (97) mL], also after adjustment ($p \leq 0.02$). In the healthy controls, higher cardiac adipose tissue volume was associated with lower myocardial flow reserve and higher coronary artery calcium score ($p \leq 0.008$). Cardiac adipose tissue was not associated with myocardial flow reserve or coronary artery calcium score in persons with type 1 or type 2 diabetes ($p \geq 0.50$)(38).

Discussion

In this section the pathophysiology of cardiovascular disease was explored in type 1 diabetes using different methods to investigate the importance of calcification and myocardial microvascular dysfunction (Figure 4).

Figure 4. Pathophysiology of cardiovascular disease in type 1 diabetes



Vascular calcification is a known consequence of aging, diabetes, dyslipidaemia and kidney disease. New insights continue to emerge and deepen our understanding of the mechanisms underlying vascular calcification, and attention has turned towards the impact of osteogenic regulation on vascular calcification and large arterial stiffening(3, 49). In a cross-sectional study in type 1 diabetes (paper 1, study A), we observed an independent relationship between higher PTH and stiffening of the large arteries. PTH is a regulator of calcium homeostasis and secretion of PTH from the parathyroid gland, triggered by low serum calcium, results in calcium release from the bones, reduced calcium excretion by the kidneys and increased calcium absorption by the small intestine(50). Aside from its well-established role in calcium homeostasis, PTH excess might be related to development of cardiovascular disease. A meta-analysis from 2013 including 12 observational studies showed that PTH excess (quartile 4) was associated with an 1.45-fold increased (95% CI: 1.24-1.71) incidence of cardiovascular disease compared to low levels (quartile 1)(51). The Atherosclerosis Risk in Communities (ARIC) study, including 10.392 adults followed for a median of 19 years, did not find PTH levels (across quartiles) to be associated with cardiovascular outcome(52). However, when the authors added their data to the before mentioned meta-analysis(52), the pooled hazard ratios remained statistically significant, although slightly reduced to 1.39 (95% CI: 1.17-1.64). In 6.545 persons

without cardiovascular disease participating in the multi-ethnic study of atherosclerosis, higher aortic pulse pressure (a surrogate measure of arterial stiffness) was significantly associated with higher PTH (p<0.001) in adjusted linear regression models, however, significance was lost after further adjustment for mean arterial pressure(53). We assessed arterial stiffness as carotid-femoral pulse wave velocity which serves as the reference standard technique to quantify arterial stiffness(54). Paper I highlight PTH as a promising petitioner in the cross-talk between bone and vascular disease, and the results suggests that a possible link between PTH and cardiovascular disease is an adverse effect of PTH on arterial function. Study A was cross-sectional and not able to evaluate causal relationship or longitudinal changes. The relationship between PTH and cardiovascular outcome in type 1 diabetes should be evaluated in future longitudinal studies, to further clarify the role of PTH in cardiovascular disease in diabetes.

From bone-markers to study B, where we investigated myocardial microvascular dysfunction in type 1 diabetes in relation to kidney function. In a cross-sectional study including persons with type 1 diabetes and sex and age matched healthy controls (paper 2, study B) we observed presence of myocardial microvascular injury in persons with type 1 diabetes and macroalbuminuria, that was not seen in persons with type 1 diabetes and normoalbuminuria. This microvascular injury was demonstrated as a lower (impaired) myocardial flow reserve. These findings were confirmed in a separate set of analysis where we excluded participants with known coronary artery disease and/or ischemia revealed by cardiac PET/CT. Similar associations between presence of albuminuria and reduced myocardial flow reserve has previously been demonstrated in type 2 diabetes by our group(6) and others(55). That albuminuria reflects widespread vascular damage including myocardial microvascular injury in both type 1 and type 2 diabetes, is in line with the Steno hypothesis(56).

Impaired myocardial flow reserve is an independent risk factor of cardiovascular disease as demonstrated by Murthy et al. in 2783 persons (of whom 1172 had diabetes) referred for cardiac PET/CT(5), and supported by findings in a recent study in 451 persons with diabetes and 451 without diabetes, all without overt coronary artery disease(57). Few small randomized clinical trials have evaluated whether myocardial flow reserve can be improved by treatment. Neither pravastatin in type 1 diabetes(58) or pioglitazone in insulin-requiring type 2 diabetes(59) improved myocardial flow reserve as compared to placebo. Interestingly, Hesse et al. have demonstrated that acute iv infusion of an angiotensin-converting enzyme inhibitor improved the myocardial flow reserve in persons with type 2 diabetes(60), and another study demonstrated that long-term (1 year) treatment with angiotensin-converting enzyme inhibition increased the myocardial flow reserve as compared to β-blockade in persons with hypertension(61). In a small randomized study treatment with eplerenone improved myocardial flow reserve as compared to treatment

with hydrochlorothiazide in 16 persons with diabetes (mix of type 1 and 2 diabetes) and albuminuria(62). In 64 persons with type 2 diabetes on stable angiotensin converter enzyme inhibition, addition of spironolactone improved myocardial flow reserve as compared to hydrochlorothiazide or placebo(63). That renin angiotensin aldosterone blockade, known to reduce albuminuria in diabetes, also seem to positively affect the myocardial microvascular function, further strengthens the notion that microvascular damage in different vascular beds in diabetes are linked. It remains to be proven that an increase in myocardial flow reserve reduce cardiovascular events. The new nonsteroidal mineralocorticoid receptor antagonist (MRA) finerenone has recently demonstrated in the first MRA kidney outcome study a reduced progression of kidney disease as well as less cardiovascular disease(64).

In the same cross-sectional study as mentioned above (paper 2, study B), we observed that coronary artery calcium score is higher in persons with type 1 diabetes than in healthy controls, in line with the literature(65). Coronary artery calcium score is a well-established, non-invasive measure of the atherosclerotic burden. Our results further indicate a higher coronary artery calcium score in persons with type 1 diabetes and macroalbuminuria compared to normoalbuminuria, but due to the highly skewed distribution of the coronary artery calcium score, we had limited power to detect a difference between the groups.

In the same cross-sectional study in type 1 diabetes we further explored the possible association between sympathetic dysfunction and alterations in myocardial microvascular function and perfusion (paper 3, study B). The pathophysiology linking cardiac autonomic neuropathy and cardiovascular disease in type 1 diabetes has been highlighted as an area in which more research is needed(2). Cardiac ^{123}I-MIBG scintigraphy enable direct characterization of cardiac sympathetic integrity, using a radiolabeled-norepinephrine analogue that shares uptake mechanisms with norepinephrine(66). In paper 3, we demonstrated that an impaired cardiac autonomic function correlated with reduced myocardial flow reserve. This association was strongest when cardiac autonomic function was evaluated as late heart-to-mediastinum ratio (from the cardiac ^{123}I-MIBG scintigraphy) and the 30-to-15 ratio (a cardiovascular autonomic reflex test, predominantly reflecting parasympathetic tonus(67)). When evaluating the association between cardiac autonomic function and myocardial flow reserve, presence of ischemic coronary heart disease is a confounder which potentially can affect both measures. An advantage of using cardiac PET/CT is the identification of ischemia, which enabled us to confirm our findings in a separate set of analyses, where we excluded participants with both known coronary artery disease and/or ischemia identified by the cardiac PET/CT. This indicates that impaired cardiac autonomic function is linked to impaired stimulated blood flow, also when the influence of coronary artery disease is eliminated. Very few

studies have evaluated cardiac autonomic function and myocardial blood flow in type 1 diabetes(68, 69). In type 2 diabetes, our group has previously demonstrated similar associations between myocardial flow reserve and the late heart-to-mediastinum ratio(70), indicating a link in both type 1 and type 2 diabetes between sympathetic dysfunction and alterations in myocardial microvascular function and perfusion. It has been hypothesized that sympathetic dysfunction through decreased endothelial nitric oxide activity reduce the myocardial flow reserve, resulting in impairment of the vascular function of the myocardium in diabetes(71). Although our study in type 1 diabetes is large compared to previous studies on this topic, we acknowledge that the small sample size increases the risk of a type 2 error and confirmation in larger studies is warranted. The cross-sectional design prevents us for concluding about a causal relationship, and this should be explored in longitudinal studies.

From sympathetic nerve dysfunction to cardiac adipose tissue measured in the same cross-sectional study in type 1 diabetes and in a previous, similar study in type 2 diabetes. Cardiac adipose tissue has been suggested as an important link when coupling diabetes, obesity and cardiovascular disease. Traditionally focus has been on obesity as a risk factor for cardiovascular disease, but in recognition of the heterogeneity of the fat depots, focus has turned towards visceral fat and the epicardial and pericardial adipose tissue (together named total cardiac adipose tissue) surrounding the myocardium and the coronary arteries. Epicardial adipose tissue is a source of inflammatory mediators(72), the direct contact with the myocardium allows direct communication between the two, and the shared microcirculation enables vasocrine crosstalk, all of which could suggest a role for epicardial adipose tissue in cardiovascular disease pathology(73). In paper 4, we analyzed total cardiac adipose tissue in the 60 persons with type 1 diabetes from study B and in 60 persons with type 2 diabetes and 30 healthy controls from a previous study(6). The two studies were very suited for comparisons as the protocols were identical, equipment was the same, and the three groups were matched on sex and age. We showed that the volume of cardiac adipose tissue was higher in type 2 diabetes than in type 1 diabetes and in the control group, and comparable between the latter two. This is in line with the literature(74). We could not establish an association between cardiac adipose tissue and coronary artery calcium score or myocardial flow reserve in type 1 diabetes or in type 2 diabetes. In the control group cardiac adipose tissue volume was positively associated with coronary artery calcium score and negatively associated with myocardial flow reserve. The automatic quantification of cardiac adipose tissue we used has the advantage of being observer independent and ensure consistency of the volumes measured within our cohort. However, it is a limitation that we are not able to differentiate between epicardial and pericardial adipose tissue, and we cannot rule out that this can have affected our results. Most studies on cardiac fat address epicardial adipose tissue and not total cardiac adipose tissue. In other cross-sectional studies epicardial adipose tissue was not associated with coronary atherosclerosis in

type 1 diabetes(74), and in type 2 diabetes it remains uncertain if epicardial adipose tissue and coronary calcification are associated independent from traditional cardiovascular risk factors(75, 76). The few studies that have investigated the association between cardiac fat and myocardial flow reserve are in line with our divergent findings in diabetes and controls(77, 78). Taken together, these findings raise uncertainty of the functional importance of cardiac adipose tissue including epicardial adipose tissue in both type 1 and type 2 diabetes(73).

In this section the pathophysiology of cardiovascular disease was explored in type 1 diabetes (and to a lesser extent in type 2 diabetes) focusing on the importance of calcification and myocardial microvascular dysfunction (Figure 4). We found an independent relationship between higher PTH and stiffer large arteries, thus highlighting PTH as a petitioner in a possible link between decalcification of the bones and vascular calcification. However, decalcification of the bones is probably a minor contributor to vascular calcification in type 1 diabetes, but this remains to be confirmed in future studies. We also found that myocardial microvascular impairment was linked to microvascular disease in the kidneys, and that sympathetic dysfunction in type 1 diabetes was linked to impaired myocardial microvascular function, whereas amount of cardiac adipose tissue was not. Myocardial microvascular dysfunction may be an important contributor to cardiovascular disease in type 1 diabetes, but our findings needs confirmation in future studies. Ultimately follow-up studies and intervention trials are needed to fully understand the role of myocardial microvascular dysfunction in cardiovascular disease in diabetes.

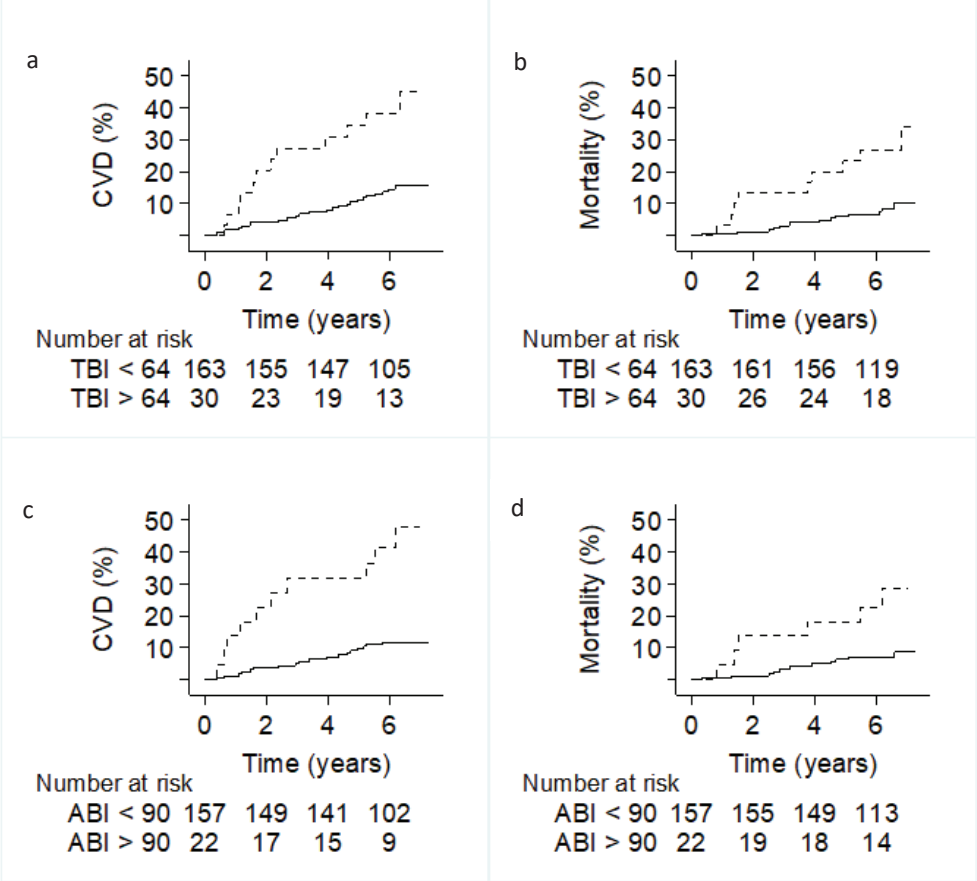
II) Identifying high-risk individuals with type 2 diabetes

In this part of the thesis, we explored two clinical measurements (toe-brachial index and ankle-brachial index) and two circulating biomarkers (SDMA and ADMA) as risk markers for cardiovascular disease in type 2 diabetes. Moreover, in a methodological study we compared two different devices for measurement of 24-hour ambulatory blood pressure, an established risk marker in type 2 diabetes.

Results

In a prospective, follow-up study (study C, Table 1), 200 persons with type 2 diabetes and microalbuminuria were followed for a median of 6.1 years in order to find markers of cardiovascular risk, and 40 cardiovascular disease events and 26 deaths were recorded. We demonstrated in paper 5 that lower toe-brachial index and ankle-brachial index was associated with higher risk of cardiovascular disease and all-cause mortality in this study also following adjustments for traditional cardiovascular risk factors (Figure 5). Moreover, lower toe-brachial index remained a significant risk factor for cardiovascular disease after additional adjustment for NT-proBNP and coronary artery calcium score. When toe-brachial index was added to traditional risk factors the area under the Reciever Operating Characteristic (ROC) curve increased significantly for cardiovascular disease. Toe-brachial index and ankle-brachial index added predictive value for both cardiovascular disease and all-cause mortality, when reclassification was evaluated using integrated discrimination improvement (IDI) statistics(39).

Figure 5. Toe-brachial index and ankle-brachial index and risk of cardiovascular disease and all-cause mortality

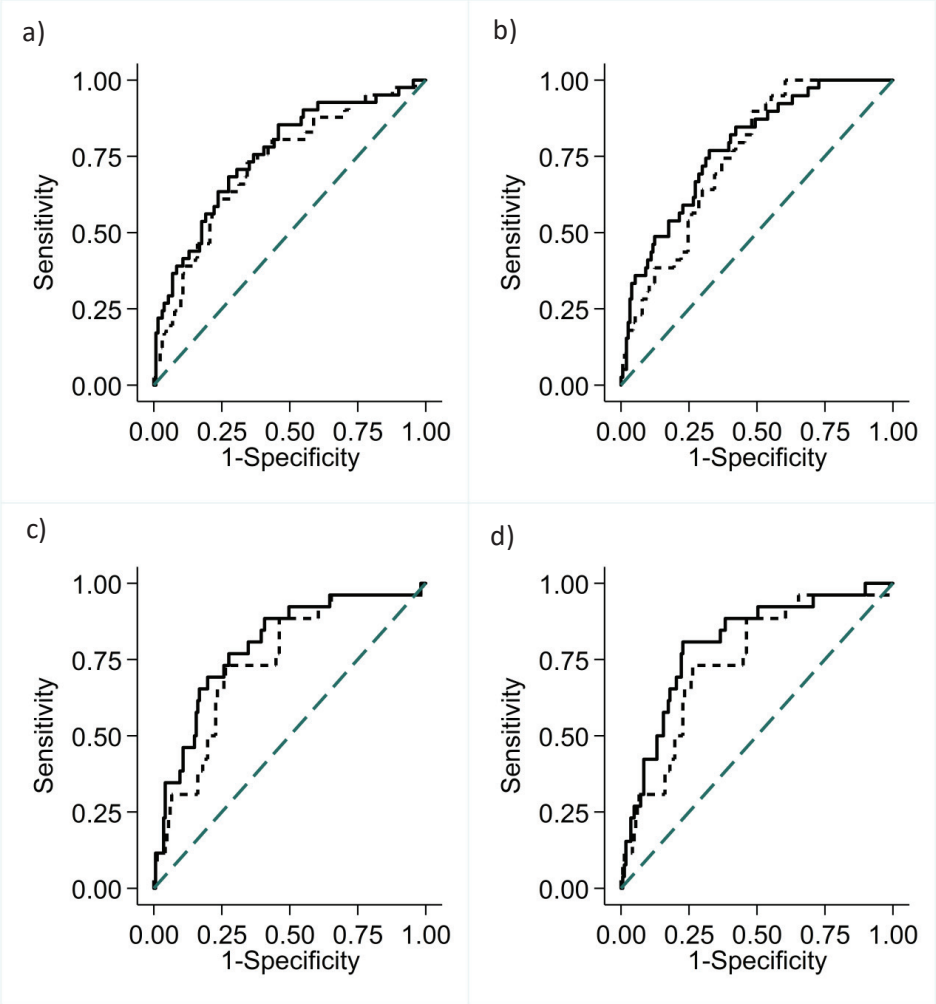


Kaplan–Meier failure function estimates. Toe-brachial index in two categories above or below cut-off of 0.64 for (a) the cardiovascular disease endpoint (hazard ratio 3.4 (95% CI 1.7, 6.9), p<0.0001) and (b) all-cause mortality (3.8 (1.6, 8.8), p=0.002); ankle-brachial index in two categories above or below cut-off of 0.9 for (c) the cardiovascular disease endpoint (4.9 (2.2, 10.5), p<0.0001) and (d) all-cause mortality (3.9 (1.5, 10.3), p=0.007). Numbers refer to participants at risk in each category at the beginning of each 2 year interval. Dashed line, below cut-off; solid line, above cut-off. ABI: ankle-brachial index. CVD: cardiovascular disease. TBI: toe-brachial index. From Zobel et al, Diabetologia, 2017(39).

In paper 6 we tested two circulating biomarkers in the same cohort and demonstrated that higher levels of SDMA was a risk factor for cardiovascular disease, all-cause mortality and decline in renal function, also following adjustments for traditional cardiovascular risk factors. Higher levels of ADMA was associated with increased risk of all-cause mortality in unadjusted and adjusted analysis. When SDMA or ADMA were added to traditional risk factors the area under the ROC curve did not increase significantly for any endpoints

(Figure 6). Using IDI statistics, SDMA and ADMA added predictive value for all-cause mortality, and SDMA for decline in renal function(40).

Figure 6. SDMA and ADMA receiver operating characteristic (ROC) curves

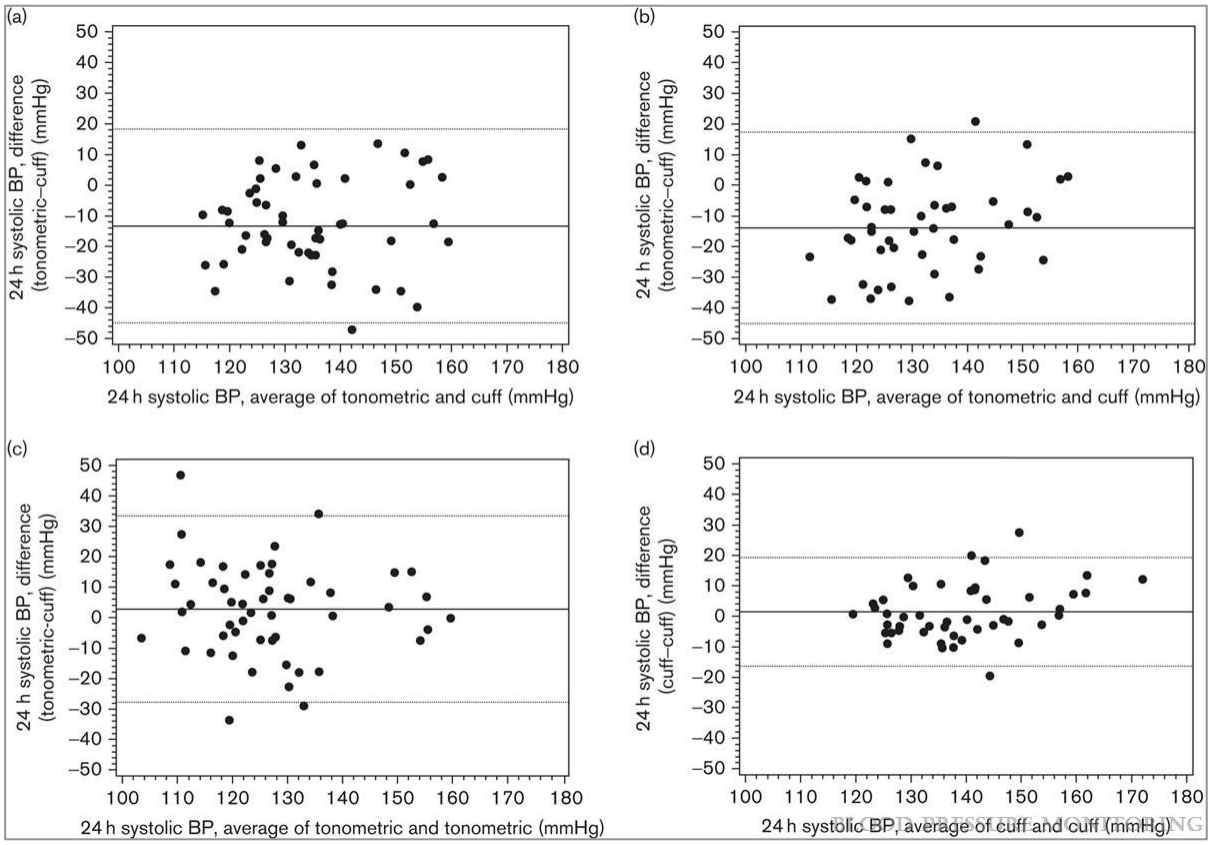


	AUC (95% CI)	p-value
Panel a: cardiovascular disease endpoint		
Base model	0.745 (0.668, 0.822)	0.24
Base model + symmetric dimethylarginine	0.768 (0.686, 0.850)	
Panel b: all-cause mortality		
Base model	0.743 (0.644, 0.843)	0.10
Base model + symmetric dimethylarginine	0.803 (0.713, 0.893)	
Panel c: deterioration in kidney function		
Base model	0.722 (0.631, 0.813)	0.16
Base model + symmetric dimethylarginine	0.752 (0.664, 0.840)	
Panel d: all-cause mortality		
Base model	0.743 (0.644, 0.843)	0.11
Base model + asymmetric dimethylarginine	0.794 (0.699, 0.889)	

Included in the base model: sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, creatinine and urinary albumin excretion rate. Dashed line: reference; Dotted line: Base model; Full line: Base model + symmetric dimethylarginine/asymmetric dimethylarginine. From Zobel et al., Cardiovascular Diabetology, 2017(40).

In a methodological study (study D, Table 1) we compared two devices that measure 24-hour ambulatory blood pressure, an already established risk marker in type 2 diabetes, with the aim to explore whether cuff-inflations affect the blood pressure measurements and to compare the precision of the two devices. We compared 24-hour and nighttime blood pressure measured by a cuff-less tonometric device (BPro, HealthStats, Singapore) and a standard upper-arm cuff-device (Takeda, TM2430, Japan) in 53 participants with type 2 diabetes. In paper 7 we report that mean 24-hour systolic blood pressure was significantly higher measured with the cuff *vs* tonometric device (mean+SD: 142±15 *vs* 128±15 mmHg), as was mean nighttime systolic blood pressure (130±17 *vs* 123±16 mmHg) and nocturnal systolic blood pressure decline was significantly lower measured with the tonometric *vs* cuff device (6.7±5.3 *vs* 10.3±7.6 %), $p \leq 0.01$ for all three comparisons (Figure 7). We further assessed the reproducibility of both devices for two independent recordings and found no significant difference in 24-hour or nighttime systolic blood pressure between the two tonometric recordings ($p \geq 0.19$) or the two cuff recordings ($p \geq 0.09$). Participants rated the cuff device more uncomfortable than the tonometric device, both during day and nighttime. However, accelerometer assessed sleep quality did not differ between nights wearing the two different devices, and nocturnal systolic blood pressure decline was not associated with the subjectively evaluated influence of the device on sleep quality(41).

Figure 7. Comparison of 24-hour systolic blood pressure measured with a cuff and a tonometric device

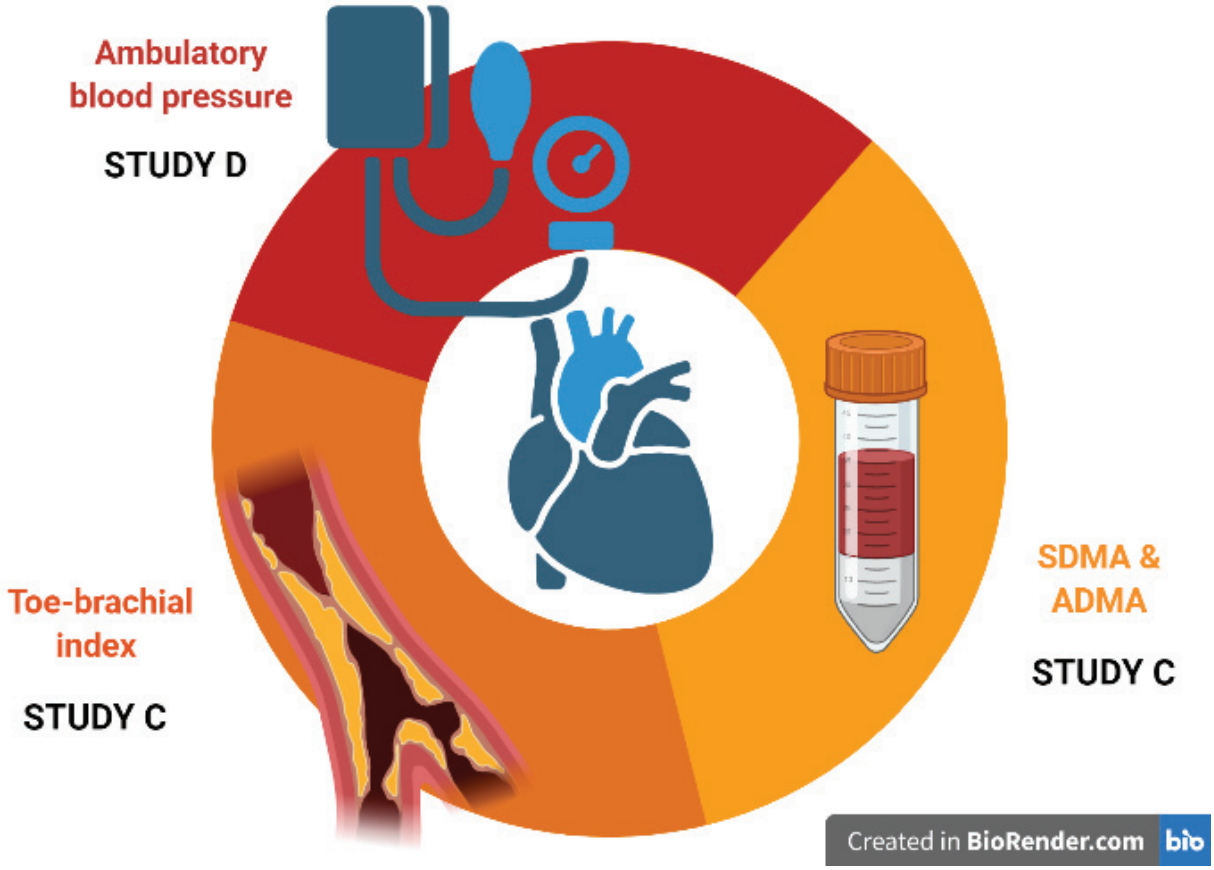


Bland–Altman plots of 24-hour systolic blood pressure comparing (a) tonometric vs cuff device at two different visits (b) tonometric vs cuff device at the same visit – on different arms (c) tonometric device first versus second recording (d) cuff device first versus second recording. From Petersen et al., Blood Pressure Monitoring, 2015(41).

Discussion

Identifying high-risk individuals with type 2 diabetes is an important component of preventing cardiovascular complications (Figure 8). Biomarkers, ranging from single biomarkers to omics, can help improve cardiovascular risk prediction in diabetes and help tailor treatment to the underlying pathophysiology. Moreover, biomarkers related to different pathways may be additive or supplementary.

Figure 8. Cardiovascular risk prediction in type 2 diabetes.



Measurement of the toe-brachial index is a diagnostic test for peripheral arterial disease and a prognostic test for healing of foot ulcers(79, 80). Results from paper 5 provide support for the use of toe-brachial index as a tool to refine the risk prediction of cardiovascular disease in persons with type 2 diabetes and microalbuminuria, without known coronary artery disease. Moreover, we demonstrated correlations between toe-brachial index, carotid intima-media thickness and coronary artery calcium score, which illustrates that atherosclerosis is a universal disease affecting multiple arterial beds, and that toe-brachial index is not just a measure of peripheral disease. That lower toe-brachial index could predict cardiovascular disease in addition to the risk information provided by traditional cardiovascular risk factors, and by both NT-ProBNP and coronary artery calcium score, highlights the strength of toe-brachial index in risk prediction. Our results are in line with findings in populations with type 2 diabetes and cardiovascular disease(8, 81) or with clinically suspected atherosclerotic peripheral arterial disease(82). In contrast to atherosclerosis measured in the carotid or coronary arteries, toe-brachial index has the advantage of being a simple test that easily can be performed in most clinical settings. Toe-brachial index is to be preferred

over ankle-brachial index, because the ankle-brachial index can be falsely elevated in diabetes due to medial calcification(83, 84). The arteries in the toe are less susceptible to medial calcification(83).

Compared to the toe-brachial index, the two circulating biomarkers SDMA and ADMA are markers of a different aspect of the pathophysiology underlying vascular disease in diabetes. ADMA is an endogenous inhibitor of the nitric oxide production(85) and SDMA is a competitive inhibitor of the substrate L-arginine for nitic oxide synthase(86, 87). Thus, ADMA and SDMA affect the endothelial function, and increased levels may lead to vascular disease. Results from paper 6, support the use of SDMA to improve risk prediction for cardiovascular disease, all-cause mortality and renal function decline. In contrast to the toe-brachial index that increased the area under the ROC curve when added to traditional risk factors, SDMA and ADMA was not able to improve risk prediction when applying c-statistics to compare the area-under-the ROC curve for a model including traditional cardiovascular risk factors *vs* a model including traditional cardiovascular risk factors plus SDMA or ADMA. Sole reliance on c-statistics to evaluate novel biomarkers has been disputed(88), and IDI statistics has been suggested by Pencina et al. as a more powerful method to evaluate a new biomarker's diagnostic performance(89). Using IDI statistics, we demonstrated added predictive value for all-cause mortality for SDMA and ADMA, and for renal function decline for SDMA. Both toe-brachial index and ankle-brachial index added predictive value for cardiovascular disease and all-cause mortality, when evaluated using IDI statistics.

A challenge for all new biomarkers is to reach implementation into the clinic, where the traditional risk factors including sex, age, diabetes duration, smoking, blood pressure, lipids, urinary albumin excretion and measures of kidney function have been dominating for risk prediction in decades. The slowness of introduction of new biomarkers into the clinic is probably both a result of cost, but also the unavoidable question about the therapeutic consequences of identifying high risk individuals. Screening of asymptomatic persons with type 2 diabetes at high risk of atherosclerotic cardiovascular disease is not recommended in the most recent ADA standards of medical care in diabetes(90), in part because intensive medical treatment should already be prescribed to this group if indicated by traditional risk factors. Over the recent years, many new treatments options have become available for cardio-renal complications in diabetes, including Sodium-Glucose Cotransporter-2 (SGLT2) inhibitors(91), GLP-1 RAs(19), PCSK9-inhibitors(92) and non-steroidal mineralocorticoid receptor antagonists(64). Combining imaging and biomarker-based risk prediction open new possibilities for identifying high-risk individuals and for guiding selection of a treatment option including the above-mentioned treatments using a precision medicine approach. Such exploration of an individualized treatment approach is expected to be the new frontier in diabetes research(93), and may bring biomarkers from different pathways into play.

In contrast to toe-brachial-index and the circulating biomarkers SDMA and ADMA, 24-hour ambulatory blood pressure measurement is a clinical risk marker already recommended in guidelines and applied in daily clinic(94). Discomfort related to cuff inflation has created a need for alternative methods for measuring 24-hour ambulatory blood pressure and we therefore compared a traditional cuff-based device to a tonometric device and evaluated both discomfort and precision. We demonstrated (paper 7), that participants both during daytime and night-time rated the cuff device significantly more uncomfortable than the tonometric device. Surprisingly, there was no association between nocturnal systolic blood pressure decline and subjectively evaluated device discomfort or objectively evaluated sleep quality. Despite participants being subjectively disturbed by the cuff, nocturnal systolic blood pressure decline was higher measured with the cuff *vs* the tonometric device. This concurs with a study by Trapeano et al. where objective sleep-quality on the day of 24-hour ambulatory blood pressure monitoring was similar to the sleep-quality the following 3 days without blood pressure monitoring, although subjective sleep-quality differed(95). Bridgen et al. conducted 24-hour intra-arterial blood pressure monitoring, followed by 24-hour combined intra-arterial and arm cuff blood pressure monitoring in 13 hospitalized patients, demonstrating that cuff inflations did not influence nighttime blood pressure(96). In our study, the cuff device measured systematically higher 24-hours and nighttime systolic blood pressures than the tonometric device. The difference was not explained by variance in objective or subjective sleep quality. We speculate that the tonometric device selectively fails to measure high blood pressure values associated with movement. Our study design does not allow us to determine which of the two devices that measure the “true” blood pressure. This can only be achieved by simultaneous measurement with both devices and synchronized intra-arterial blood pressure recording (the “gold standard”). To the best of our knowledge, the tonometric BPro device has not been tested against intra-arterial brachial blood pressure monitoring while oscillometric devices have(97). The tonometric device has been validated for ambulatory use in non-diabetic individuals according to the modified European Society of hypertension (ESH) protocol and Advancement of Medical instrumentation (AAMI) standard(98), but has not been validated according to current protocols and is not recommended by the DABL Educational trust(99). Others have reported disagreement between 24-hour ambulatory blood pressure measurement using the tonometric BPro device and an oscillometric cuff device(100, 101), including a recent study in 100 hypertensive persons from a renal outpatient clinic. In this study the tonometric BPro device measured lower 24-hour and daytime systolic blood pressure compared to an oscillometric device (129±19 *vs* 136±15 and 133±20 *vs* 139±14 mmHg), and comparable nighttime systolic blood pressures (100). The authors concluded that 49% of the participants would be classified as normotensive using the tonometric device, and uncontrolled hypertensive using the oscillometric device, and that 53% of the participants classified as nondippers

(defined as a relative nocturnal systolic BP decrease ≤10%) using the tonometric measurements were true nondippers, 47% were dippers(100).

24-hour ambulatory blood pressure measurement is an important tool for better cardiovascular risk stratification. In diabetes, 24-hour ambulatory blood pressure has been demonstrated to correlate better with organ damage(102, 103) and risk of cardiovascular disease(15, 104) than blood pressure measured in the office. Compared to office blood pressure, 24-hour ambulatory blood pressure better quantify the blood pressure in daily life. Altered 24-hour blood pressure profiles, including lack of nocturnal blood pressure decline, or increased short term blood pressure variability can be detected by a 24-hour ambulatory blood pressure measurement. Lack of nocturnal blood pressure decline is common in diabetes (prevalence of up to 30%) and particularly in the presence of concomitant nephropathy or autonomic neuropathy (prevalence of up to 80%)(105-109), and is considered a cause of renal damage(110) and associated with increased mortality(111, 112).

In this section, new and established risk markers for cardiovascular disease in diabetes were explored. Lower toe-brachial index could predict cardiovascular disease in type 2 diabetes in addition to the risk information provided by traditional risk factors, and by both NT-proBNP and coronary artery calcium score. We explored toe-brachial index as a risk predictor as it is an easily accessible measure of systemic atherosclerosis, that most often is evaluated using imaging. We argue based on the observed strength of toe-brachial index in risk prediction and the fact that it can be assessed in most clinical settings that it should be implemented into the clinic as a risk measure of cardiovascular disease in type 2 diabetes. SDMA and ADMA are two interesting circulating biomarkers that are available for cardiovascular risk prediction in type 2 diabetes, however competing with many other candidates, and in contrast to the toe-brachial index, SDMA and ADMA were not able to improve risk prediction when applying c-statistics. We therefore consider that more studies are needed if SDMA and ADMA should be implemented as biomarkers of cardiovascular risk to candidate implementation in the clinic. Finally, 24-hour ambulatory blood pressure measurement is a clinical risk marker recommended in guidelines, however discomfort related to the cuff-inflations may affect its application in clinic practice. We demonstrated in a methodological study that a traditional cuff device was significantly more uncomfortable than a tonometric device, but the cuff-inflations did not affect the blood pressure measurements. The tonometric device was less precise and cannot be recommended for clinical use. Going forward there is an unmet need for more comfortable solutions for 24-hour ambulatory blood pressure measurements, without compromising the precision.

III) Understanding the clinical effects and the mode-of-action of GLP-1 RAs

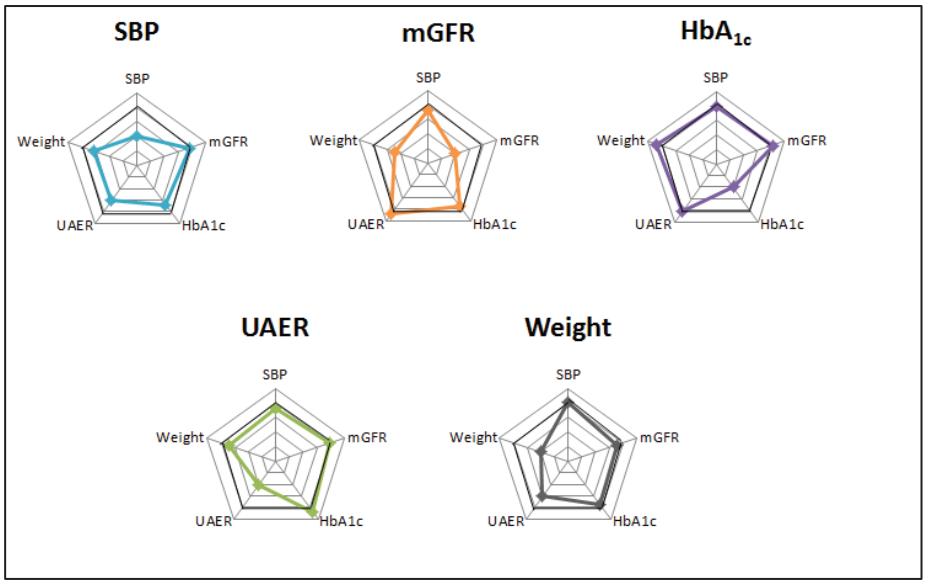
This part of the thesis aimed to understand the clinical effects and the mode-of-action of GLP-1 RAs. We therefore explored the effect of GLP-1 RAs on 1) clinical cardiovascular risk factors; 2) arterial inflammation assessed as [^{18}F]FDG uptake and [^{64}Cu]DOTATATE uptake, a new tracer added due to discussions of specificity; and 3) the lipidome.

Results

In post-hoc analyses of a small open-label study (paper 8, study E, Table 1) and of a larger randomized clinical trial (paper 9, study F, Table 1), we explored liraglutides effect on cardio-renal risk factors including HbA_{1c}, body weight, systolic blood pressure, LDL-cholesterol, urinary albumin excretion rate and measured/estimated GFR on an individual patient level. A good responder was defined as an individual with a change from baseline to week 7 (study E) / week 26 (study F) within the best quartile for the respective cardio-renal risk factor.

The post-hoc analysis of the open-label study, including 31 individuals with type 2 diabetes, revealed no cross-dependency in the response in the risk factors after 7 weeks of treatment with liraglutide. On a patient level, a good treatment response in one risk factor was not linked to a good response in the other risk factors (Figure 9)(42).

Figure 9. Cross-dependency in risk factor response

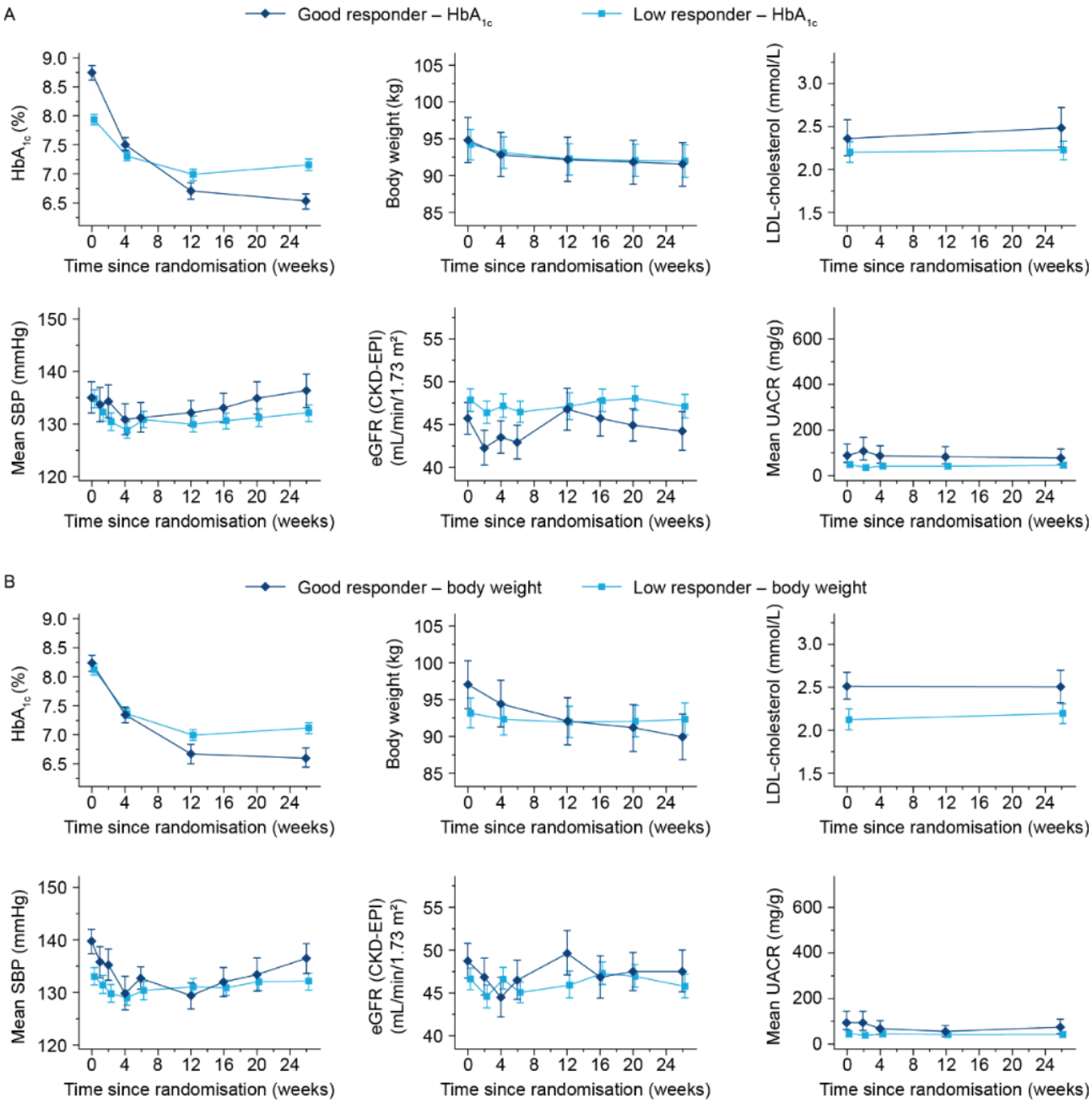


The radar-charts reveal how a good responder responds in the other risk factors compared to a low responder. The marked line represents no difference between the two responder groups. A point close to the center equals a larger reduction in the risk factor for a high compared to a low responder, and a point outside the marked line an increase / lesser reduction.

From Zobel et al, Journal of Diabetes and Its Complications, 2017(42).

In the post-hoc analysis of the randomized clinical trial we pursued the same hypothesis in an analysis of 109 individuals with type 2 diabetes and chronic kidney disease treated with liraglutide for 26 weeks. Good responders in HbA_{1c} (reduction≥1.7%) had similar changes in the other risk factors compared to low responders (p≥0.17, Figure 10A). Good body weight responders (reduction≥4.6kg) had a significantly larger response in HbA_{1c} than low responders (mean±SD: -1.6%±0.94 *vs* -1.0±0.82%; p=0.003), whereas response in the other risk factors were similar between the two responder groups (p≥0.12, Figure 10B). Good responders in systolic blood pressure (reduction≥10 mmHg), urinary albumin creatinine rate (≤54% of baseline value), LDL-cholesterol (≤85% of baseline value) and estimated GFR (≥107% of baseline value) had a response in the other risk factors similar to the low responders (p≥0.07)(43).

Figure 10. Cross-dependency in risk factor response to liraglutide treatment

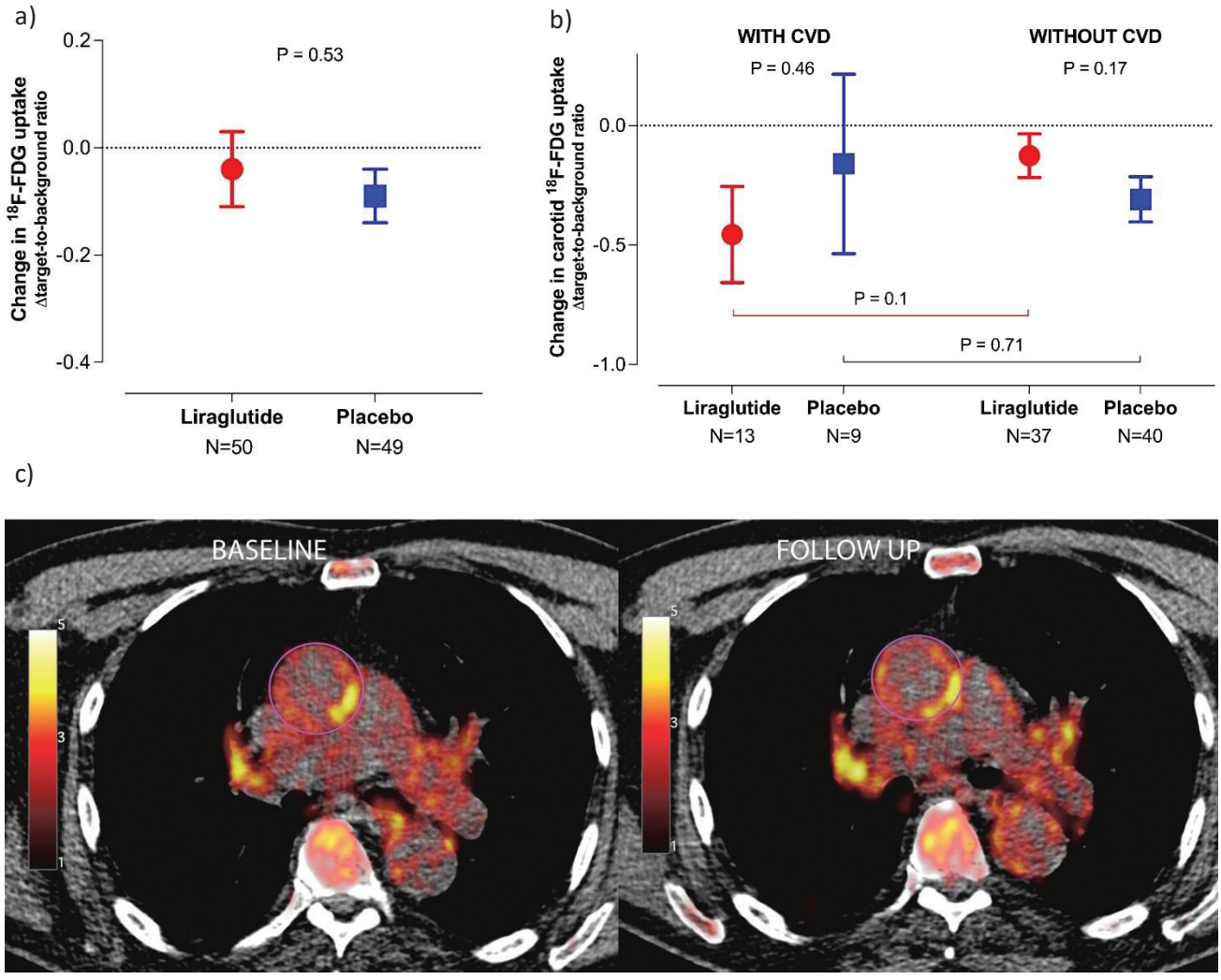


Changes in other risk factors for A, Good vs low HbA_{1c} responders. B, Good vs low body weight responders. A liraglutide treated participant with a change from baseline to week 26 within the best quartile was defined as a good responder. Observed mean ± standard error. From Zobel et al., Diabetes Obesity and Metabolism, 2019(43).

From clinical effects of GLP-1 to a randomized double-blinded placebo controlled clinical trial with the aim of understanding the mode-of-action of GLP-1 RAs (study G, Table 1). In a randomized trial including 102 participants with type 2 diabetes, 26-weeks of liraglutide treatment did not reduce the [^{18}F]FDG-PET

measure of vascular inflammation (active-segment target-to-background ratio) compared to placebo (p=0.53, Figure 11a, paper 10). Participants with a history of cardiovascular disease (n=23) had a 10.4% higher carotid [^{18}F]FDG uptake at baseline than the participants without cardiovascular disease (n=79) (p=0.16). We compared change in carotid [^{18}F]FDG uptake in the participants with and without a history of cardiovascular disease and observed a borderline significant interaction (p=0.052) between treatment group and history of cardiovascular disease. In analyses only including the participants with a history of cardiovascular disease, we observed a significant reduction in [^{18}F]FDG uptake for the participants treated with liraglutide (p=0.04, n=13), but not for the participants treated with placebo (p=0.68, n=9), but the difference between the two groups did not reach statistical significance (p=0.46, Figure 11b)(44).

Figure 11. Arterial inflammation assessed using [^{18}F]FDG-PET



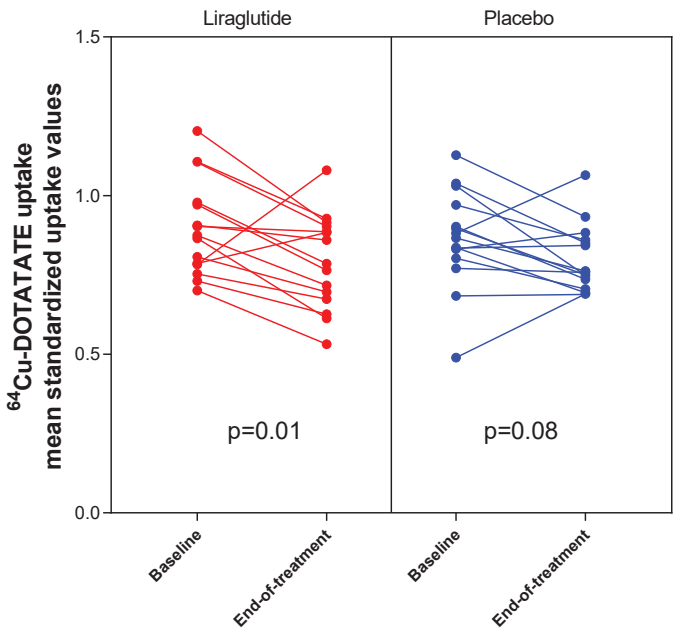
Mean change from baseline to end-of-treatment in a) active segments target-to-background ratio for the liraglutide and the placebo treated groups (primary endpoint). b) Most diseased segment target-to-

background ratio for the liraglutide and the placebo treated groups in sub-analyses of persons with or without cardiovascular disease (CVD) at baseline. The plots represent mean with standard error, unpaired t-test was used for comparison of the change from baseline to end-of-treatment between the groups. c) Representative [^{18}F]FDG-PET/CT images at baseline and at end-of-treatment from a participant treated with liraglutide. From Zobel et al., Circulation: Cardiovascular Imaging, 2021(44).

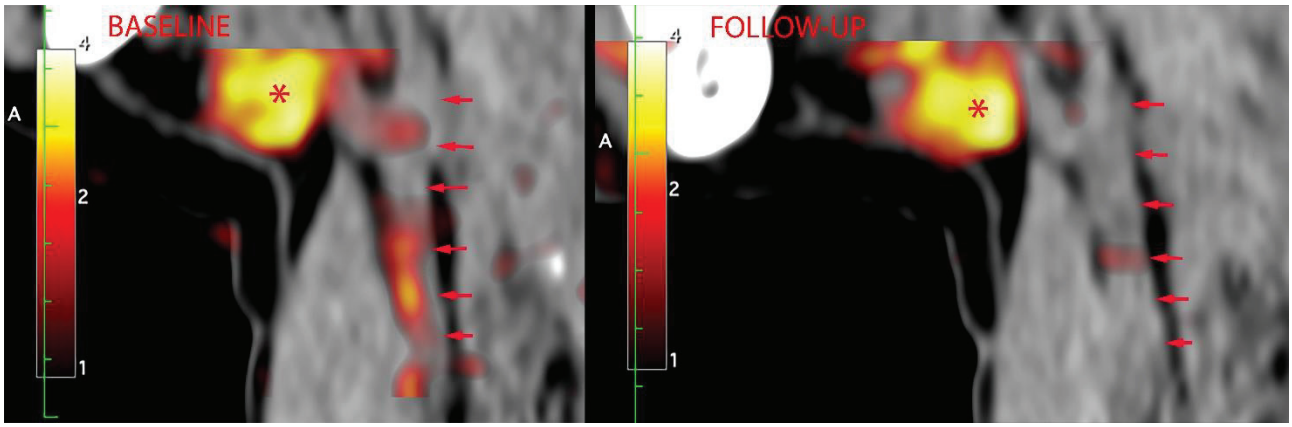
[^{18}F]FDG is a glucose based PET tracer, and to avoid that blood sugar affected uptake, we conducted a substudy in 30 participants included in study G, where we assessed liraglutides effect on vascular inflammation using [^{64}Cu]DOTATATE uptake (paper 11). We observed that the [^{64}Cu]DOTATATE uptake (mean standardized uptake values) was reduced in the liraglutide treated group (n=15, p=0.01) and unchanged in the placebo group (n=15, p=0.08, Figure 12). However, the mean difference between treatment groups did not reach statistically significance (p=0.44)(45).

Figure 12. Arterial inflammation assessed using [^{64}Cu]DOTATATE uptake

a)



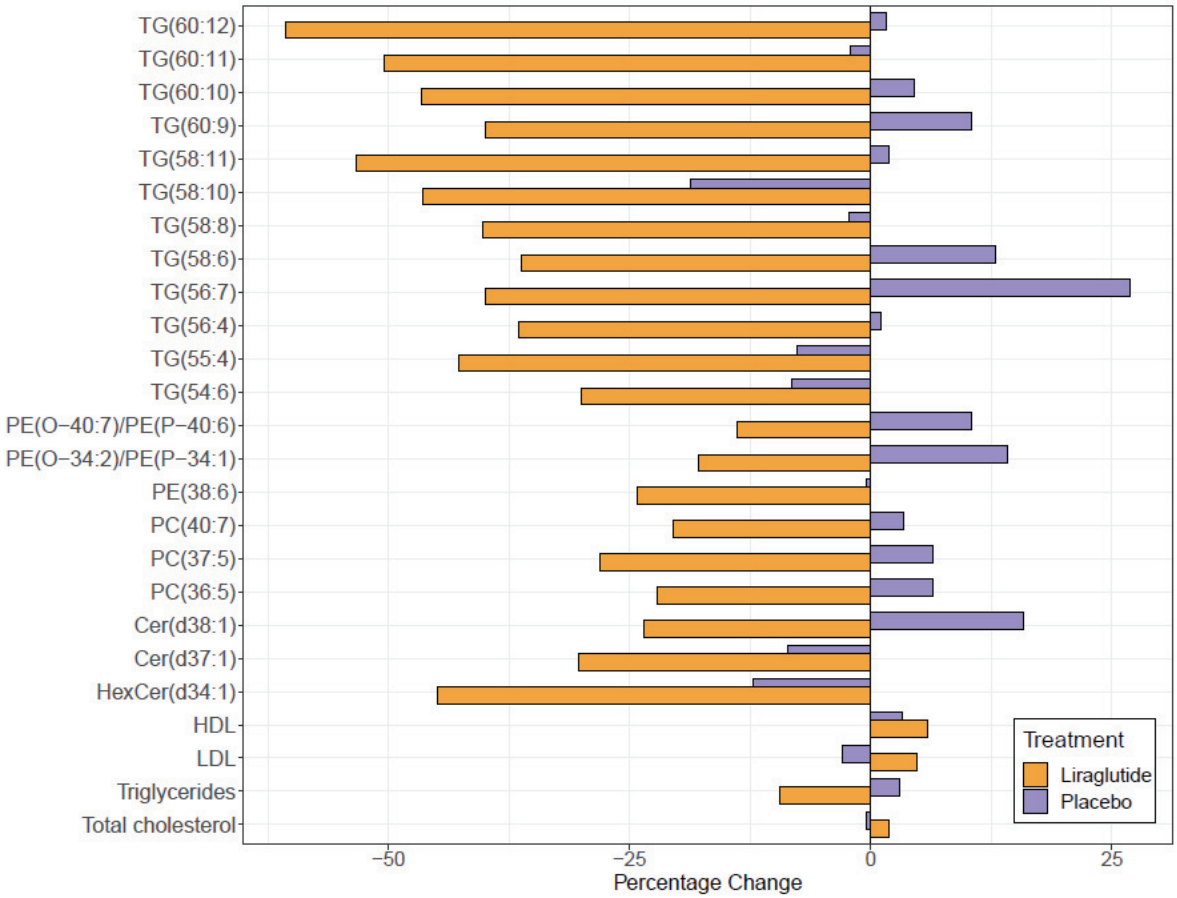
b)



*a) [^{64}Cu]DOTATATE uptake evaluated as mean standardized uptake values at baseline and end-of-treatment for the individual participants. Paired t-test was used for comparisons. b) Representative saggital [^{64}Cu]DOTATATE-PET/CT images of the left carotid artery (marked with arrows) at baseline (SUV_{mean}=1.2) and at end-of-treatment (SUV_{mean}=0.92) from a participant treated with liraglutide. *Physiological uptake in a submandibular gland. From Zobel et al., Diagnostics, 2021(45).*

In the same randomized trial, we assessed liraglutides effect on the lipidome with the aim of identifying possible down-stream effects of liraglutide on atherogenic lipids (paper 12, study G, Table 1). We measured 260 lipids covering 11 lipid families before and after treatment with liraglutide in study G. We observed that liraglutide compared to placebo significantly reduced the level of 21 individual lipids from the following lipid families: ceramides, hexocyl-ceramides, phosphatidylcholines, phosphatidylethanolamines and triglycerides (Figure 13)(46).

Figure 13. Percentage change in average amount of individual lipids and traditional clinical lipids following 26 weeks treatment with liraglutide or placebo



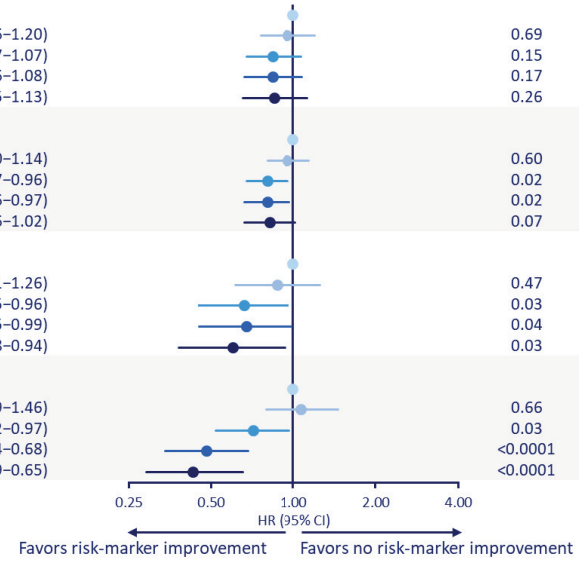
Percentage change in mean lipid amount after 26 weeks liraglutide/placebo treatment. The plot presents the 21 individual lipids significantly reduced by liraglutide compared to placebo and four traditional clinical lipid measurements (not significantly reduced by liraglutide compared to placebo treatment), including HDL-cholesterol, LDL-cholesterol, triglycerides and total-cholesterol. Cer=Ceramides, HexCer=Hexocyl-ceramides, PC=Phosphatidylcholines, PE=Phosphatidylethanolamines, TG=Triglycerides. From Zobel et. al, BMJ Open Diabetes Research and Care, 2021(46).

Lastly, we explored if multiple risk marker response affects outcome in type 2 diabetes. In a post-hoc analysis of the cardiovascular outcome trials LEADER (n=8638; median follow-up 3.8 years) and SUSTAIN 6 (n=3040; median follow-up 2.1 years), we investigated to what extent multiple risk marker improvement conferred higher risk reduction than none or one risk marker improvement (paper 13, study H and I, Table 1). The liraglutide/semaglutide- and placebo-treated groups were pooled for analysis and categorized by number of risk markers with a clinically relevant improvement after 1 year of study participation. We

demonstrated that in persons with type 2 diabetes, improvements in ≥ 2 risk markers conferred higher cardiovascular risk reduction compared to none or 1 improved risk marker (Figure 14). Moreover, the risk of nephropathy was lowered stepwise with the numbers of improved risk markers(47).

Figure 14. Outcomes according to number of risk marker improvements* among persons with type 2 diabetes

	Number of people with event/subgroup total (%)	IR	HR (95% CI)†	*P*-value	*P*-value$_{\text{test for trend}}$‡
MACE					
G0 (reference)	104/1055 (9.9)	2.9	1		
G1	305/3162 (9.6)	2.9	0.95 (0.76–1.20)	0.69	
G2	336/3540 (9.5)	2.8	0.84 (0.67–1.07)	0.15	0.08
G3	239/2515 (9.5)	2.8	0.84 (0.66–1.08)	0.17	
G4	127/1406 (9.0)	2.8	0.85 (0.65–1.13)	0.26	
Expanded MACE					
G0 (reference)	171/1055 (16.2)	4.8	1		
G1	511/3162 (16.2)	4.8	0.95 (0.80–1.14)	0.60	
G2	525/3540 (14.8)	4.4	0.80 (0.67–0.96)	0.02	0.004
G3	365/2515 (14.5)	4.3	0.80 (0.66–0.97)	0.02	
G4	199/1406 (14.2)	4.4	0.82 (0.66–1.02)	0.07	
CV death					
G0 (reference)	40/1055 (3.8)	1.1	1		
G1	119/3162 (3.8)	1.1	0.87 (0.61–1.26)	0.47	
G2	118/3540 (3.3)	1.0	0.66 (0.45–0.96)	0.03	0.005
G3	88/2515 (3.5)	1.0	0.67 (0.45–0.99)	0.04	
G4	41/1406 (2.9)	0.9	0.60 (0.38–0.94)	0.03	
Nephropathy					
G0 (reference)	60/1055 (5.7)	1.7	1		
G1	213/3162 (6.7)	2.0	1.07 (0.79–1.46)	0.66	
G2	193/3540 (5.5)	1.6	0.71 (0.52–0.97)	0.03	<0.0001
G3	101/2515 (4.0)	1.2	0.48 (0.34–0.68)	<0.0001	
G4	54/1406 (3.8)	1.2	0.43 (0.29–0.65)	<0.0001	

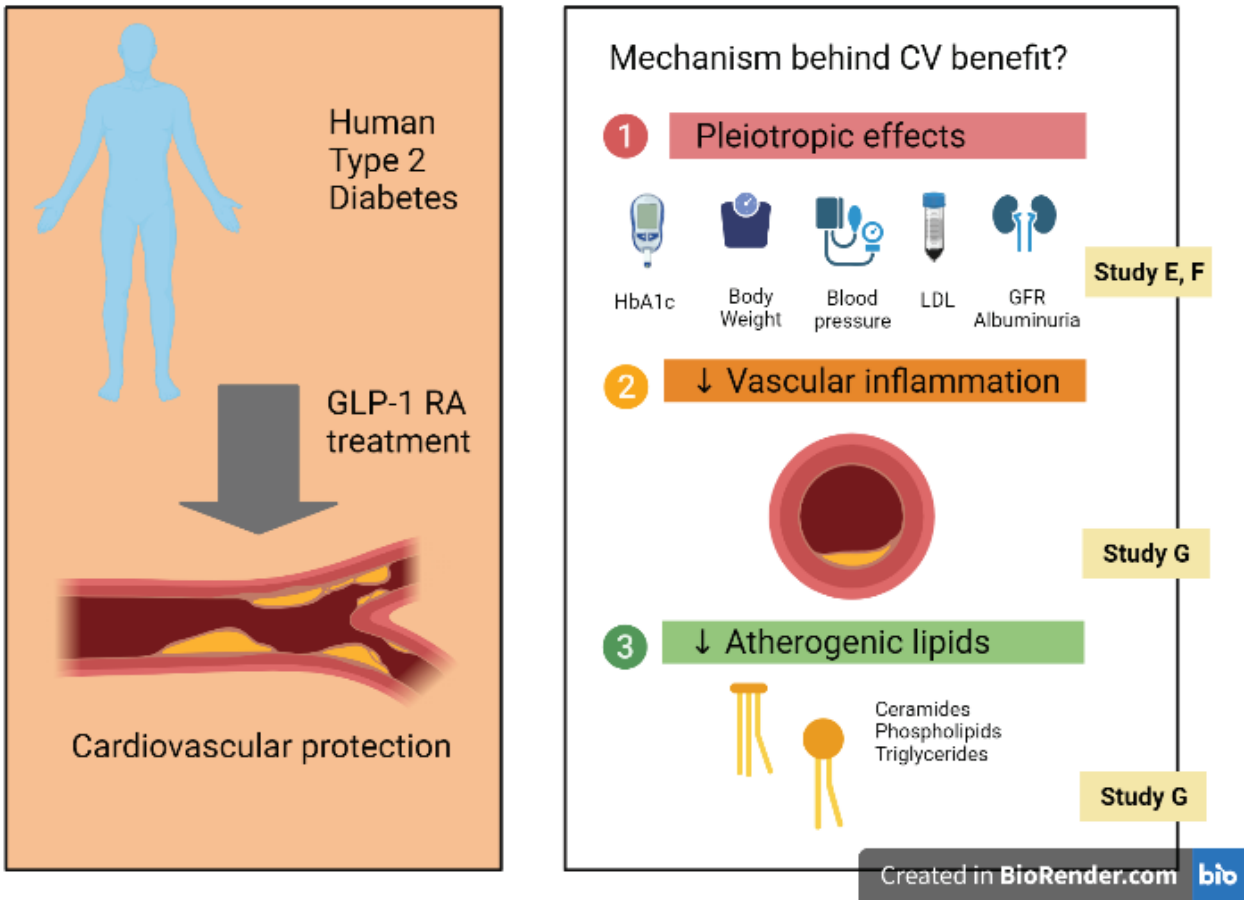


*The risk for each of the four outcomes is shown as hazard ratios according to number of risk markers with a clinically relevant improvement at year 1 [none (group G0), 1 (G1), 2 (G2), 3 (G3) and ≥4 (G4)]. Post hoc analysis of data from 11,678 persons with type 2 diabetes included in the LEADER or SUSTAIN 6 trials. *Adjusted for baseline variables; †Compared G1–G4 to G0 (the reference group); ‡Test for trend. CI, confidence interval; CV, cardiovascular; HR, hazard ratio; IR, incidence rate per 100 person years of observation; MACE, major adverse cardiovascular events. From Zobel et al., Diabetes Obesity and Metabolism, 2021(47).*

Discussion

In this section the clinical effects and mode-of-action of liraglutide was explored in type 2 diabetes using different methods to investigate the effect on clinical cardiovascular risk factors, vascular inflammation and the lipidome (Figure 15).

Figure 15. Clinical effects and the mode-of-action of GLP-1 RAs



Liraglutide treatment has several favorable effects on cardio-renal risk factors. These multiple effects are referred to as the pleiotropic effects of liraglutide. Originally, we hypothesized that liraglutide treatment would lead to a response in all risk factors in some individuals, while others would not respond in any risk factors. This hypothesis was not supported by our analyses of cross-dependencies in the response to liraglutide treatment in two different populations. We first demonstrated in paper 7 that treatment response to liraglutide in type 2 diabetes is largely individual. The findings from this study should be interpreted with the limitations of a post-hoc analysis in a small, open-label study. Therefore, we went on to confirm the results in a post-hoc analysis of a multicenter randomized trial in paper 8. We confirmed that aside from a positive association between reduction in body weight and HbA_{1c} there are no obvious cross-dependencies in the individual risk factor response to liraglutide treatment. An HbA_{1c} reduction following a reduction in body weight may be more evident after 26 compared to 7 weeks treatment with liraglutide, and we speculate that the difference in study duration and size between the open-label study end the randomized trial partly explain why we observed a link between body weight and HbA_{1c} reduction in one study and not the other. These analyses though overall confirmative are is still exploratory, and an

important limitation is the day-to-day variation in the risk factors, particularly in albuminuria, systolic blood pressure and serum creatinine, which may hamper detection of true correlations.

The lack of cross-dependency in risk factor response to liraglutide treatment is interesting and suggests that a pronounced concurrent improvement in several cardiovascular risk factors following liraglutide treatment is maybe not a major contributor to the cardiovascular benefits observed with GLP-1 RAs in outcome studies. It is a challenge to pin-point the relevant mechanisms underlying the cardiovascular benefits observed with liraglutide in outcome studies. A recent mediation analysis of the LEADER trial aimed to identify potential mediators(31). Candidates included HbA_{1c}, body weight, urinary albumin-to-creatinine ratio, systolic blood pressure, LDL-cholesterol, confirmed hypoglycemia, and use of sulfonylurea or insulin, all known cardiovascular risk factors positively affected by liraglutide in the LEADER trial. The analysis identified HbA_{1c} and to a lesser extent urinary albumin-to-creatinine ratio as potential mediators of liraglutides effect on major adverse cardiovascular events, while mediation by the other candidates was small(31). A mediation analysis only allows investigation of possible associations among known, measured risk factors and outcomes, but do not necessarily identify causality. Recent cardiovascular outcome trials have shown clear benefits for other GLP-1 RAs, including semaglutide(20), albiglutide(21) and dulaglutide(22), with modest changes in HbA_{1c}. As an example, in the Researching Cardiovascular Events With a Weekly Incretin in Diabetes (REWIND) trial, the difference in HbA_{1c} between the dulaglutide and the placebo treated groups was modest (-0.5 %) at week 16, but a 22% reduced risk of major adverse cardiovascular events was observed with dulaglutide treatment(22). Other modern cardiovascular outcome trials failed to demonstrate cardiovascular benefit for lixisenatide(113), alogliptin(114), saxagliptin(115), sitagliptin(116) and insulin glargine(117), despite reductions in HbA_{1c} in the active compared to the placebo groups. Whether HbA_{1c} is a true mediator of the cardiovascular protection observed with liraglutide or a marker of an unmeasured factor remains a key question.

In several animal studies, GLP-1 inhibits the atherosclerotic process (32, 118-122). In humans, it has been demonstrated that carotid plaques ex vivo were less inflamed and thereby more stable in persons treated with GLP-1 RAs(123), which together with findings of reduced serum concentrations of the inflammatory marker sCD163 and fewer inflammatory macrophages after liraglutide treatment(124) support a hypothesis that liraglutide reduce vascular inflammation. The late divergence of the Kaplan-Meier curves in the LEADER trial(19) is also compatible with a more indirect pleiotropic cardiovascular effect of liraglutide rather than a direct antithrombotic effect. We designed the LiraFlame trial to evaluate the hypothesis that liraglutide reduce arterial inflammation, which could contribute to the known cardiovascular benefit with liraglutide(19). As our primary end-point, we used [^{18}F]FDG uptake to asses change in arterial inflammation

following liraglutide treatment, as several studies have shown that this method can be used to directly quantify arterial inflammation reliably and noninvasively(125). Furthermore, [^{18}F]FDG has successfully been applied to assess the impact of pharmaceutical interventions on arterial inflammation in a number of clinical trials(126-128). In 102 persons with type 2 diabetes randomized to liraglutide or placebo for 26 weeks, we could not demonstrate a reduced [^{18}F]FDG uptake following liraglutide treatment compared to placebo. The included population was above 50 years of age and diagnosed with type 2 diabetes, but the population was not enriched with persons at high cardiovascular risk, and thus only included 23% with a history of cardiovascular disease compared to 81% in the LEADER trial(19). Thus, most of the included persons had a relatively low cardiovascular risk, and we speculate that low arterial inflammation at baseline reduced the potential for attenuation of the signal. In support of this hypothesis we observed a borderline significant interaction between treatment and history of cardiovascular disease. The included persons with a history of cardiovascular disease had 10% higher carotid [^{18}F]FDG uptake at baseline than the persons without cardiovascular disease, and in an exploratory analysis only including the persons with a history of cardiovascular disease, we observed a numeric difference in [^{18}F]FDG uptake in the persons treated with liraglutide compared to placebo.

An alternative hypothesis that could explain the unchanged [^{18}F]FDG uptake following liraglutide treatment in our population is that the specificity of [^{18}F]FDG uptake may hamper the possibility to sense a signal in persons with lower levels of arterial inflammation(129, 130). It is important to emphasize that positive results in clinical studies of anti-inflammatory drugs, like statins(127), indicate that the [^{18}F]FDG uptake does hold clinically relevant information, despite limited specificity. Still, the lack of cell specificity is one limitation of the [^{18}F]FDG tracer, that has fueled interest in more cell-specific probes including [^{64}Cu]DOTATATE, that directly targets the G-protein-coupled receptor somatostatin receptor subtype-2 selectively expressed on the surface of activated macrophages. In a substudy we demonstrated that the carotid [^{64}Cu]DOTATATE uptake was reduced over 26 weeks in the 15 persons treated with liraglutide, and unchanged in the 15 persons treated with placebo, but without significant difference between the two groups.

Taken together, our findings indicate that 26 weeks liraglutide treatment compared to placebo does not significantly reduce arterial inflammation assessed as [^{18}F]FDG or [^{64}Cu]DOTATATE uptake in persons with type 2 diabetes and with low level of arterial inflammation. However, there are trends in our data that indicate that the hypothesis that liraglutide reduce arterial inflammation should be pursued in future studies including a population with type 2 diabetes and history of cardiovascular disease or with PET verified high level of arterial inflammation as an inclusion criteria.

It the attempt to pin-point the relevant mechanisms underlying the cardiovascular benefits observed with liraglutide in outcome studies, there has also been interest in liraglutides effect on atherogenic lipids. The aforementioned mediation analysis of the LEADER trial did not identify LDL-cholesterol as an important mediator of liraglutides effect on major adverse cardiovascular events(31). However, the lipid metabolism in type 2 diabetes and cardiovascular disease is complex and other lipids are involved in the development of atherosclerosis. With new lipidomic technologies using mass spectrometry we identified and evaluated 260 individual lipids before and after 26 weeks treatment with liraglutide *vs* placebo. As a novel finding, we observed that individual ceramide and phospholipid lipid species were reduced in the liraglutide treated group compared to placebo. We further confirm what others have demonstrated before, that triglyceride lipid species were reduced in the liraglutide treated group compared to placebo(33, 131, 132), and we add that this was primarily by a downregulation of large, poly-unsaturated triglycerides. Ceramides and phospholipids are of interest, because lower levels could be a pathway to reduced cardiovascular risk. Circulating ceramide and phospholipid levels are both risk factors for future cardiovascular disease and death(133-136). In animal models, inhibiting the biosynthesis of ceramides ameliorates atherosclerosis and arterial stiffness(137, 138). In humans, evidence that reduction in ceramides translate into fewer cardiovascular events is sparse, but a strong association between ceramides, and risk of cardiovascular disease was mitigated by a Mediterranean dietary intervention in the recent PREDIMED trial(139). Our study was hypothesis-generating and by using lipidomics we were able to investigate liraglutide's possible effect on a broad panel of lipid families and individual lipids. The lipid regulating effect of liraglutide should be examined further, and we hope future research can confirm our findings and deepen the understanding of the single lipids affected by liraglutide's role in larger causal networks in atherosclerosis.

The exploration of the pleiotropic effects of liraglutide on several clinical cardiovascular risk factors in paper 7 and 8, inspired us to investigate whether multiple risk factor response matter for outcome in persons with type 2 diabetes. The risk factors evaluated included HbA_{1c}, body weight, systolic blood pressure, LDL-cholesterol, urinary albumin creatinine ratio and estimated GFR. We demonstrated that the number of improved risk factors impact both cardiovascular and renal outcomes. This is in line with findings from the Steno 2 study (n=160), that combined lifestyle intervention targeting exercise, obesity, diet and smoking, as well as pharmacological therapy targeting glucose, lipids and blood pressure, including angiotensin-converting enzyme inhibitors and aspirin in persons with type 2 diabetes and microalbuminuria. Steno 2 demonstrated a 50% reduction in cardiovascular events after 8 years(34) and 20% reduction in mortality after 13 years(140), and a median increase in survival of 7.9 years after 21 years of follow-up, compared to standard of care(141). Recent epidemiological data also support that the number of well-controlled risk markers matters for outcome in type 2 diabetes(142). Our findings imply that, in a clinical setting, it is

important to consider multiple risk factors and underscore the benefit of pleiotropic antidiabetic treatments that target more than one risk factor. This was a post-hoc analysis of LEADER and SUSTAIN-6, two cardiovascular outcome trials that have clearly demonstrated that liraglutide and semaglutide reduce the risk of major adverse cardiovascular events in type 2 diabetes, however, these analyses do not inform us on the underlying mechanisms.

In this section, we explored the clinical effects and mode-of-action of liraglutide using different methods to examine the effect on clinical cardiovascular risk factors, arterial inflammation and the lipidome. We demonstrated in a post-hoc analysis of a small open-label study and a large randomized trial, that the treatment response to liraglutide is largely individual, in terms of response in HbA_{1c}, body weight, blood pressure, LDL-cholesterol, urinary albumin excretion and measures of kidney function. In a clinical trial we demonstrated that 26 weeks liraglutide treatment compared to placebo did not significantly reduce arterial inflammation assessed as [^{18}F]FDG or [^{64}Cu]DOTATATE uptake in persons with type 2 diabetes and with overall low level of arterial inflammation. Trends in our data indicate that the hypothesis that GLP-1 RAs reduce arterial inflammation should be pursued in future studies including a population with type 2 diabetes and history of established cardiovascular disease. In the same clinical trial, we observed that liraglutide reduced the level of ceramides, phospholipids and triglycerides, all lipids linked to risk of cardiovascular disease. This effect of liraglutide should be pursued in future studies. Finally, we demonstrated in a post-hoc analysis of two cardiovascular outcome trials, that multiple risk factor response reduced the risk of cardiovascular disease and nephropathy in type 2 diabetes.

Conclusion and future perspectives

In the first part of this thesis, the pathophysiology of cardiovascular disease was explored in type 1 diabetes. Using different methods, we investigated the importance of calcification and myocardial microvascular dysfunction. We identified PTH as petitioner in a possible link between decalcification of the bones and vascular calcification. We observed that myocardial microvascular impairment was linked to microvascular disease in the kidneys, and that sympathetic dysfunction in type 1 diabetes was linked to myocardial microvascular impairment, whereas amount of cardiac adipose tissue was not. In the second part of this thesis, new and established risk markers for cardiovascular disease in diabetes were explored. We identified lower toe-brachial index as a promising risk marker of cardiovascular disease in type 2 diabetes, whereas the circulating biomarkers SDMA and ADMA had less predictive strengths. We investigated methods for 24-hour ambulatory blood pressure measurement, a well-established cardiovascular risk marker also recommended in guidelines, and we demonstrated in a methodological study that a traditional cuff device was significantly more uncomfortable than a cuff-less tonometric device, but the tonometric device was less precise and thus, cannot be recommended for clinical use. In the third part of this thesis, we explored the clinical effects and mode-of-action of liraglutide. Using different methods, we explored the effect on clinical cardiovascular risk factors and demonstrated that the treatment response to liraglutide is largely individual. We explored liraglutides effect on arterial inflammation and observed that [^{18}F]FDG and [^{64}Cu]DOTATATE uptake was unchanged by liraglutide compared to placebo in a randomized trial, and lastly we explored liraglutides effect on the lipidome and observed a reduction in the level of ceramides, phospholipids and triglycerides compared to placebo. Finally, we demonstrated that multiple risk factor response matters for outcome in type 2 diabetes.

In future perspectives, we are especially interested in myocardial microvascular dysfunction, the toe-brachial index, the need for more convenient 24-hour ambulatory blood pressure devices, and in pursuing the mechanism behind GLP-1 RA's cardioprotective effects.

We aim to pursue the role of myocardial flow reserve as a measure of myocardial microvascular function in diabetes as early identification of myocardial microvascular damage may provide new prospects for risk stratification in diabetes. In an ongoing prospective study, we aim to evaluate the predictive value of the myocardial flow reserve beyond traditional cardiovascular risk factors and coronary artery calcium score. We will include 900 persons with type 2 diabetes without known cardiovascular disease, stratified into groups of normo-, micro-, and macroalbuminuria, and follow them for up to 5 years. Hopefully, this study will help clarify if myocardial flow reserve assessed by cardiac PET/CT should have a role in future risk stratification in clinical care. In addition to a role in risk stratification, it will be interesting to see future

research uncover if the myocardial flow reserve is improved by some of the new treatment options available for cardio-renal complications in diabetes. As mentioned the new nonsteroidal MRA has demonstrated benefits on cardiac and renal outcomes in type 2 diabetes with chronic kidney disease, and from preclinical studies this seems linked to an effect on inflammation and fibrosis in heart and kidney, thus it would be of great interest to study the effect of finerenone on myocardial microvascular function(64). Sorensen et al noticed in a recent cross-sectional study in type 2 diabetes, a crude trend towards a lower resting myocardial blood flow in persons treated with sodium–glucose cotransporter 2 (SGLT2) inhibitor than in those treated with other anti-diabetics(55). SGLT-2 inhibitors have cardiovascular benefits in large outcome studies(91), and it has been hypothesized that this partly could be explained by induction of a mild ketosis and a shift toward oxidation of ketone bodies instead of free fatty acids, which would improve the cardiac energetics(143). This would yield an overall decrease in cardiac oxygen demand and presumably a lower resting myocardial blood flow, and SGLT-2 inhibitor treatment could thereby increase the myocardial flow reserve. It will be interesting to see the results of an ongoing study evaluating this hypothesis [ClinicalTrials.gov Identifier: NCT03151343].

In relation to cardiovascular risk prediction, toe-brachial index is a measure of peripheral arterial disease, that we suggest used as a measure of systemic atherosclerosis to improve risk prediction in type 2 diabetes. Peripheral arterial disease and related wounds and amputations are among the most expensive and invalidating complications to diabetes. The LEADER trial demonstrated liraglutide's effect on cardiovascular outcomes(19), but in addition identified a decreased risk of amputations with liraglutide compared to placebo(144). It will be interesting to see the results of the ongoing 'A Research Study to Compare a Medicine Called Semaglutide Against Placebo in People With Peripheral Arterial Disease and Type 2 Diabetes trial (STRIDE)', which evaluates the effect of semaglutide on walking ability compared to placebo in 800 participants with type 2 diabetes and peripheral arterial disease [ClinicalTrials.gov Identifier: NCT04560998].

From a new cardiovascular risk marker to a very well-established risk marker in diabetes. 24-hour ambulatory blood pressure measurements are recommended in guidelines, however the discomfort related to the cuff-inflations may affect compliance and the use in clinic practice, even if blood pressure measurements are not affected by the inflations as demonstrated in our methodological study. Although the participants rated the cuff-less tonometric device (BPro) more comfortable, it cannot be recommended for clinical use going forward. Thus, there exist an unmet need for a new convenient and comfortable method for ambulatory blood pressure measurement with a precision like the conventional cuff-based

methods. Ideally, such a solution could be monitored wireless and feed results directly into electronic health records.

GLP-1 RAs with proven cardiovascular benefits are recommended as part of the glycemic management in persons with type 2 diabetes with established atherosclerotic cardiovascular disease(24). Going forward, there is a great interest in better understanding the mechanisms underlying the cardiovascular protection observed with GLP-1 RA treatment. It will be interesting to see the results of the ongoing study, 'A Trial Investigating the Effect of Semaglutide on Atherosclerosis in Patients With Cardiovascular Disease and Type 2 Diabetes' [ClinicalTrials.gov Identifier: NCT04032197], sponsored by Novo Nordisk and inspired by the LiraFlame trial. The trial will as the LiraFlame trial include 100 participants with type 2 diabetes. The primary endpoint is change in [^{18}F]FDG uptake in the carotid arteries from baseline to week 26, and a key secondary endpoint is change in [^{68}Ga]DOTATATE uptake in the carotid arteries from baseline to week 26. Informed by the results of the LiraFlame trial, a key inclusion criterion is established cardiovascular disease. Steno Diabetes Center Copenhagen is one of three recruiting centers, and study completion is estimated to 2022.

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Paper I

Higher Parathyroid Hormone Level Is Associated With Increased Arterial Stiffness in Type 1 Diabetes

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Vascular calcification is a common consequence of aging, hypercholesterolemia, chronic renal insufficiency, and diabetes (1). Stiffening of the large arteries is a result of vascular calcification. Carotid–femoral pulse wave velocity (cfPWV) is considered the gold standard measure of arterial stiffness (2) and has been shown to be a strong predictor of mortality and cardiovascular outcome.

We evaluated the association between arterial stiffness (evaluated by cfPWV) and bone mass density (evaluated with dual-energy X-ray absorptiometry at the femoral neck), as well as a comprehensive panel of bone markers, in a well-characterized cohort of 347 persons with type 1 diabetes. We hypothesized that vascular calcification is linked to decalcification of the bones.

The participants were recruited from the outpatient clinic at Steno Diabetes Center, Gentofte, Denmark. Written informed patient consent and ethical approval of the study were obtained.

A total of 164 (47%) participants were women, mean ± SD age was 55.8 ± 9.6 years and cfPWV was 11.0 ± 3.4 m/s, and median (interquartile range) parathyroid hormone (PTH) was 39.1 (29.1, 57.3) pg/mL.

Table 1 shows the unadjusted and stepwise adjusted associations between cfPWV and bone mineral density, clinical bone markers (PTH, 25-hydroxyvitamin D, calcium, and phosphorus) and markers of bone mineral metabolism (endostatin, sclerostin, Dickkopf 1, and osteoprotegerin). In unadjusted analyses, bone mineral density, all clinical bone markers, and markers of mineral metabolism—except calcium, phosphorus, and Dickkopf 1—were associated with cfPWV ($P \leq 0.041$). After adjustment for age, sex, and mean arterial pressure, the level of bone mineral density, PTH, and sclerostin remained associated with cfPWV ($P \leq 0.027$). After adjustment for additional risk factors (HbA_{1c}, total cholesterol, BMI, antihypertensive treatment, urinary albumin excretion rate, estimated glomerular filtration rate, and smoking), PTH remained positively associated with cfPWV ($P = 0.014$).

In the search for a link between bone and vascular disease, we demonstrated an association between arterial stiffness and bone mineral density, clinical bone markers, and markers of mineral metabolism. These associations lost significance after comprehensive adjustment, except for the relationship between higher PTH and increased arterial stiffness.

PTH is one of the main regulators of calcium homeostasis. Secretion of PTH from the parathyroid gland is triggered by low serum calcium. PTH secretion results in raised serum calcium through its release from the bones, reduced renal excretion, and increased small intestine absorption (3). Aside from its well-established role in calcium homeostasis, elevated PTH has been linked to presence of hypertension and cardiac hypertrophy (4), and PTH excess may be related to development of cardiovascular disease (5).

Our study consisted of a well-characterized group of persons with type 1 diabetes. Arterial stiffness was evaluated using the gold standard method, analyzed as a continuous variable and with proper adjustment. Bone mineral density was evaluated with robust methods, and all bone markers were analyzed as continuous variables; no arbitrary cutoffs were applied.

Our findings highlight PTH as a potential mediator for the cross talk between bone and vascular disease. However, our findings need validation, and prospective studies investigating the relationship between PTH and cardiovascular outcome in type 1 diabetes are warranted. Depending on the results of such studies, therapies known to reduce PTH (e.g., cinacalcet) could potentially reduce the cardiovascular risk in

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Table 1—Unadjusted and stepwise-adjusted associations between cfPWV and measures of bone/mineral metabolism

	Unadjusted		Adjusted for age, sex, and mean arterial pressure		Adjusted for age, sex, mean arterial pressure, and other risk factors	
	β	*P*	β	*P*	β	*P*
Bone mineral density						
Femoral neck bone mineral density	−0.23	<0.001	−0.15	0.027	−0.06	0.43
Clinical bone markers						
PTH*	0.23	<0.001	0.28	<0.001	0.17	0.014
25-Hydroxyvitamin D	0.12	0.041	−0.12	0.09	−0.10	0.17
Ionized calcium	−0.01	0.08	−0.06	0.37	−0.02	0.77
Phosphorus	0.07	0.19	0.11	0.13	0.001	0.98
Markers of bone mineral metabolism						
Endostatin*	0.16	0.003	0.12	0.09	0.05	0.49
Sclerostin*	0.14	0.011	0.16	0.022	0.01	0.85
Dickkopf 1*	0.007	0.90	0.06	0.43	0.12	0.15
Osteoprotegerin*	0.36	<0.001	0.13	0.08	0.02	0.77

Other risk factors included HbA_{1c}, total cholesterol, BMI, antihypertensive treatment, urinary albumin excretion rate, estimated glomerular filtration rate, and smoking. cfPWV was measured with the SphygmoCor (AtCor Medical, Sydney, Australia). Plasma vitamin D [25(OH)D3] levels were determined by high-performance liquid chromatography–tandem mass spectrometry. Plasma PTH levels were analyzed using a second-generation electrochemiluminescence immunoassay (Cobas e601, Roche Diagnostics). Serum endostatin, sclerostin, Dickkopf 1, and osteoprotegerin were measured by sandwich ELISA (Biomedica Medizinprodukte, Austria). *Log_2 transformed for analyses. The β estimates represent standardized effect.

subjects with type 1 diabetes with elevated PTH.

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Paper 2

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Myocardial flow reserve assessed by cardiac ^{82}Rb positron emission tomography/computed tomography is associated with albumin excretion in patients with Type 1 diabetes

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Aims To evaluate myocardial flow reserve (MFR) and coronary artery calcium (CAC) in persons with Type 1 diabetes with or without albuminuria and in non-diabetic controls. MFR reflects the function of large epicardial arteries and myocardial microcirculation. CAC represents structural aspects of atherosclerosis. In addition, we evaluated the association of MFR and CAC with retinopathy, another microvascular complication.

Methods and results Cross-sectional study in Type 1 diabetes, stratified by normoalbuminuria (NORMO; $n = 30$) and macroalbuminuria (MACRO; $n = 30$), and in non-diabetic controls ($n = 30$). MFR (pharmacological stress flow/rest flow) was evaluated by cardiac ^{82}Rb positron emission tomography/computed tomography. MFR was similar in patients with NORMO and controls (3.1 ± 0.79 vs. 3.0 ± 0.79; $P = 0.74$). Patients with MACRO had lower (impaired) MFR when compared with NORMO (2.1 ± 0.92 vs. 3.1 ± 0.79; $P < 0.0001$). The CAC score [median (interquartile range)] was higher in NORMO when compared with controls [72 (22–247) vs. 0 (0–81), $P = 0.03$], and comparable between MACRO and NORMO. MFR was comparable in patients with diabetes and simplex or no retinopathy ($n = 24$ and $n = 12$, 2.8 ± 0.84 vs. 3.3 ± 0.77, $P = 0.11$), but lower in proliferative ($n = 24$) compared with simplex retinopathy (2.1 ± 0.97 vs. 2.8 ± 0.84, $P = 0.02$). The CAC score was comparable between groups of retinopathy.

Conclusion Myocardial microvascular function was comparable in non-diabetic controls and patients with Type 1 diabetes and NORMO; but impaired in the presence of microvascular complications (MACRO and proliferative retinopathy). Coronary calcification was elevated in diabetes, however, not explained by albuminuria.

Keywords cardiovascular disease • coronary artery calcium score • myocardial flow reserve • macroalbuminuria • cardiac PET/CT • Type 1 diabetes

Introduction

Approximately 35% of persons with Type 1 diabetes will develop albuminuria. Deckert *et al.*[1] proposed in 1989 in the Steno hypothesis that albuminuria reflects widespread vascular damage. The hypothesis links leaky renal vessels to a general impaired vascular endothelial function. Many subsequent studies have confirmed the association between albuminuria, retinopathy, and risk of cardiovascular disease[2,3] supporting the hypothesis of widespread vascular damage in Type 1 diabetes.

Major advances in non-invasive imaging enable the investigation of new aspects of the microcirculation. Among these methods is

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quantitative cardiac positron emission tomography (PET) which allows the measurement of myocardial blood flow at rest and during pharmacologically induced hyperaemic conditions. The ratio between resting and maximal induced myocardial blood flow is termed the myocardial flow reserve (MFR) and reflects to what extend the flow can increase during stress. MFR mirrors the function of the large epicardial arteries and the microcirculation of the myocardium.[4] A hybrid scanner can combine cardiac PET with computed tomography (CT) to estimate coronary artery calcium (CAC), a specific marker of atherosclerosis.

Knowledge about cardiac microvascular function in Type 1 diabetes is scarce. Only two small studies have reported impaired MFR in young men with Type 1 diabetes as compared to healthy controls.[5,6]

In a recent study in persons with Type 2 diabetes free of overt cardiovascular disease, we demonstrated lower (impaired) MFR and higher CAC score (increased calcification) to be associated with concomitant albuminuria.[7] It is not clear to what extent these findings can be extrapolated to Type 1 diabetes.

Taking advantages of similar cardiac PET/CT imaging as in our study of persons with Type 2 diabetes, we undertook a cross-sectional study in healthy controls and persons with Type 1 diabetes stratified by albumin excretion. We aimed to gain information of the prevalence and predictors of reduced MFR and increased CAC in persons with Type 1 diabetes (with or without albuminuria) while comparing them to non-diabetic controls, and in addition, we evaluated the association of MFR and CAC with diabetic retinopathy, another microvascular complication.

Methods

Study population

We included 60 persons with Type 1 diabetes according to the WHO criteria. A priori, we decided to include 30 participants with normoalbuminuria (NORMO) (<30 mg/24 h or 30 mg/g creatinine) and 30 participants with a history of macroalbuminuria (MACRO) (≥300 mg/24 h or 300 mg/g creatinine in two out of three consecutive urine collections; $n = 30$). Persons with NORMO and MACRO were matched on age and sex. Persons classified with NORMO did not have any history of microalbuminuria or MACRO prior to enrolment in the study. The participants were recruited from the Steno Diabetes Center Copenhagen among subjects participating in a cross-sectional study focusing on detailed phenotyping of Type 1 diabetes patients with or without progressive renal complications. None of the participants included were symptomatic for angina and the reason of the cardiac PET/CT was exclusively research. Exclusion criteria were (i) non-diabetic kidney disease; (ii) renal failure [estimated glomerular filtration rate (eGFR) <15 mL/min/1.73 m^2], dialysis, or kidney transplantation; (iii) change in renin–angiotensin aldosterone system blocking treatment during the last month; and (iv) contraindications for cardiac PET/CT. Moreover, 30 healthy non-diabetic persons studied earlier with an identical protocol using the same equipment at our centre in 2013[7] were used as controls.

Power calculation was performed using the power statement implemented in the SAS software, version 9.3. Calculations were based on our previous results on MFR in persons with Type 2 diabetes performed in the same centre using the same equipment.[7] In the assumption of a mean difference in MFR of 0.6 [and a standard deviation (SD) of 0.8] between persons with NORMO vs. MACRO, a total of 54 persons (27 in each group) were needed to provide 80% power for a Type 1 error of 5%. We included 30 persons in each group to account for technical difficulties and incomplete investigations.

The study was performed from August 2016 to January 2018. The study was conducted in accordance with the Helsinki protocol and all participants gave informed written consent and the protocol was approved by the local ethics committee.

Clinical measurements

HbA_{1c} was measured by high performance liquid chromatography. Plasma creatinine was measured by an enzymatic method (Hitachi 912, Roche Diagnostics, Mannheim, Germany). The CKD-EPI equation was used to calculate eGFR.[8] Urinary albumin creatinine rate (UACR) was measured by an enzyme immunoassay in three consecutive morning urine samples. Measurements of 24-h blood pressure were recorded using a cuff-device (Takeda, TM2430, Japan[9]) programmed to measure blood pressure every 15 min between 7 am and 10 pm and every 30 min between 10 pm and 7 am. Standard resting 12-lead electrocardiogram was obtained. Height and weight was measured, and body mass index was calculated as weight/height (kg/m^2). A detailed medical history including treatment and previous cardiovascular disease was obtained from all participants and cross referenced with electronic patient records. Current smoking was defined as one or more cigarettes/cigars/pipes a day.

For the control subjects, all clinical measurements were assessed and defined as described above with the exceptions that (i) urinary albumin excretion rate (UAER) was measured in two 24-h urine collections by an enzyme immunoassay; and (ii) 24-h blood pressure was recorded using BPro (HealthStats, Singapore), a tonometric wrist-device that records brachial blood pressure derived from radial pulse waves.[10] The device captured the blood pressure every 15 min for 24 h.

Hybrid cardiac PET/CT imaging

A dynamic, gated cardiac PET/CT study was performed using a hybrid PET/CT scanner in 3D mode (Siemens Biograph mCT 128, Siemens, Munich, Germany) after administration of 1100 MBq ^{82}Rb (CardioGen-82, Bracco Diagnostics, Monroe Township, NJ, USA). The procedure has previously been described in detail.[7] Cardiac PET/CT was performed at rest and at stress after adenosine was infused at 140 μg/kg/min for 6 min to induce maximum myocardial hyperaemia. Myocardial blood flow was automatically calculated using the Siemens Syngo MBF 2.3 (Siemens Medical Solutions, Malvern, PA, USA) with one-compartment tracer kinetic models for ^{82}Rb[11] and the extraction curve from Lortie *et al.*[12] Myocardial perfusion abnormalities were assessed semi quantitative by two experienced operators. Before the examination, phosphodiesterase 5 inhibitors were discontinued for 72 h, dipyridamole-containing medications were discontinued for 36 h, and nitrates were discontinued for 12 h. Moreover, all subjects refrained from caffeine-containing beverages for 18 h before examination, and theophylline-containing medications were discontinued for 24 h. Angiotensin converting enzyme inhibitors were not discontinued,[13,14] and we did not set therapy with beta-blocker on hold due to possible discomfort for the patients and it has been demonstrated that beta-blocker can be continued without loss of the essential interpretation of the results.[15] CAC score was calculated as the sum of CAC content in the three main coronary arteries using the method described by Agatston *et al.*[16] and semiautomated commercially available software (Corridor4DM, INVIA, Ann Arbor, MI, USA).

Different cut-off for MFR has been applied depending on the characteristic of the study population; and a cut-off of 2.5 has been suggested in patients without obstructive coronary artery disease (CAD).[17,18] We, therefore, prespecified a cut-off of 2.5. An elevated CAC was defined as an Agatston score >300.[19]

Table 1 Clinical characteristics of participants

Characteristics	Controls ($n = 30$)	Normoalbuminuria ($n = 30$)	Macroalbuminuria ($n = 30$)	*P*-value controls vs. normoalbuminuria	*P*-value normoalbuminuria vs. macroalbuminuria
Female (%)	40	40	43	1.0	0.79
Age (years)	59.8 ± 9.9	59.8 ± 9.1	58.2 ± 9.9	0.99	0.52
Known diabetes duration (years)		32.6 ± 12.7	41.4 ± 13.3		0.01
Body mass index (kg/m^2)	24.7 ± 3.4	25.6 ± 4.1	27.2 ± 4.2	0.38	0.15
HbA_{1c} (mmol/mol)	35.8 ± 1.9	61.3 ± 8.3	66.3 ± 11.7	<0.0001	0.70
HbA_{1c} (%)	5.4 ± 0.17	7.8 ± 0.76	8.2 ± 1.07	<0.0001	0.70
LDL cholesterol (mmol/L)	3.4 ± 0.7	2.3 ± 0.7	2.1 ± 0.8	<0.0001	0.32
Total cholesterol (mmol/L)	5.5 ± 0.7	4.5 ± 0.8	4.4 ± 0.9	<0.0001	0.73
eGFR (mL·min^{-1} 1.73·m^{-2})	82.8 ± 13.1	89.1 ± 10.4	62.5 ± 23.1	0.043	<0.0001
Urinary albumin creatinine rate (mg/g)[a]	6 (5–10.5)	3 (3–5)	121 (53–283)	0.0002	<0.0001
Smokers (%)	13	14	14	0.96	1.0
Alcohol (beverages/week)	8.5 (4–14)	7 (4–18)	6.5 (1–14)	0.87	0.91
Antihypertensive treatment (%)	10	57	100	0.0001	<0.0001
RAAS inhibition treatment (%)	10	47	97	0.002	<0.0001
Beta-blocker treatment (%)	0	3	27	1.0	0.026
Aspirin treatment (%)	3	37	63	0.0012	0.039
Lipid-lowering treatment (%)	0	70	80	<0.0001	0.37
Retinopathy (no/simplex/proliferative) (%)		37/53/10	3/27/70		
Known coronary artery disease (%)	0	0	12		0.01

Data are expressed as *n* (%), mean ± SD, or geometric mean (IQR). *P* values from independent samples *t*-test and χ^2 test or Fisher's exact test.
eGFR, estimated glomerular filtration rate; RAAS, renin–angiotensin aldosterone system.
[a]Urinary albumin excretion rate (mg/24 h) for the 30 controls.

Retinopathy

Retinopathy status was obtained from medical records for the diabetic participants. All persons attending the outpatient clinic at Steno Diabetes Center Copenhagen have regular ophthalmology examinations (approximately every 1–2 years) where retinal photographies are taken through a dilated pupil by certified eye nurses. Retinopathy was graded as nil, presence of or historical simplex, proliferative, or blind based on the worst eye.

Statistical analysis

For continuous variables, the normal distributed are given as mean and SD, and the non-normal distributed (CAC, UACR, and alcohol intake) as median with interquartile range (IQR). Categorical variables are provided as total numbers in percent. When analysing differences between two groups, we applied independent samples *t*-test when comparing continuous variables, and the χ^2 test or Fisher's exact test as appropriate when comparing categorical variables. The non-normal distributed variables were $\log_2$ transformed in all analyses. However, the CAC score distribution was highly skewed and hence not amenable to transform into normal distribution and was analysed using the Mann–Whitney *U* test. When analyzing differences in MFR and CAC in adjusted analysis, analysis of covariance was applied, ensuring normal distribution of the residuals for CAC after $\log_2$ transformation. Linear regression was used to analyse correlations between MFR, CAC, and other covariates. We provide R^2 to present the proportion of variability in the dependent variable explained by the model and the *F*-test was applied to determine whether this relationship was statistically significant.

Participants with a history of percutaneous coronary intervention ($n = 5$) were excluded from the analyses of CAC. Results were considered to be significant at a two-tailed *P*-value <0.05. Statistical analyses were performed using SAS software (version 9.3; SAS Institute).

Rationale for selection of covariates

We adjusted for traditional cardiovascular risk factors based on prior evidence. In analysis comparing the level of MFR and CAC between controls, patients with diabetes and NORMO or MACRO we adjusted for sex, age, 24-h systolic blood pressure, eGFR, and smoking. The groups were pre-categorized according to UACR, HbA_{1c}, and diabetes duration and these covariates were therefore omitted as was medical treatment due to risk of bias by indication. We did not adjust for low density lipoprotein (LDL) cholesterol, since the level was higher in controls when compared with the diabetic participants most likely because the controls were not receiving lipid-lowering treatment. In analyses restricted to the participants with diabetes, we applied additionally adjustment for HbA_{1c}, LDL cholesterol and diabetes duration, and for the analyses of retinopathy also for UACR.

Results

Clinical characteristics

Characteristics for the total cohort are presented in *Table 1*. The 90 participants included 37 (41%) females and mean ± SD age was 59.3 ± 9.5 years. Compared to the persons with NORMO the

Table 2 Cardiac PET/CT and albuminuria

Variables	Controls ($n = 30$)	Normoalbuminuria ($n = 30$)	Macroalbuminuria ($n = 30$)	*P*-value controls vs. normoalbuminuria	*P*-value normoalbuminuria vs. macroalbuminuria
24-h systolic blood pressure (mmHg)	127 ± 13	135 ± 9	138 ± 11	0.009	0.26
24-h diastolic blood pressure (mmHg)	79 ± 8	78 ± 6	77 ± 5	0.88	0.48
24-h pulse pressure (mmHg)	48 ± 10	57 ± 6	61 ± 9	0.0002	0.04
Heart rate (bpm)	61 ± 8	69 ± 12	72 ± 12	0.003	0.41
Myocardial blood flow rest (mL/g/min)	1.1 ± 0.25	1.0 ± 0.25	1.2 ± 0.35	0.82	0.01
Myocardial blood flow stress (mL/g/min)	3.0 ± 0.53	3.0 ± 0.51	2.5 ± 0.82	0.73	0.004
MFR	3.0 ± 0.79	3.1 ± 0.79	2.1 ± 0.92	0.74	<0.0001
MFR <2.5 (%)	17	23	77	0.52	<0.0001
CAC score[a]	0 (0–81)	72 (22–247)	263 (23–1315)	0.028	0.17
CAC score >300 (%)	7	17	44	0.71	0.026

Data are expressed as total numbers in percent, mean ± SD, or geometric mean (IQR). *P*-values from independent samples *t*-test, χ^2test, and Mann–Whitney *U* test[a] or Fisher's exact test.
CAC, coronary artery calcium; MFR, myocardial flow reserve.

controls had lower 24-h systolic blood pressure, HbA_{1c}, and eGFR but higher LDL cholesterol and UACR. Persons with MACRO had longer diabetes duration and lower eGFR compared with persons with NORMO. Of the 60 participants with diabetes, 7 (12%) had known CAD (five had a history of percutaneous coronary intervention and two had coronary artery bypass graft). These participants were all in the group with MACRO. Simplex retinopathy was present in 16 (53%) and proliferative retinopathy in 3 (10%) of the persons with NORMO. Simplex retinopathy was present in 8 (27%) and proliferative retinopathy in 21 (70%) of the persons with MACRO. In the total population, the mean left ventricular ejection fraction at rest was 64.2% with a range of 39–85%.

Cardiac PET/CT in controls and in Type 1 diabetes stratified by urinary albumin excretion

The MFR and the frequency of reduced MFR (<2.5) were similar in persons with NORMO and in controls (3.1 ± 0.79 vs. 3.0 ± 0.79 and 23 vs. 17%, $P \geq 0.52$). Persons with MACRO had a lower MFR and the frequency of reduced MFR was higher when compared with persons with NORMO (2.1 ± 0.92 vs. 3.1 ± 0.79 and 77 vs. 23%, $P < 0.0001$). When we adjusted the MFR for CAC score, MFR remained lower and the frequency of reduced MFR higher in persons with MACRO compared to NORMO ($P \leq 0.001$).

The CAC score was higher in persons with NORMO compared to controls [72 (22–247) vs. 0 (0–81), $P = 0.028$], and comparable between persons with MACRO and NORMO [263 (23–1315) vs. 72 (22–247), $P = 0.17$]. The frequency of high CAC was similar in persons with NORMO and the controls (17 vs. 7%, $P = 0.71$), but higher in persons with MACRO compared to NORMO (44 vs. 17%, $P = 0.026$). Results are summarized in *Table 2*.

After adjustment for sex, age, 24-h systolic blood pressure, eGFR, and smoking, MFR remained lower and the frequency of reduced MFR higher in persons with MACRO compared to NORMO ($P \leq 0.0077$, *Figure 1A*), and these differences persisted after additional adjustment for HbA_{1c}, LDL cholesterol, and diabetes duration ($P \leq 0.038$). The CAC remained higher in the persons with NORMO compared to controls after adjustment ($P = 0.03$), but the difference between persons with MACRO and NORMO lost significance.

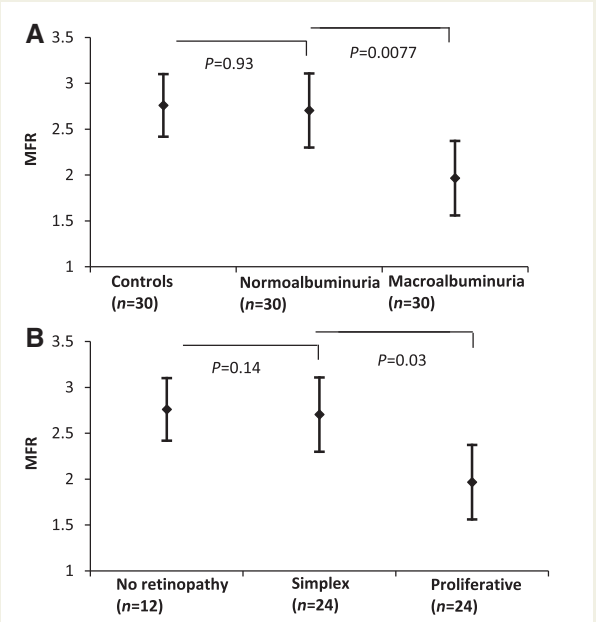

Figure 1 Estimated marginal means with 95% CIs for myocardial flow reserve (MFR) adjusted for sex, age, 24-h systolic blood pressure, eGFR, and smoking according to groups [(*A*) Controls, normoalbuminuria and macroalbuminuria; (*B*) Retinopathy].

The myocardial blood flow during stress were similar in persons with NORMO and in controls (3.0 ± 0.51 vs. 3.0 ± 0.53 mL/g/min, $P = 0.73$). Persons with MACRO had lower myocardial blood flow during stress compared with persons with NORMO (2.5 ± 0.82 vs. 3.0 ± 0.51 mL/g/min, $P = 0.004$). Results are summarized in *Table 2*.

Table 3 Cardiac PET/CT and retinopathy

Variables	No retinopathy ($n = 12$)	Simplex ($n = 24$)	Proliferative ($n = 24$)	*P*-value no retinopathy vs. simplex	*P*-value simplex vs. proliferative
MFR	3.3 ± 0.77	2.8 ± 0.84	2.1 ± 0.97	0.11	0.02
MFR <2.5 (%)	8	46	75	0.03	0.04
CAC score[a]	45 (0–435)	72 (23–247)	299 (51–1651)	0.42	0.25
CAC score >300 (%)	25	14	48	0.64	0.04

Data are expressed as total numbers in percent, mean ± SD, or geometric mean (IQR). *P*-values from independent samples *t*-test, χ^2test, and Mann–Whitney *U* test[a] or Fisher's exact test.
CAC, coronary artery calcium; MFR, myocardial flow reserve.

The difference between persons with MACRO and NORMO lost significance following adjustment ($P = 0.19$).

A total of 10 participants had ischaemia on the PET/CT, seven of which were known with CAD. All 10 had reversible ischaemia (Nine with MACRO and one healthy control), median (IQR) extent was 24 (14–29)%. In five of these participants, irreversible ischaemia (fixed perfusions defects) was also observed, all had MACRO and the median extent was 19 (16–25)%.

Cardiac PET/CT in persons with Type 1 diabetes stratified by retinopathy stage

In persons with simplex retinopathy ($n = 24$) compared to persons without retinopathy ($n = 12$) MFR was comparable (2.8 ± 0.84 vs. 3.3 ± 0.77, $P = 0.11$), while reduced MFR was more frequent (46 vs. 8%, $P = 0.03$). In persons with proliferative ($n = 24$) compared to simplex retinopathy the MFR was lower (2.1 ± 0.97 vs 2.8 ± 0.84, $P = 0.02$) and the frequency of reduced MFR was higher (75 vs. 46%, $P = 0.04$). The CAC score was comparable between stages of retinopathy, but high CAC was more frequent in persons with proliferative compared to simplex retinopathy (48 vs. 14%, $P = 0.04$). Results are summarized in *Table 3*.

The MFR remained lower in persons with proliferative compared to simplex retinopathy after initial adjustment ($P = 0.03$, *Figure 1B*), but lost significance after further adjustment for HbA_{1c}, LDL cholesterol, diabetes duration, and UACR ($P = 0.27$). The differences in frequency of reduced MFR and high CAC between retinopathy groups lost significance after adjustment.

Variables correlated with MFR and CAC

In the total population, MFR was negatively correlated with CAC ($R^2 = 0.20$, $P < 0.0001$, *Figure 2A*), UACR ($R^2 = 0.25$, $P < 0.0001$, *Figure 2B*), age ($R^2 = 0.07$, $P = 0.01$), 24-h systolic blood pressure ($R^2 = 0.05$, $P = 0.04$), and positively correlated with eGFR ($R^2 = 0.22$, $P < 0.0001$, *Figure 2C*). In analyses restricted to the persons with diabetes, MFR was negatively correlated with CAC ($R^2 = 0.15$, $P = 0.004$), UACR ($R^2 = 0.25$, $P < 0.0001$), age ($R^2 = 0.07$, $P = 0.04$), and diabetes duration ($R^2 = 0.19$, $P = 0.0005$), and positively correlated with eGFR ($R^2 = 0.22$, $P = 0.0002$).

In the total population, CAC was negatively correlated with eGFR ($R^2 = 0.06$, $P = 0.02$) and positively correlated with UACR ($R^2 = 0.05$, $P = 0.04$), age ($R^2 = 0.21$, $P < 0.0001$), and 24-h systolic blood pressure ($R^2 = 0.10$, $P = 0.004$). In the diabetic population, CAC was positively correlated with age ($R^2 = 0.30$, $P < 0.0001$) and diabetes duration ($R^2 = 0.17$, $P = 0.002$) but not UACR ($R^2 = 0.009$, $P = 0.50$).

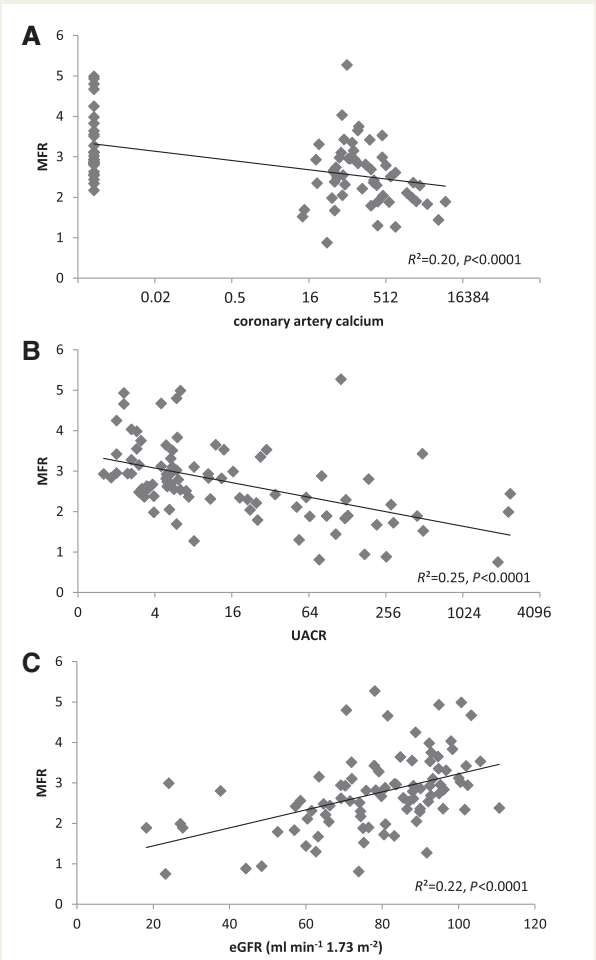

Figure 2 Correlations between myocardial flow reserve (MFR) and (*A*) coronary artery calcium, (*B*) urinary albumin creatinine ratio (UACR) and (*C*) estimated glomerular filtration rate (eGFR).

Variables association with MFR in adjusted analyses

In the total cohort, higher UACR ($P = 0.02$), higher age ($P = 0.009$), lower eGFR ($P = 0.03$), and smoking ($P = 0.02$) were associated with lower MFR in multivariate linear regression ($R^2 = 0.39$). In analyses restricted to the persons with diabetes, higher UACR ($P = 0.02$), female sex ($P = 0.02$), higher age ($P = 0.05$), and longer diabetes duration ($P = 0.02$) were independently associated with lower MFR ($R^2 = 0.54$).

Variables association with CAC in adjusted analysis

Higher age and 24-h systolic blood pressure were associated with higher CAC in multivariate linear regression ($R^2 = 0.34$) in the total cohort ($P \leq 0.03$). Further inclusion of MFR added to the model ($R^2 = 0.41$) and was significantly associated with higher CAC ($P = 0.004$). In analyses restricted to the persons with diabetes, longer diabetes duration ($P = 0.03$) and higher age ($P = 0.0002$) were associated with higher CAC ($R^2 = 0.44$). Further inclusion of MFR added to the model ($R^2 = 0.46$), but was not significantly associated with CAC ($P = 0.22$).

Additional analysis

To avoid the potential confounding effect of epicardial stenosis on MFR, we performed a sensitivity analysis excluding all participants with known CAD or reversible and/or irreversible ischaemia revealed by cardiac PET ($n = 10$). MFR was 3.0 ± 0.79; 3.1 ± 0.79, and 2.3 ± 0.89 in controls, in persons with NORMO, and in persons with MACRO, respectively. These results are presented in Supplementary data online, *Table S1*. The results were confirmatory as the differences in MFR and in MFR <2.5 between normoalbuminuric participants and controls were non-significant, and the differences between macroalbuminuric and normoalbuminuric participants were significant ($P \leq 0.002$). After adjustment for sex, age, 24-h systolic blood pressure, eGFR, and smoking, MFR remained lower and the frequency of reduced MFR higher in persons with MACRO compared to NORMO ($P \leq 0.03$). After additional adjustment for HbA_{1c}, LDL cholesterol, and diabetes duration, the difference in MFR lost significance ($P = 0.10$), however, the difference in reduced MFR (<2.5) persisted ($P = 0.007$).

Discussion

The main findings in this study were (i) the myocardial microvascular function was comparable in the healthy controls and the persons with Type 1 diabetes and NORMO; but impaired in the presence of MACRO and also in the presence of another microvascular complication: retinopathy; (ii) the coronary calcification was high in persons with Type 1 diabetes, but was to a larger extent explained by age and diabetes duration than presence of albuminuria. Our findings signify that microvascular impairment occurs in multiple microvascular beds and we detected microvascular injury in the heart which potentially affects how heart disease in Type 1 diabetes should be understood and treated.

Very little data on myocardial blood flow in Type 1 diabetes has been published until now. Older studies in a limited number of diabetic persons (mix of Types 1 and 2) referred for coronary arteriography have shown reduced MFR in persons with diabetes compared to persons without diabetes.[20–22] The ability to measure MFR non-invasively by cardiac PET allows examinations of larger and less selected populations. In 2783 consecutive patients referred for cardiac PET Murthy *et al.*[23] demonstrated that the rate of cardiac death for the diabetes patients ($n = 1172$, type of diabetes not specified) without CAD but with impaired MFR was comparable to that for non-diabetic patients with CAD. The cardiac mortality rate for persons with diabetes but without CAD and preserved MFR was very low. This emphasizes impaired MFR as a powerful, independent predictor of cardiac death in diabetes.

In this study by Murthy *et al.*, the patients had symptoms of chest pain and/or dyspnoea and more than 60% had previous cardiovascular disease. Therefore, the median values for MFR was very low (1.6 for the patients with diabetes and 1.9 for the non-diabetics) and the cut-off for impaired MFR was chosen accordingly. In our population, we anticipated that the prevalence of cardiovascular disease was considerable lower, and therefore, that the median MFR was higher. In accordance with our expectations, the median value for MFR was 2.7 in our study.

Knowledge about the myocardial microvascular function in Type 1 diabetes is sparse. Our study showed that myocardial microvascular function was comparable in the healthy controls and the persons with Type 1 diabetes and NORMO. Pitkänen *et al.*[6] reported impaired MFR quantified by cardiac PET in 12 young men with Type 1 diabetes and NORMO compared with 12 healthy matched volunteers (3.76 ± 1.69 vs. 5.31 ± 1.86, $P < 0.05$). Similarly, a study including 35 young subjects with diabetes (18 with Type 1 and 17 with Type 2) and 11 age-matched healthy controls, showed that the MFR was comparable in the subjects with Type 1 and Type 2 diabetes, but lower than in the controls.[24] In comparison, our study population consisted of older patients with a longer duration of diabetes, which may account in part for the different results.

In our study, myocardial microvascular function was impaired in the presence of MACRO and this association persisted after adjustment for traditional cardiovascular risk factors including the variables associated with lower MFR in our cohort (higher UACR, female sex, higher age, and longer duration of diabetes). MFR may capture both epicardial artery disease as well as microvascular disease. When we used CAC score as a co-variate (in absence of direct atheroquantification such as CT-angiogram-derived plaque burden) to test whether there was a true difference in microvascular disease between the participants with MACRO compared to NORMO, MFR remained lower and the frequency of reduced MFR higher in persons with MACRO compared to NORMO. To further disentangle if the association between albuminuria and MFR simply reflected existing clinically diagnosed CAD, we performed a separate set of analysis excluding the 10 participants with known CAD and/or ischaemia revealed by cardiac PET/CT. Our results were confirmatory indicating that there is a separate microvascular injury in the heart in Type 1

diabetes and it is found especially in the persons with further microvascular damage. Our findings in Type 1 diabetes are concordant with results from a study published in 2004 that demonstrated impaired MFR, measured by cardiac PET, in 16 persons with Type 1 diabetes with microangiopathy as compared to 12 without ($P < 0.05$). Microangiopathy was defined as presence of non-proliferative diabetic retinopathy ($n = 14$), microalbuminuria ($n = 2$), and/or peripheral neuropathy ($n = 10$).[25] We have previously reported impaired MFR in persons with Type 2 diabetes and albuminuria (UAER ≥30 mg/24 h, $n = 30$) as compared to NORMO ($n = 30$).[7] Recently, these results have been confirmed in a larger study including 118 patients with Type 2 diabetes demonstrating that MFR decreased progressively in relation to higher urinary albumin excretion.[26]

Knowledge about the myocardial microvascular function in Type 1 diabetes and the link to retinopathy is also limited. A cardiac PET study in 21 young men with Type 1 diabetes and NORMO found no difference in MFR in participants with or without retinopathy nor between patients with diabetes without retinopathy and 12 non-diabetic controls ($P \geq 0.2$).[5] Comparable results were shown in another small study including 12 young men with Type 1 diabetes and NORMO; here, MFR was similar in the presence of mild background retinopathy ($n = 5$) as in patients without retinopathy.[6] However, these results are in contrast to a small study ($n = 29$) in Type 2 diabetes demonstrating reduced MFR, measured by cardiac catheterization, in the presence of retinopathy compared with no retinopathy (1.9 ± 0.4 vs. 2.8 ± 0.3, $P < 0.01$). Data on renal function or albumin excretion were not presented.[27] We demonstrated an association between impaired microcirculation in the heart and proliferative retinopathy. In contrast to the two small studies in young men with Type 1 diabetes, our study population was larger, older, and with longer diabetes duration.

Since diabetic retinal disease often coexists with diabetic renal disease, it has been difficult to discern their individual association to cardiovascular disease in Type 1 diabetes. Our study was intentionally stratified by albuminuria and the association between MFR and retinopathy was partly explained by the albumin excretion rate, signifying that retinopathy had no independent association to the myocardial microcirculation. Ninety-seven percent of the persons with MACRO in our study had retinopathy underlining the close relation between retinal and renal disease in Type 1 diabetes.

CAC is an accepted non-invasive measure of atherosclerotic burden. Our findings of higher CAC in people with Type 1 diabetes than in non-diabetic control subjects are in concordance with the results from the Coronary Artery Calcification in Type 1 Diabetes Study.[28] We report comparable CAC score between persons with MACRO and NORMO, however, investigating the association dichotomized at a CAC score >300, we found a higher frequency of elevated CAC score in persons with MACRO compared to NORMO. It may be that there is an association between CAC and albuminuria in Type 1 diabetes, but we might have limited power to detect it because of the skewed distribution of CAC scores.

Clinical implications

Our findings suggest microvascular myocardial damage is common in Type 1 patients and can be identified with MACRO. How best to treat the microvascular damage in the heart remains to be established in future studies in contrast to the macrovascular damage known to respond to antihypertensive and lipid-lowering medication.

Strengths and limitations

The strength of this study is that to our knowledge this is the first evaluation of MFR and CAC in Type 1 diabetes in a cohort stratified by albuminuria by design. Potential limitations include that (i) the power calculation was based on differences in MFR between albuminuric groups and not retinopathy; and (ii) the cross-sectional nature of our study precludes the assessment of causal relationships.

Conclusion

In persons with Type 1 diabetes, we have demonstrated (i) impaired MFR in persons with MACRO compared to NORMO; and (ii) impaired MFR in persons with proliferative compared to simplex retinopathy. Our findings support the hypothesis that albuminuria reflects widespread vascular damage in Type 1 diabetes and that microvascular impairment occurs in multiple microvascular beds. Prospective studies are needed to establish the role of MFR in cardiovascular disease risk prediction in Type 1 diabetes.

Supplementary data

Supplementary data are available at *European Heart Journal - Cardiovascular Imaging* online.

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Conflict of interest: None declared.

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Paper 3

Cardiac Autonomic Function Is Associated With Myocardial Flow Reserve in Type 1 Diabetes

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The link between cardiac autonomic neuropathy and risk of cardiovascular disease is highlighted as an area in which research is needed. This study was undertaken to evaluate the association between measures of cardiac autonomic function and cardiac vascular function in type 1 diabetes using new and sensitive methods. This was a cross-sectional study in patients with type 1 diabetes, stratified by normoalbuminuria (*n* = 30) and macroalbuminuria (*n* = 30), and in healthy control subjects (*n* = 30). Cardiac autonomic function was evaluated using heart rate variability (HRV) indices, cardiovascular autonomic reflex tests (CARTs), and cardiac ^{123}I-metaiodobenzylguanidine (MIBG) imaging. Cardiac vascular function was assessed as myocardial flow reserve (MFR) measured by cardiac ^{82}Rb-positron emission tomography/computed tomography. The measures of cardiac autonomic function (except low frequency–to–high frequency ratio and the Valsalva test ratio) were positively correlated to MFR in unadjusted analysis. All the HRV indices lost significance after adjustment for age and heart rate. After further adjustment for relevant cardiovascular risk factors, the late heart-to-mediastinum ratio directly measuring the function of adrenergic receptors and sympathetic integrity (from the MIBG scintigraphy) and the 30-to-15 ratio (a CART), remained positively associated with MFR ($P \leq 0.04$). Cardiac autonomic dysfunction, including loss of cardiac sympathetic integrity in type 1 diabetes, is associated with and may contribute to impaired myocardial blood flow regulation.

Cardiac autonomic neuropathy (CAN) is defined as impairment of autonomic control of the cardiovascular system. CAN involves damage to the autonomic nerve fibers that innervate the heart and blood vessels, resulting in abnormalities in heart rate control and vascular dynamics (1).

The cardiac autonomic function can be evaluated with simple bedside tests using heart rate variability (HRV) indices or response in heart rate to standing, slow breathing, or the Valsalva maneuver (cardiovascular autonomic reflex tests [CARTs]). These indirect tests can reveal altered sympathetic and parasympathetic activity. Cardiac radionuclide imaging using the nonmetabolized norepinephrine analog metaiodobenzylguanidine (MIBG) allows direct assessment of cardiac sympathetic integrity and may reveal CAN in the early stages before it can be detected by the HRV indices and the CARTs (2).

CAN is a severe and often overlooked complication in diabetes associated with increased mortality and silent myocardial ischemia (2). In a recent scientific statement from the American Heart Association and the American Diabetes Association, the pathophysiology linking CAN to the risk of cardiovascular disease in type 1 diabetes was highlighted as an area in which research is needed (3). We have demonstrated an association between impaired cardiac autonomic function and reduced MFR in type 2 diabetes (4). In the current study, we examine whether this association extends to type 1 diabetes.

The myocardial flow reserve (MFR) reflects the function of the large epicardial arteries and the microcirculation of the myocardium. MFR is a powerful independent predictor of cardiac death and nonfatal myocardial infarction in diabetes (5), and can be calculated using cardiac

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^{82}Rb-positron emission tomography (PET)/computed tomography (CT) imaging.

Using MIBG imaging, HRV indices, and CARTs, this study was undertaken to evaluate the association between the cardiac autonomic function and cardiac vascular function assessed as the MFR measured by cardiac ^{82}Rb-PET/CT.

RESEARCH DESIGN AND METHODS

Study Population

We included 60 participants with type 1 diabetes stratified by albuminuria: 30 with normoalbuminuria (<30 mg/24 h or 30 mg/g creatinine and without a history of albuminuria) and 30 with the presence of or a history of macroalbuminuria (≥300 mg/24 h or 300 mg/g creatinine in two of three consecutive urine collections). History of macroalbuminuria was defined as albuminuria ≥300 mg/24 h or 300 mg/g creatinine in two of three consecutive measurements documented in the electronic medical files at any time point.

These participants were recruited from the Steno Diabetes Center Copenhagen among subjects participating in a cross-sectional study focusing on detailed phenotyping of patients with type 1 diabetes with or without progressive renal complications. We stratified participants according to normoalbuminuria or macroalbuminuria, as one of the overall aims of the study was to examine the prevalence of impaired MFR in *1*) patients with type 1 diabetes and normoalbuminuria compared with *2*) patients with type 1 diabetes and macroalbuminuria (6). We matched the persons with normoalbuminuria and macroalbuminuria by sex and age. Exclusion criteria included the following: *1*) nondiabetic kidney disease; *2*) renal failure (estimated glomerular filtration rate [eGFR] <15 mL/min/1.73 m^2), kidney transplantation, or dialysis; *3*) change in renin angiotensin aldosterone system blocking treatment 1 month prior to study participation; and *4*) contraindications for cardiac PET/CT.

As control subjects, we used 30 healthy persons without diabetes recruited from a newspaper advertisement. These participants were studied at our center in 2013 using an identical protocol and the same equipment; results on these control subjects have previously been reported (4,7).

As in our study in type 2 diabetes, the sample size calculation was based on MFR (6,7). Data were not available in a comparable cohort of individuals with type 1 diabetes. Participants were grouped according to disease status (diabetes vs. control subjects) and albuminuria analogous to our previous study in type 2 diabetes; however, we allowed participants to have a history of cardiovascular disease in the current study. In our previous study in type 2 diabetes, the sample size sufficient to detect differences in MFR was also adequate to detect associations between MFR and cardiac autonomic function assessed by MIBG (4). We therefore anticipated the sample size to be sufficient to detect these associations in the current study as well.

The study was conducted in compliance with the Declaration of Helsinki, and the protocol was reviewed and approved by the appropriate independent ethics committee. All participants provided written informed consent.

Clinical Measurements

We used high-performance liquid chromatography to measure HbA_{1c}, an enzymatic method to measure plasma creatinine (Hitachi 912; Roche Diagnostics, Mannheim, Germany), and the CKD-EPI equation was used to calculate eGFR (8). An enzyme immunoassay was used to measure the urinary albumin creatinine rate (UACR) in three consecutive morning urine samples. A cuff device (model TM2430; Takeda, Tokyo, Japan) (9) measured 24-h blood pressure. Height and weight were measured and used to calculate BMI (weight in kilograms divided by the square of height in meters). We obtained a detailed medical history, including treatment, previous cardiovascular disease, and smoking status (current smoking was defined as one or more cigarettes/cigars/pipes a day). Information on physical activity during leisure time (hours/week) was obtained from a questionnaire. The medical history was cross-referenced with electronic patient records.

All clinical measurements for control subjects were assessed and defined as described above with the following two exceptions: *1*) urinary albumin excretion rate (UAER) was measured by an enzyme immunoassay in two 24-h urine collections and *2*) 24-h blood pressure was recorded using a tonometric wrist device (BPro; HealthStats, Singapore) (10).

Hybrid Cardiac PET/CT Imaging

All participants underwent a dynamic, gated cardiac PET/CT after administration of 1,100 MBq of ^{82}Rb (CardioGen-82; Bracco Diagnostics, Monroe Township, NJ). We used a hybrid PET/CT scanner in 3D mode (Siemens Biograph mCT 128; Siemens, Munich, Germany). Cardiac PET/CT was performed at rest and during stress. Stress was assessed by adenosine infusion at 140 μg/kg/min for 6 min to induce maximum myocardial hyperemia. Myocardial blood flow was automatically calculated as previously described in detail (7).

Cardiac Autonomic Function by ^{123}I-MIBG Scintigraphy

All 60 participants with diabetes and 14 of the control subjects (the first consecutively included) underwent cardiac ^{123}I-MIBG scintigraphy. One hour before intravenous tracer injection (200 MBq ^{123}I-MIBG), participants were given potassium iodine to block thyroid iodine uptake. Planar anterior-posterior images were taken after 15 min (early) and after 240 min (late). We used a Philips SKYLight Gamma Camera with JETstream software (Philips Medical Systems, Best, the Netherlands) with medium energy collimator, 256 × 256 matrix, and an acquisition time of 600 s. A 15% energy window set symmetrically over the 159 keV photo peak was used to image ^{123}I. One

experienced observer assessed the images using the Extended Brilliance Workspace NM Application Suite version 4.5.3.40140 (Philips Medical Systems). According to guidelines, a region of interest was drawn *1*) following the epicardial contour and *2*) as a rectangle above the mediastinum (11). The mean count within these two regions of interest was reported for early and late anterior images.

The myocardial washout rate from early to late images was calculated according to previously published guidelines (11). Evidence supports the use of the late heart-to-mediastinum ratio for assessment of symptomatic autonomic neuropathy (12).

Cardiac Autonomic Function by Heart Rate Analyses

We used the Vagus device (Medicus Engineering, Aarhus, Denmark) to measure HRV in time and frequency domains and during conventional CARTs. The Vagus device automatically measures electrocardiographic signals with a sampling frequency of 1,000 Hz. All tests were performed between 8:00 A.M. and 3:00 P.M. in a quiet examination room by trained staff. In accordance with recommendations, we used a standard protocol (13), and participants were fasted for 4 h and advised to abstain from hard physical activity for 24 h before the examination.

HRV Indices

Resting heart rate was calculated from 5-min measures of R-R interval. We calculated the SD of normal-to-normal (SDNN) intervals and the root mean square of successive differences (RMSSD) as time-domain HRV indices. SDNN is a measure of combined sympathetic and parasympathetic activity, and RMSSD is predominantly a measure of sympathetic activity.

The following frequency-domain HRV indices were calculated using fast Fourier transformation: low-frequency (LF) power (0.04–0.15 Hz), high-frequency (HF) power (0.15–0.4 Hz), and total power (≤0.4 Hz). We also calculated the ratio of LF-to-HF power. HF power predominantly reflects parasympathetic activity, whereas LF power is influenced by sympathetic and parasympathetic tone and baroreflex sensitivity, and LF power mainly contributes to total power (2,14,15).

CARTs

The CARTs included the following: *1*) response to standing (30-to-15 ratio), calculated as the ratio between longest (around the 30th heartbeat after the rise) and the shortest R-R interval (around the 15th heartbeat after the rise) during and shortly after standing from a supine position; *2*) deep breathing test for 1 min (expiration-to-inspiration [E-to-I] ratio) while sitting (the E-to-I ratio is calculated as the mean of the longest R-R interval divided by the mean of the shortest R-R interval during deep breathing during a deep breathing respiratory cycle; and *3*) the Valsalva test ratio, conducted as a 15-s exhalation through a 40 mmHg resistance mouthpiece while sitting. The Valsalva test ratio is calculated as the ratio of the longest and shortest R-R intervals during and immediately after the Valsalva maneuver. The 30-to-15 ratio and the E-to-I ratio are predominantly measures of parasympathetic tonus, whereas the Valsalva test ratio is influenced by sympathetic and parasympathetic tone (1,2). The CARTs were evaluated according to age-related reference intervals (16). CAN was defined using the American Diabetes Association criteria (17), and we defined CAN as *1*) "no CAN" when no pathological CARTs were detected or if only one abnormal CART was detected and *2*) definite CAN if two or three abnormal CARTs were detected. A diagnosis of CAN based on the CARTs was not evaluated in the participants with fewer than two CART results (n = 7) or in participants with two discrepant CART results (one normal and one pathological) (n = 6).

Sudomotor Function

In the 60 participants with diabetes, we assessed the function of small autonomic fibers using an electrochemical skin conduction test on the hands and feet (Sudoscan; Impeto Medical, Paris, France) (18). We applied age- and sex-stratified electrochemical skin conduction thresholds for hands and feet (19). Sudomotor dysfunction contributes to detection of autonomic dysfunction in peripheral diabetic neuropathy (2).

Statistical Analysis

The distribution of UACR, UAER, alcohol intake, and the time and frequency domain HRV indices was skewed, and these variables were $\log_2$ transformed for analyses and presented as the median with interquartile range (IQR). Continuous data with approximately normal distributions are given as the mean and SD. Categorical variables are provided as total numbers and percentages. Independent-samples t test was applied when comparing differences in continuous variables between two groups (e.g., control subjects vs. normoalbuminuric participants and normoalbuminuric vs. macroalbuminuric participants) and the χ^2 test or Fisher exact test, as appropriate, when analyzing categorical variables. We pooled all participants in the linear regression analyses and applied stepwise adjustment. Unadjusted models were used to determine whether an association existed between each of the measures of cardiac autonomic function and MFR (model 1). Subsequent adjustment included age (model 2); age and heart rate (model 3); age, heart rate, sex, 24-h systolic blood pressure, BMI, HbA_{1c}, UACR or UAER, and smoking (model 4); and additional adjustment of model 4 for physical activity and the prescribed drugs that could influence HRV or organ uptake of MIBG (β-blocker, amlodipine, tramadol, tricyclic antidepressants, and chlorpromazine) in a final model, model 5. The CARTs were not adjusted for heart rate since heart rate can influence these tests (13). Due to the risk of bias by indication, we did not adjust for total cholesterol, since it was highest in the healthy control subjects, most likely due to medical treatment of the participants with diabetes. Standardized regression coefficients are reported.

Pearson correlations were used to explore associations of the measures of cardiac autonomic function with each other, and ANCOVA was applied to determine if these correlations persisted after adjustment.

A two-tailed $P < 0.05$ was interpreted as significant. Statistical analyses were performed using SAS software (version 9.4; SAS Institute).

RESULTS

Clinical Characteristics

In the total cohort, the mean ± SD age was 59.3 ± 9.5 years and 41% were female. Clinical characteristics for the participants in the three groups are summarized in Table 1.

Among medications influencing HRV or organ uptake of MIBG (Table 1) (11), treatment with amlodipine was prescribed in 11 participants (18%), β-blockers in 9 participants (15%), tramadol in 3 participants (5%), and both a tricyclic antidepressant and chlorpromazine in 1 participant.

Seven participants with macroalbuminuria had a history of coronary artery disease (two had coronary artery bypass graft and five had percutaneous coronary intervention). Quantified by the PET/CT, 10 participants (9 with macroalbuminuria [of which 7 had a history of coronary artery disease] and 1 healthy control subject) had reversible ischemia. The median (IQR) extent was 24% (14–29%). In five of these participants, irreversible ischemia (fixed perfusions defects) was also observed (all five had macroalbuminuria). Median extent was 19% (16–25%).

All three CARTs and the late heart-to-mediastinum ratio were higher in the control subjects compared with the normoalbuminuric participants with diabetes ($P \leq 0.02$). The CARTs and the late heart-to-mediastinum ratio were comparable between participants with diabetes and normoalbuminuria or macroalbuminuria ($P \geq 0.05$). The HRV indices were comparable between groups ($P \geq 0.05$), except total power, which was higher in the control subjects compared with the normoalbuminuric participants ($P = 0.03$). More persons with macroalbuminuria had bilateral sudomotor dysfunction in hands and feet compared with persons with normoalbuminuria ($P = 0.006$ for both).

Prevalence of Impaired Cardiac Autonomic Function

Defined based on the CARTs, 14 participants (18%) had definite CAN and 63 had no CAN (82%). In our population, the mean ± SD late heart-to-mediastinum ratio was 2.9 ± 0.39 in the healthy control subjects compared with 2.5 ± 0.45 in the participants with diabetes. The lower 5% percentile for the late heart-to-mediastinum ratio in the healthy control subjects was 2.4. With this applied as a cutoff to the participants with diabetes, a total of 9 participants (30%) with normoalbuminuria and 15 participants (50%) with macroalbuminuria had impaired cardiac autonomic function.

Correlations Between the Measures of Cardiac Autonomic Function and MFR

Correlations between the measures of cardiac autonomic function and MFR are presented in Table 2. All measures, except LF-to-HF ratio and the Valsalva test ratio, were positively correlated to MFR in unadjusted analysis (model 1, $P \leq 0.004$) and after adjustment for age (model 2, $P \leq 0.01$). After further adjustment for heart rate, the late heart-to-mediastinum ratio was positively associated with MFR ($P = 0.003$), but all the HRV indices lost significance ($P \geq 0.07$). After additional adjustment for other risk factors (model 4), the late heart-to-mediastinum ratio (β per 1 SD increase, 0.24; $P = 0.03$) and the 30-to-15 ratio (β per 1 SD increase, 0.25; $P = 0.04$) remained positively associated with MFR. In the final model, model 5, the late heart-to-mediastinum ratio (β per 1 SD increase, 0.28; $P = 0.01$) and the 30-to-15 ratio (β per 1 SD increase, 0.30; $P = 0.01$) remained positively associated with MFR.

Figure 1 illustrates the unadjusted correlation of MFR to the late heart-to-mediastinum ratio (Fig. 1*A*) and the 30-to-15 ratio (Fig. 1*B*). The levels of MFR according to CAN status based on the CARTs are illustrated in Fig. 2*A*.

Agreement Between the Measures of Cardiac Autonomic Function

Pearson correlation coefficients between the measures of cardiac autonomic function are presented in Supplementary Table 1. Of the HRV indices and CARTs, only the 30-to-15 ratio correlated positively with the late heart-to-mediastinum ratio (unadjusted, $P = 0.002$). This association persisted after adjustment for age ($P = 0.003$), but lost significance after adjustment for additional risk factors ($P = 0.72$). The HRV indices and CARTs were, with few exceptions, strongly correlated.

Exclusion of Participants With Known Coronary Artery Disease or Ischemia Identified by Cardiac PET

To avoid the potential confounding effect of ischemic heart disease, we performed all analyses excluding the participants with known coronary artery disease or reversible and/or irreversible ischemia identified by cardiac PET ($n = 10$).

For the comparisons of late heart-to-mediastinum ratio, CARTs, and HRV indices among the three groups, results were confirmatory.

When evaluating the prevalence of impaired cardiac autonomic function using CARTs, 10 participants had definite CAN (14%) and 59 had no CAN (86%). Applying a cutoff for the late heart-to-mediastinum ratio of 2.4 (as described previously) showed that 9 participants with normoalbuminuria (30%) and 9 participants with macroalbuminuria (42%) had impaired cardiac autonomic function.

In analysis of the correlations between the measures of cardiac autonomic function and MFR, the late heart-to-mediastinum ratio and the 30-to-15 ratio were positively

Table 1—Clinical characteristics and measures of cardiac autonomic function

	Control subjects (n = 30)	Normoalbuminuria (n = 30)	Macroalbuminuria (n = 30)	P (control subject vs. normoalbuminuria)	P (normoalbuminuria vs. macroalbuminuria)
Female	12 (40)	12 (40)	13 (43)	1.0	0.79
Age (years)	59.8 ± 9.9	59.8 ± 9.1	58.2 ± 9.9	0.99	0.52
Known diabetes duration (years)		32.6 ± 12.7	41.4 ± 13.3		0.01
BMI (kg/m^2)	24.7 ± 3.4	25.6 ± 4.1	27.2 ± 4.2	0.38	0.15
24-h systolic blood pressure (mmHg)	127 ± 13	135 ± 9	138 ± 11	0.009	0.26
24-h diastolic blood pressure (mmHg)	79 ± 8	78 ± 6	77 ± 5	0.88	0.48
Heart rate (bpm)	61 ± 8	69 ± 12	72 ± 12	0.003	0.41
HbA_{1c} (mmol/mol)	35.8 ± 1.9	61.3 ± 8.3	66.3 ± 11.7	<0.0001	0.70
HbA_{1c} (%)	5.4 ± 0.17	7.8 ± 0.76	8.2 ± 1.07	<0.0001	0.70
LDL cholesterol (mmol/L)	3.4 ± 0.7	2.3 ± 0.7	2.1 ± 0.8	<0.0001	0.32
eGFR (mL min^{-1} 1.73 m^{-2})	82.8 ± 13.1	89.1 ± 10.4	62.5 ± 23.1	0.043	<0.0001
UACR (mg/g)*	6 [5–10.5]	3 (3–5)	121 [53–283]	0.0002	<0.0001
Smokers	4 (13)	4 (14)	4 (14)	0.96	1.0
Alcohol (beverages/week)	8.5 (4–14)	7 (4–18)	6.5 (1–14)	0.87	0.91
Physical activity (hours/week)	5.0 ± 4.2	4.9 ± 5.3	6.1 ± 11.4	0.93	0.61
Treatment					
Antihypertensive	3 (10)	17 (57)	30 (100)	0.0001	<0.0001
RAAS inhibition	3 (10)	14 (47)	29 (97)	0.002	<0.0001
β-Blocker	0 (0)	1 (3)	8 (27)	1.0	0.03
Aspirin	1 (3)	11 (37)	19 (63)	0.0012	0.04
Lipid-lowering	0 (0)	21 (70)	24 (80)	<0.0001	0.37
Amlodipine	0 (0)	4 (13)	10 (33)	0.11	0.07
Known coronary artery disease	0 (0)	0 (0)	7 (23)	—	0.01
MFR	3.0 ± 0.79	3.1 ± 0.79	2.1 ± 0.92	0.74	<0.0001
HRV measures					
Time and frequency domains					
SDNN intervals (ms)	39.4 [28.6–53.0]	30.0 [20.5–49.6]	18.5 [13.3–34.8]	0.057	0.057
RMSSD (ms)	25 [20.4–39.45]	19.25 [10.1–30.9]	13.0 [6.5–20.1]	0.065	0.21
LF power (ms^2)	196.7 [78.3–308.7]	73.1 [24.7–238.3]	21.1 [14.6–92.5]	0.045	0.099
HF power (ms^2)	70.9 [45.9–131.5]	43.2 [12.0–87.1]]	22.3 [5.3–66.0]	0.077	0.20
LF/HF (ratio)	2.05 [1.38–4.25]	2.07 [1.34–3.52]	2.06 [0.89–3.46]	0.64	0.44
Total power (ms^2)	606.1 [253.6–1,106.2]	291.7 [118.6–644.5]	101.9 [54.6–447.4]	0.03	0.08
CARTs					
30-to-15 ratio (response to standing)	1.24 ± 0.17	1.12 ± 0.11	1.03 ± 0.25	0.0002	0.12
E-to-I ratio (deep breathing)	1.24 ± 0.15	1.15 ± 0.12	1.12 ± 0.0	0.02	0.28
Valsalva test ratio	1.77 ± 0.41	1.48 ± 0.19	1.39 ± 0.34	0.0045	0.33
CAN					
No CAN	28 (100)	22 (85)	13 (43)	0.047	0.03
CAN	0 (0)	4 (15)	10 (57)		

Continued on p. 1282

Table 1—Continued

	Control subjects (n = 30)	Normoalbuminuria (n = 30)	Macroalbuminuria (n = 30)	P (control subject vs. normoalbuminuria)	P (normoalbuminuria vs. macroalbuminuria)
^{123}I-MIBG imaging	n = 14	n = 30	n = 30		
Late heart-to-mediastinum ratio	2.9 ± 0.39	2.6 ± 0.38	2.3 ± 0.50	0.01	0.05
Sudomotor function					
Feet (μS)		69.9 ± 14.4	55.2 ± 24.4		0.008
Sudomotor dysfunction**		11 (38)	18 (62)		0.006
Hands (μS)		58.6 ± 17.0	47.5 ± 18.7		0.02
Sudomotor dysfunction**		8 (27)	18 (62)		0.006

Data represent total number (%), mean ± SD, or median [IQR]. CAN was defined as no CAN when no pathological CART results were detected or if only one abnormal CART result was detected and as definite if two or three abnormal CART results were detected, according to the American Diabetes Association criteria. RAAS, renin-angiotensin-aldosterone system. *UAER for control subjects. **We applied age- and sex-stratified electrochemical skin conduction thresholds when evaluating sudomotor dysfunction in hands and feet.

associated with MFR unadjusted (model 1; P = 0.002) and after adjustment for age (model 2; $P \leq 0.006$). The association between MFR and the late heart-to-mediastinum ratio remained significant after adjustment for additional risk factors (model 4) (β per 1 SD increase, 0.25; P = 0.046), whereas the 30-to-15 ratio lost significance (P = 0.08).

Supplementary Fig. 1 illustrates the correlation of MFR to the late heart-to-mediastinum ratio (Supplementary Fig. 1*A*) and the 30-to-15 ratio (Supplementary Fig. 1*B*). The levels of MFR according to CAN status based on the CARTs are shown in Fig. 2*B*.

Pearson correlation coefficients between the measures of cardiac autonomic function are presented in Supplementary Table 2. Results were largely confirmatory of the results presented for all participants (Supplementary Table 1), with the exception that the late heart-to-mediastinum ratio was not correlated with the 30-to-15 ratio, and HF power was not correlated with SDNN or LF-to-HF ratio.

Additional Analyses

Analyses only including the participants with diabetes were generally confirmatory; MFR was positively correlated with late heart-to-mediastinum ratio (P = 0.01), total power ($P < 0.001$), and 30-to-15 ratio ($P < 0.001$). These associations persisted after adjustment for age (model 2, $P < 0.001$). Only the association between MFR and the

Table 2—Unadjusted and stepwise adjusted associations between measures of cardiac autonomic function and MFR

	Model 1 unadjusted		Model 2 adjusted for age		Model 3 adjusted for age and heart rate		Model 4 adjusted for age, heart rate, and other risk factors**		Model 5 adjusted as model 4 + physical activity and medication***	
	β	P	β	P	β	P	β	P	β	P
^{123}I-MIBG imaging (n = 74)										
Late heart-to-mediastinum ratio	0.39	0.0004	0.38	0.0005	0.32	0.0027	0.24	0.03	0.28	0.01
HRV measures (n = 84)										
Time and frequency domains*										
SDNN intervals (ms)	0.34	0.0006	0.32	0.001	0.19	0.10	0.14	0.29	0.12	0.37
RMSSD (ms)	0.33	0.0009	0.30	0.002	0.16	0.20	0.14	0.30	0.12	0.39
LF power (ms^2)	0.34	0.0007	0.31	0.001	0.20	0.07	0.14	0.25	0.11	0.38
HF power (ms^2)	0.29	0.004	0.25	0.01	0.09	0.46	0.05	0.71	0.06	0.65
LF/HF (ratio)	0.10	0.32	0.13	0.18	0.15	0.10	0.09	0.30	0.06	0.55
Total power (ms^2)	0.32	0.001	0.30	0.002	0.18	0.14	0.12	0.36	0.10	0.47
CARTs										
30-to-15 ratio (response to standing, n = 83)	0.30	0.001	0.27	0.004			0.25	0.04	0.30	0.01
E-to-I ratio (deep breathing, n = 84)	0.30	0.002	0.26	0.006			0.15	0.11	0.19	0.05
Valsalva test ratio (n = 60)	0.14	0.19	0.14	0.19			−0.01	0.92	−0.002	0.99

*Log2 transformed for analyses. **Not included in adjustment for the CARTs. The β-estimates represent standardized effect. Other risk factors included sex, 24-h systolic blood pressure, BMI, HbA_{1c}, UACR (UAER in control subjects), and smoking. ***Treatment with β-blockers, amlodipine, tramadol, tricyclic antidepressants, or chlorpromazine.

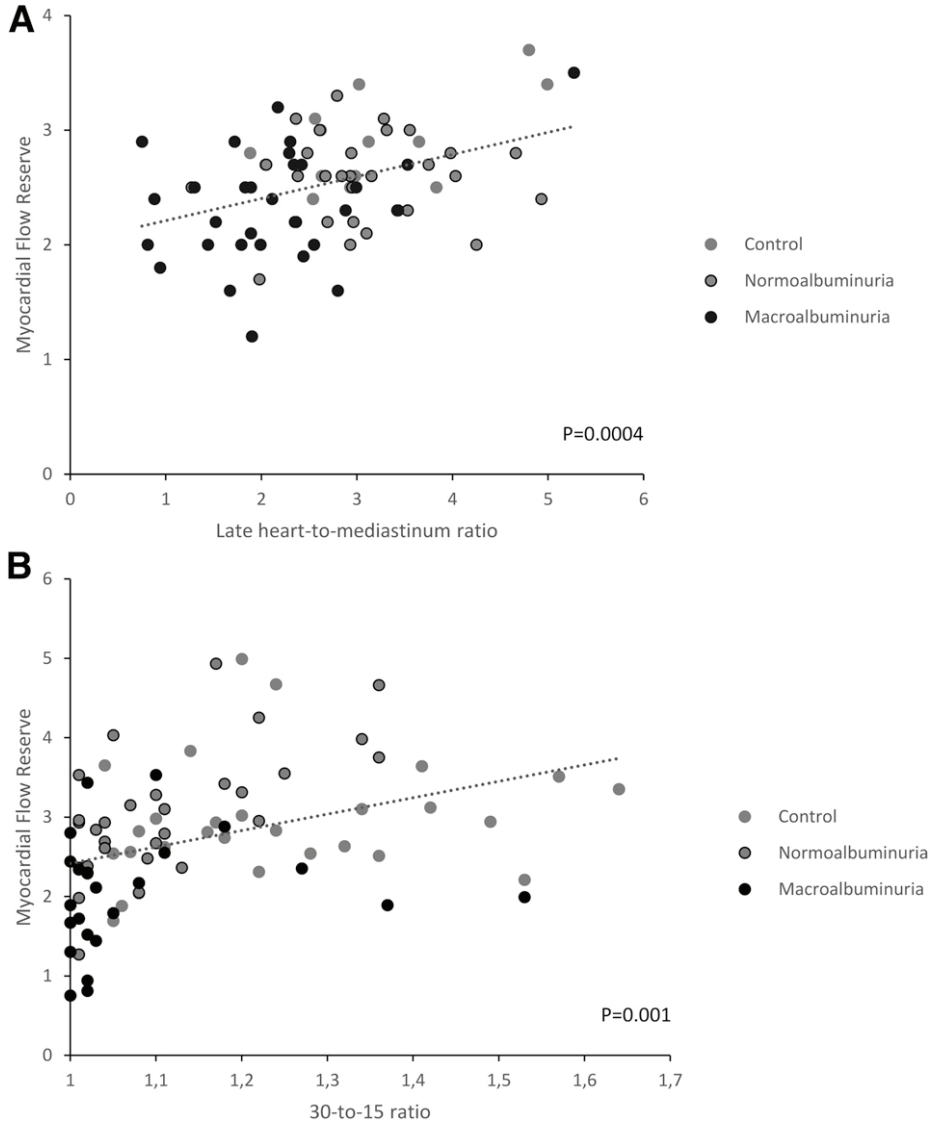

Figure 1— Correlations between MFR and measures of cardiac autonomic function: late heart-to-mediastinum ratio (*A*) and 30-to-15 ratio (*B*). All correlations were significant, $P \leq 0.001$.

30-to-15 ratio persisted after adjustment for additional risk factors (model 4, $P = 0.01$).

The myocardial washout rate of MIBG was not associated with the MFR ($P = 0.41$) or the late heart-to-mediastinum ratio ($P = 0.27$).

DISCUSSION

In this cross-sectional study in type 1 diabetes and healthy control subjects, we demonstrated a positive association between MFR and a comprehensive panel of cardiac autonomic function measures, including HRV indices, cardiac autonomic reflex tests, and the late heart-to-mediastinum ratio. We confirmed the positive association between MFR and the late heart-to-mediastinum ratio in analyses restricted to the participants free of coronary artery disease.

CAN affects both the sympathetic and parasympathetic part of the autonomic nervous system. When testing for CAN, it is therefore advised that a battery of examinations should be used to evaluate both branches of the autonomic nervous system, rather than a single test. The cardiovascular reflex tests recommended by the American Diabetes Association for the diagnosis of CAN and the HRV indices evaluate the function of both branches of the autonomic nervous system indirectly (20,21). With the introduction of radiolabeled analogs of norepinephrine, which are actively taken up by the sympathetic nerve terminals, it is now possible to directly assess the integrity of cardiac sympathetic nerve fibers (22). MIBG is an analog of norepinephrine labeled with ^{123}I to allow Gamma Camera imaging. A high heart-to-mediastinum ratio indicates an intact sympathetic system in the myocardium, whereas a low heart-to-mediastinum ratio is an indicator of impaired cardiac sympathetic integrity (23).

The aim of our study was to explore the potential link between cardiac autonomic function and impaired cardiac

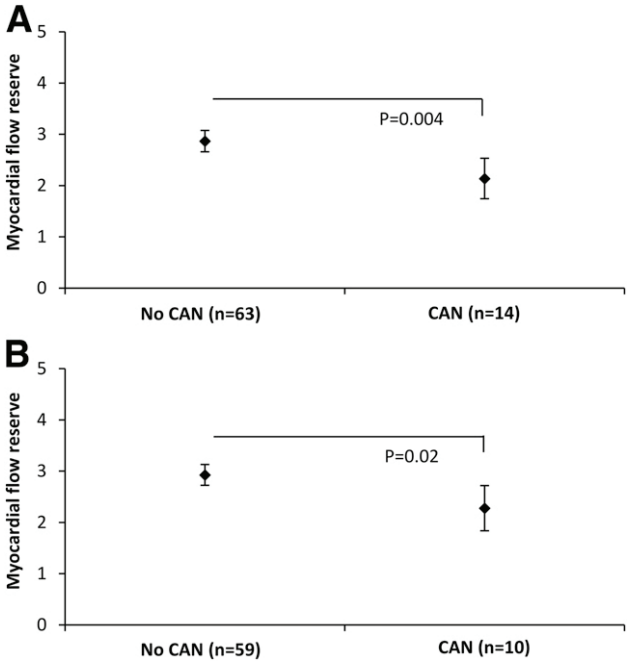

Figure 2—MFR according to CAN status: all participants (*A*) and participants with known coronary artery disease or reversible and/or irreversible ischemia identified by cardiac PET (*B*) (n = 10) were excluded. Data are presented as the mean with 95% CI. CAN diagnosis was based on CARTs using the American Diabetes Association criteria; we defined CAN as *1*) no CAN when no pathological CART results were detected or if only one abnormal CART result was detected and *2*) definite CAN if two or three abnormal CART results were detected. Differences between groups were analyzed with independent-samples t tests.

vascular function, as this could contribute to a better understanding of the pathophysiology linking CAN to risk of cardiovascular disease. The gold standard for assessing the myocardial blood flow is PET imaging (24). We used cardiac ^{82}Rb PET/CT for the assessment of MFR. MFR is calculated as the ratio between resting and maximal induced myocardial blood flow. It reflects to what extent the myocardial blood flow can increase during pharmacologically induced stress. MFR mirrors the function of the large epicardial arteries and the microcirculation of the myocardium and is a strong predictor of cardiovascular disease and death, even in the absence of known coronary artery disease (5,25). Moreover, we used the results from the ^{82}Rb PET/CT to accurately identify participants with myocardial perfusion defects, enabling us to address the potential confounding effect of coronary artery disease on the association between cardiac autonomic function and impaired cardiac vascular function.

We demonstrated associations between a wide range of different measures of cardiac autonomic function and MFR. The associations between the late heart-to-mediastinum ratio and MFR and between the 30-to-15 ratio and MFR were strongest, as they persisted even after adjustment for appropriate risk factors. A major challenge in studies investigating the possible association between cardiac autonomic function and myocardial blood flow is to address the potential confounding by the presence of ischemic heart disease, which might affect both the cardiac autonomic function and MFR. To further disentangle whether the association between cardiac autonomic function and MFR simply reflected existing clinically diagnosed coronary artery disease, we performed a separate set of analyses excluding the 10 participants with known coronary artery disease and/or ischemia revealed by cardiac PET/CT. The association between the late heart-to-mediastinum ratio and MFR remained significant in these analyses even after comprehensive adjustment. These findings implicate that cardiac autonomic dysfunction may be associated with impaired stimulated blood flow in type 1 diabetes even after eliminating the influence of coronary artery disease.

A study in persons undergoing cardiac transplantation was the first to demonstrate a role of cardiac adrenergic signals in the regulation of myocardial blood flow (26). The findings provoked speculations on how impaired MFR could aggravate existing endothelial dysfunction or atherosclerotic lesions and, in periods of increased oxygen demand, could lead to myocardial ischemia and left ventricular dysfunction, even in the absence of atherosclerotic lesions (27). Very few data on cardiac autonomic function and myocardial blood flow in type 1 diabetes have been published until now. A study in 28 persons with either type 1 or type 2 diabetes (27) reported impaired vasodilator response of coronary resistance vessels in the presence of sympathetic nerve dysfunction. Likewise, a study in 14 persons with type 1 diabetes (28) demonstrated that the myocardial blood flow differed regionally in relation to islets of persistent cardiac sympathetic integrity. Later on, the same authors could not confirm the regional differences in vasodilator reserve, but showed a relation between sympathetic dysfunction (assessed by ^{11}C hydroxyephedrine PET) and a wide range of abnormalities in myocardial blood flow (assessed by ^{13}N ammonia PET) in 28 persons with type 1 diabetes (29). Myocardial injury is an advanced condition involving several mechanisms that may or may not be linked with sympathetic integrity. More studies are needed to confirm our observed association between cardiac autonomic function and impaired cardiac vascular function in type 1 diabetes using a methodology that directly addresses the confounding effect of ischemia.

Another measure obtained from the MIBG scintigraphy is the myocardial washout rate, calculated as the difference in myocardial counts between the early and the late image, normalized to the mediastinum counts. The washout rate is considered to reflect adrenergic activity with a high washout rate, indicating high adrenergic activity. The clinical relevance of the washout rate is not clear in diabetes. The washout rate in our study population was not related to MFR or to late heart-to-mediastinum ratio.

We demonstrated a general impaired function of the cardiac autonomic system in persons with type 1 diabetes and normoalbuminuria compared with healthy control subjects, which is in line with previous findings (30). Furthermore, we demonstrated CAN to be more frequent

in persons with type 1 diabetes and macroalbuminuria compared with normoalbuminuria. Autonomic imbalance has been linked to chronic kidney disease and its progression in type 1 diabetes (31), and our findings add to the literature, with previous studies in type 1 diabetes (32,33) demonstrating a lower cardiac autonomic function in persons with macroalbuminuria compared with normoalbuminuria.

In relation to peripheral autonomic dysfunction, we demonstrated significantly higher prevalence of bilateral sudomotor dysfunction in the feet or hands in the participants with macroalbuminuria compared with normoalbuminuria.

For cardiac autonomic integrity assessed as the late heart-to-mediastinum ratio, our findings in type 1 diabetes are in concordance with our previous observations in type 2 diabetes, where we demonstrated the late heart-to-mediastinum ratio to be comparable in persons with type 2 diabetes and normoalbuminuria or albuminuria, but lower in healthy control subjects compared with the persons with diabetes and normoalbuminuria (4).

We found agreement between the HRV indices and the CARTs, and between the individual measures of cardiac autonomic function within these two groups. The late heart-to-mediastinum ratio was positively correlated with the 30-to-15 ratio, but not with the other CARTs or HRV indices. This correlation lost significance following adjustment for cardiovascular risk factors.

Strengths and Limitations

The strengths of this study include the robust measures of cardiac autonomic function and MFR assessed by PET imaging. Although the study is large compared with previous studies on this topic, the relatively small sample size increases the risk of type II errors. The sample size was anticipated, based on results from a previous study in type 2 diabetes with the limitation that inclusion and exclusion criteria were different in the two studies. Another limitation is the cross-sectional nature of the study, preventing us from coming to a conclusion about causal relationships. Inclusion of participants with a history of microalbuminuria, covering the full continuum of albuminuria, would have enhanced the statistical evaluation of the associations between the measures of cardiac autonomic function and albuminuria, and would have allowed us to investigate whether change in autonomic function is an early or late phenomenon in diabetic nephropathy.

Conclusion

In conclusion, we found distinct associations between measures of cardiac autonomic function and MFR. Most notably, after adjustment for relevant cardiovascular risk factors and in the participants free of coronary artery disease, the late heart-to-mediastinum ratio directly measuring the integrity of cardiac sympathetic nerve fibers was positively associated with MFR. This indicates that cardiac autonomic dysfunction, including the loss of sympathetic integrity, may be associated with impaired myocardial blood flow regulation in type 1 diabetes, even after eliminating the influence of coronary artery disease.

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Author Contributions. E.H.Z. conceived and designed the research, acquired the data, performed the statistical analysis, and drafted the manuscript. P.H. acquired the data, contributed to interpretation of the results, and reviewed/edited the manuscript. S.A.W. acquired the data, contributed to interpretation of the results, and reviewed/edited the manuscript. C.S.H, J.F., B.J.v.S., L.H., and A.K. contributed to interpretation of the results and reviewed/edited the manuscript. P.R. conceived and designed the research, contributed to interpretation of the results, and reviewed/edited the manuscript. T.W.H. conceived and designed the research, acquired the data, performed the statistical analysis, contributed to interpretation of the results, drafted the manuscript, and reviewed/edited the manuscript. E.H.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data Availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

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Paper 4

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Cardiovascular Diabetology

ORIGINAL INVESTIGATION

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Relation of cardiac adipose tissue to coronary calcification and myocardial microvascular function in type 1 and type 2 diabetes

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Abstract

Background: Cardiac adipose tissue may have local paracrine effects on epicardial arteries and the underlying myocardium, promoting calcification and affecting myocardial microcirculation. We explored whether the total amount of cardiac adipose tissue was associated with coronary artery calcium score (CAC) and myocardial flow reserve in persons with type 1 or type 2 diabetes and healthy controls.

Methods: We studied three groups: (1) 30 controls, (2) 60 persons with type 1 diabetes and (3) 60 persons with type 2 diabetes. The three groups were matched for sex and age. The three groups derived from retrospective analysis of two clinical studies. All underwent cardiac ^{82}Rb positron emission tomography/computed tomography (PET/CT) scanning. Cardiac adipose tissue volume (the sum of epicardial and pericardial fat), CAC, and myocardial flow reserve (ratio of pharmacological stress flow and rest flow) were evaluated using semiautomatic software. We applied linear regression to assess the association between cardiac adipose tissue, CAC and myocardial flow reserve.

Results: Mean (SD) cardiac adipose tissue volume was 99 (61) mL in the control group, 106 (78) mL in the type 1 diabetes group and 228 (97) mL in the type 2 diabetes group. Cardiac adipose tissue was positively associated with body mass index in all three groups ($p \leq 0.02$). In the controls, cardiac adipose tissue was positively associated with CAC score ($p = 0.008$) and negatively associated with myocardial flow reserve ($p = 0.005$). However, cardiac adipose tissue was not associated with CAC or myocardial flow reserve in the groups including persons with type 1 or type 2 diabetes ($p \geq 0.50$).

Conclusions: In contrast to what was found in healthy controls, we could not establish a relation between cardiac adipose tissue and coronary calcification or myocardial microvascular function in person with type 1 or type 2 diabetes. The role of cardiac adipose tissue in cardiovascular disease in diabetes remains unclear.

Keywords: Cardiac adipose tissue, Epicardial adipose tissue, Pericardial adipose tissue, Type 1 diabetes, Type 2 diabetes, Albuminuria, Myocardial flow reserve, Coronary artery calcium score, Cardiac sympathetic innervation, MIBG, Cardiac PET/CT

Background

Cardiac adipose tissue is a highly metabolic active fat depot surrounding the heart and coronary arteries. It includes epi- and pericardial adipose tissue. There has been considerable interest in the cardiac adipose tissue

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as a risk marker [1]. High levels of epicardial adipose tissue have been associated with incident cardiovascular disease and mortality in persons (particularly men) with type 2 diabetes in our previous study [2] and was associated with increased risk of coronary artery disease in persons with a high risk of cardiovascular disease, of whom 45% had type 2 diabetes [3]. An increased epicardial adipose tissue volume has also been demonstrated to be an important determinant of atrial fibrillation recurrence following electrical cardioversion or catheter ablation [4] and to correlate with ventricular tachycardia recurrence after catheter ablation in persons undergoing ventricular tachycardia ablation [5]. In contrast, a study in persons with type 1 diabetes could not demonstrate an association between the epicardial adipose tissue volume and coronary atherosclerosis [6].

Little is known about the possible mechanisms that could link cardiac adipose tissue to an increased risk of cardiovascular disease. It has been demonstrated that adipose tissue is an active endocrine tissue, implied in the production of numerous pro-inflammatory mediators [7]. Thus, abdominal adipose tissue in persons with overweight has been linked to an over-production of inflammatory mediators with effects on the local fat, insulin resistance and there has been an increasing interest in a possible crosstalk with myocardial function [8, 9]. These effects may be reduced with hypoglycemic agents with pleiotropic effects.

Epicardial adipose tissue embeds the coronary arteries and autonomic nerve fibers, and shares microcirculation with the underlying myocardium. We were interested in whether the association between cardiac adipose tissue and cardiovascular disease might be mediated by micro- or macrovascular changes or autonomic nerve damage. Due to the proximity of the structures, cardiac adipose tissue could through paracrine signalling promote calcification or impair the myocardial microcirculatory function [7, 10]. Knowledge of these associations in persons with diabetes, who are known to have higher amounts of cardiac adipose tissue and higher risk of cardiovascular disease compared to persons without diabetes, is sparse [6, 11–16]. A study in patients with systolic heart failure demonstrated an association between epicardial adipose tissue thickness and cardiac sympathetic denervation, implying that cardiac adipose tissue could affect autonomic nerve function [17].

The aim of this retrospective analysis was to explore whether the total amount of cardiac adipose tissue (sum of epi- and pericardial adipose tissue) was associated with coronary artery calcium score (CAC), myocardial flow reserve and/or cardiac sympathetic nerve integrity in persons with type 1 or type 2 diabetes and healthy controls.

Methods

Study population

The present study is a retrospective analysis of two clinical studies performed at a single center: A study from 2013 in 60 persons with type 2 diabetes and 30 controls [18]; and a study from 2016 to 2018 in 60 persons with type 1 diabetes [19]. The two studies used identical protocols and the same equipment and were therefore suitable for comparisons. The clinical information was obtained at a visit at the Steno Diabetes Center Copenhagen and all scans were performed at the Department of Clinical Physiology, Nuclear Medicine & PET at Rigshospitalet, Copenhagen. In the present study, we analyzed all participants from the two original studies. Therefore, we had three groups to compare: (1) controls; (2) persons with type 1 diabetes; and (3) persons with type 2 diabetes. The three groups were matched for age and sex as part of the original design of the two studies.

Main exclusion criteria were renal failure and contraindications for cardiac positron emission tomography (PET)/computed tomography (CT). Cardiovascular disease [history of coronary artery disease or other cardiovascular disease (including stroke) or heart symptoms] was an exclusion criterion for the type 2 diabetes group and the controls, but not for the type 1 diabetes group. None of the participants had symptoms of angina at time of inclusion. History of cardiovascular disease was assessed from patient electronic health records and information on symptoms from the heart was obtained from thorough interviews with all participants using the WHO Rose questionnaire.

Hybrid cardiac PET/CT imaging

Cardiac adipose tissue was measured using the cardiac software, Syngo.via Frontier—Cardiac risk assessment (Siemens, AG; Healthcare Sector, Germany), which uses non-contrast CT data to automatically quantify the adipose tissue volume located on the outside of the myocardium by tracing myocardial and adipose tissue borders based on the specific density of fat (−150 to −50 Hounsfield units). The software does not distinguish epi- and pericardial fat thus total cardiac adipose tissue above the entire heart was measured. Two separate investigators examined and verified the accuracy of the automatically generated adipose tissue tracings.

All participants underwent a dynamic, gated cardiac PET/CT on a hybrid PET/CT scanner in 3D mode (Siemens Biograph mCT 128, Siemens, Munich, Germany) after administration of 1100 MBq ^{82}Rb (CardioGen-82, Bracco Diagnostics, Monroe Township, NJ, USA). Cardiac PET/CT was performed at rest and during stress (maximum myocardial hyperaemia was induced by adenosine infusion). Myocardial blood flow was calculated as

the ratio between maximal induced and resting myocardial blood flow, as previously described in detail [18]. The myocardial flow reserve reflects to what extent the myocardial blood flow can increase during stress. Using the method described by Agatston, we calculated CAC score as the sum of CAC content in the three main coronary arteries using semiautomated software (Corridor4DM, INVIA, Ann Arbor, MI, USA), as previously reported [18]. Myocardial perfusion abnormalities were assessed semi quantitatively by two experienced operators.

Cardiac sympathetic innervation by ^{123}I-MIBG scintigraphy

We performed cardiac radionuclide imaging using the nonmetabolized norepinephrine analogue metaiodobenzylguanidine (MIBG). Sympathetic nerve terminals actively take up MIBG, and by quantifying the cardiac uptake, it is possible to directly assess cardiac sympathetic nerve integrity. MIBG is radioactively labeled with ^{123}I to allow imaging. Cardiac ^{123}I-MIBG uptake was semiquantified using images taken 240 min after intravenously tracer injection (200 MBq ^{123}I-MIBG). We calculated the heart to mediastinum ratio, by drawing regions of interest following the epicardial contour and the upper mediastinum (avoiding the thyroid gland) in the planar anterior view [20]. An intact sympathetic innervation of the myocardium is reflected by a high heart-to-mediastinum ratio.

As previously described, cardiac ^{123}I-MIBG scintigraphy was performed in all 60 participants with type 1 diabetes, in 29 of the participants with type 2 diabetes, and in 14 of the controls [20, 21].

Clinical measurements

HbA_{1c} and lipids were measured by standard methods. The CKD-EPI equation was used to calculate eGFR. In the type 1 diabetes group, urine samples were taken three consecutive mornings and urinary albumin creatinine rate (UACR) was measured by an enzyme immunoassay. For the persons with type 2 diabetes and the controls, two 24-h urine collections were taken and urinary albumin excretion rate (UAER) was measured by an enzyme immunoassay. The 24-h blood pressure was recorded using a cuff-device (Takeda, TM2430, Japan) in the type 1 diabetes group and a tonometric wrist-device (BPro, HealthStats, Singapore) in the type 2 diabetes group and in the controls.

Statistical analysis

Normal distributed continuous variables are provided as mean and standard deviation (SD), non-normal distributed (UACR, UAER and CAC) as median with interquartile range (IQR) and categorical variables as total numbers and percent. UACR, UAER and CAC were log2 transformed in all analyses.

Independent samples t-test was applied to compare continuous variables between participants with cardiac adipose tissue below or above the median, and the χ^2 test or Fisher's exact test, as appropriate, when comparing categorical variables.

Linear regression analysis was used to determine whether any association existed between total cardiac adipose tissue and clinical characteristics or measures of cardiac function. The three groups were analyzed separately.

A two-tailed $p < 0.05$ was interpreted as significant. Missing data was not replaced.

Results

The characteristics of the controls, and the participants with type 1 and 2 diabetes are shown for all within the groups and according to median cardiac adipose tissue level in the three groups in Table 1. Persons with cardiac adipose tissue below the median had lower body mass index (BMI) in all three groups ($p \leq 0.02$) and higher HDL-cholesterol level in the control group and in the type 2 diabetes group ($p \leq 0.04$). The controls with cardiac adipose tissue below the median had lower CAC score and higher myocardial flow reserve ($p = 0.005$ for both).

Reversible ischemia was observed on the cardiac PET/CT in 1 control person, 10 participants with type 1 diabetes (7 of whom were known with coronary artery disease) and 11 participants with type 2 diabetes. Five participants with type 1 diabetes and three with type 2 diabetes had irreversible ischemia (fixed perfusions defects) which was not found in any control persons.

Mean (SD) cardiac adipose tissue level was 99 (61) mL in the control group; 106 (78) mL in the persons with type 1 diabetes and 228 (97) mL in the persons with type 2 diabetes. The cardiac adipose tissue level was comparable in controls and persons with type 1 diabetes, however significantly higher in persons with type 2 diabetes compared to controls ($p < 0.0001$) and type 1 diabetes ($p < 0.0001$). These differences remained significant after adjustment for age, sex, BMI and UACR/UAER ($p \leq 0.02$).

We evaluated the associations between cardiac adipose tissue and clinical characteristics separately in the three groups (Table 2 and Fig. 1). Cardiac adipose tissue was positively associated with BMI in all three groups ($p \leq 0.02$). In the control group, cardiac adipose tissue was positively associated with age and UAER ($p \leq 0.03$).

Furthermore, we evaluated the associations between cardiac adipose tissue and myocardial flow reserve, left ventricular ejection fraction, CAC score and ^{123}I-MIBG uptake, separately in the three groups (Table 2 and

Table 1 Clinical characteristics of all participants

Characteristics	Controls (n = 30)				Persons with type 1 diabetes (n = 60)				Persons with type 2 diabetes (n = 60)			
	All	Cardiac adipose tissue < 88 mL	Cardiac adipose tissue ≥ 88 mL	p	All	Cardiac adipose tissue < 71 mL	Cardiac adipose tissue ≥ 71 mL	p	All	Cardiac adipose tissue < 226 mL	Cardiac adipose tissue ≥ 226 mL	p
Numbers of participants	30	14	15	–	60	30	30	–	60	30	30	
Female, n (%)	12 (40.0)	7 (24.1)	4 (13.8)	0.20	25 (41.7)	16 (53.3)	9 (30.0)	0.07	20 (33.3)	13 (43.3)	7 (23.3)	0.1
Age (years)	60 (10)	57 (9)	63 (10)	0.14	59 (9)	58 (10)	61 (8)	0.21	63 (9)	64 (10)	63 (8)	0.76
Body mass index (kg/m^2)	24.8 (3.4)	22.2 (2.0)	27.2 (2.8)	*< 0.0001*	26.4 (4.2)	23.8 (2.6)	29.1 (3.8)	*< 0.0001*	31.6 (4.5)	30.2 (4.5)	32.9 (4.2)	*0.02*
Known diabetes duration (years)	–	–	–	–	37 (14)	37 (13)	37 (14)	0.99	14 (10)	15 (8)	13 (12)	0.68
24 h systolic blood pressure (mmHg)	126 (14)	123 (11)	132 (14)	0.06	136 (10)	136 (10)	138 (10)	0.47	134 (18)	133 (16)	135 (20)	0.62
HbA_{1c} (%)	35.8 (1.9)	35.2 (2.2)	36.3 (1.5)	0.11	61.8 (10.1)	62.1 (9.7)	61.6 (10.6)	0.84	55.5 (11.4)	56.1 (13.3)	55.0 (9.3)	0.71
LDL cholesterol (mmol/L)	3.4 (0.7)	3.3 (0.5)	3.5 (0.9)	0.42	2.2 (0.8)	2.1 (0.6)	2.2 (0.9)	0.53	2.2 (0.9)	2.1 (0.8)	2.3 (0.9)	0.30
HDL cholesterol (mmol/L)	1.6 (0.5)	1.8 (0.7)	1.4 (0.3)	*0.04*	1.8 (0.5)	1.9 (0.5)	1.7 (0.5)	0.11	1.1 (0.4)	1.2 (0.5)	1.0 (0.2)	*0.03*
Total cholesterol (mmol/L)	5.5 (0.7)	5.5 (0.8)	5.5 (0.8)	0.96	4.5 (0.9)	4.4 (0.8)	4.5 (1.0)	0.47	4.3 (0.9)	4.2 (0.9)	4.4 (0.9)	0.60
eGFR (mL min^{-1} 1.73 m^{-2})	82.8 (13.1)	85.3 (12.2)	80.2 (14.3)	0.31	75.8 (22.3)	76.9 (24.0)	74.7 (20.7)	0.71	76.0 (24.0)	80.3 (23.0)	71.8 (24.6)	0.17
Urinary albumin excretion rate (mg/24 h)*	6.0 [5.0–10.5]	5.8 [5.0–6.5]	6.5 [5.0–22.5]	0.12	11.5 [3.3–120.8]	8.5 [4.3–129.7]	18 [3.0–106.0]	0.57	32.5 [6.5–146.0]	16 [6.5–93.5]	50.5 [8.5–208.3]	0.71
Anti-hypertensive treatment, n (%)	3 (10.0)	2 (6.9)	1 (3.4)	0.45	47 (78.3)	23 (76.7)	24 (80)	0.75	55 (91.7)	25 (83.3)	30 (100)	0.05
Aspirin treatment, n (%)	1 (3.3)	1 (3.5)	0 (0)	1.0	30 (50.0)	14 (46.7)	16 (53.3)	0.61	53 (88.3)	26 (86.7)	27 (90.0)	1.0
Lipid lowering medication, n (%)	0 (0)	0	0	–	45 (75.0)	22 (73.3)	23 (76.7)	0.77	56 (93.3)	28 (93.3)	28 (93.3)	1.0
Smokers, n (%)	4 (13.3)	3 (10.3)	1 (3.5)	0.33	8 (14.0)	4 (14.3)	4 (13.8)	1.0	46 (76.7)	23 (76.7)	23 (76.7)	1.0

Table 1 (continued)

Characteristics	Controls (n = 30)				Persons with type 1 diabetes (n = 60)				Persons with type 2 diabetes (n = 60)			
	All	Cardiac adipose tissue < 88 mL	Cardiac adipose tissue ≥ 88 mL	p	All	Cardiac adipose tissue < 71 mL	Cardiac adipose tissue ≥ 71 mL	p	All	Cardiac adipose tissue < 226 mL	Cardiac adipose tissue ≥ 226 mL	p
Known coronary artery disease or ischemia identified by cardiac PET, n (%)	1 (3.3)	0	1	1.0	9 (15)	3 (10.0)	6 (20.0)	0.47	13 (21.7)	9 (30.0)	4 (13.3)	0.12
Left ventricle ejection fraction (mL)	60.0 (7.5)	59.8 (8.4)	60.2 (7.0)	0.9	66.1 (9.3)	68.2 (9.1)	64.0 (9.1)	0.08	61.6 (9.0)	60.2 (8.5)	63.0 (9.5)	0.25
Coronary artery calcium score	0 [0–81]	0 [0–0]	56 [0–176]	*0.005*	99 [22.5–434]	73 [17.5–392.5]	196.5 [23.5–1286.9]	0.63	211 [23.5–585]	300 [28–964]	163 [13–467]	0.35
Myocardial flow reserve	3.0 (0.8)	3.4 (0.7)	2.6 (0.7)	*0.005*	2.6 (1.0)	2.6 (1.1)	2.6 (0.9)	0.98	2.3 (0.7)	2.2 (0.6)	2.4 (0.8)	0.32
Late heart-to-mediastinum ratio n = 103	2.8 (0.6)	3.0 (0.5)	2.5 (0.7)	0.12	2.5 (0.5)	2.5 (0.5)	2.4 (0.4)	0.16	2.5 (0.5)	2.4 (0.6)	2.5 (0.5)	0.65

Data represents numbers, n (%), mean (SD) or median [IQR]

The three groups were analyzed separately, and p values compare values from participants with cardiac adipose tissue below or above the median within each of the three groups. p values from independent samples t-test and χ^2 test or Fisher's exact test

Italic values indicate significance of p value ($p < 0.05$)

eGFR estimated glomerular filtration rate

*Urinary albumin creatinine rate for the type 1 diabetes cohort

Table 2 Associations between cardiac adipose tissue and clinical characteristics as well as measures of cardiac function

	Controls (n = 30)		Persons with type 1 diabetes (n = 60)		Persons with type 2 diabetes (n = 60)	
	β coefficient (95% CI)	p	β coefficient (95% CI)	p	β coefficient (95% CI)	p
Clinical characteristics						
Age	0.007 (0.001; 0.013)	*0.02*	0.003 (− 0.0001; 0.006)	0.06	0.001 (− 0.001; 0.004)	0.27
Body mass index	0.034 (0.017; 0.052)	*0.0004*	0.039 (0.029; 0.049)	*< 0.0001*	0.014 (0.003; 0.026)	*0.02*
LDL cholesterol	0.00006 (− 0.006; 0.007)	0.99	0.002 (− 0.001; 0.005)	0.20	0.002 (− 0.0008; 0.004)	0.17
Total cholesterol	− 0.001 (− 0.008; 0.005)	0.66	0.002 (− 0.001; 0.006)	0.19	0.002 (− 0.0008; 0.004)	0.16
Lipid lowering treatment	–	–	− 0.00005 (− 0.002; 0.001)	0.95	0.0001 (− 0.0005; 0.0008)	0.68
Hemoglobin A_{1c}	0.0002 (− 0.004; 0.009)	0.46	− 0.0003 (− 0.004; 0.003)	0.84	0.001 (− 0.001; 0.003)	0.37
Urinary albumin excretion rate[a]	0.006 (0.0005; 0.012)	*0.03*	0.002 (− 0.002; 0.005)	0.31	0.0016 (− 0.001; 0.004)	0.38
Systolic blood pressure	0.005 (− 0.002; 0.011)	0.14	0.002 (− 0.002; 0.005)	0.29	0.0007 (− 0.002; 0.003)	0.60
Cardiac measures						
Left ventricle ejection fraction	− 0.004 (− 0.01; 0.003)	0.23	− 0.003 (− 0.006; 0.0003)	0.08	0.0005 (− 0.002; 0.003)	0.72
Coronary artery calcium score	0.008 (0.002; 0.013)	*0.008*	0.001 (− 0.002; 0.005)	0.50	− 0.0002 (− 0.003; 0.003)	0.89
Myocardial flow reserve	− 0.008 (− 0.014; − 0.003)	0.005	− 0.0007 (− 0.004; 0.003)	0.67	0.00002 (− 0.003; 0.003)	0.98
Late heart-to-mediastinum ratio[b]	− 0.009 (− 0.022; 0.003)	0.12	− 0.002 (− 0.006;0.001)	0.19	0.001 (− 0.003; 0.005)	0.59

Linear regression analysis. The three groups were analyzed separately. β coefficient represents standardized effects within groups

Italic values indicate significance of p value ($p < 0.05$)

[a] Urinary albumin creatinine rate for the persons with type 1 diabetes

[b] Available in 14 controls, 29 persons with type 2 diabetes and 60 persons with type 1 diabetes

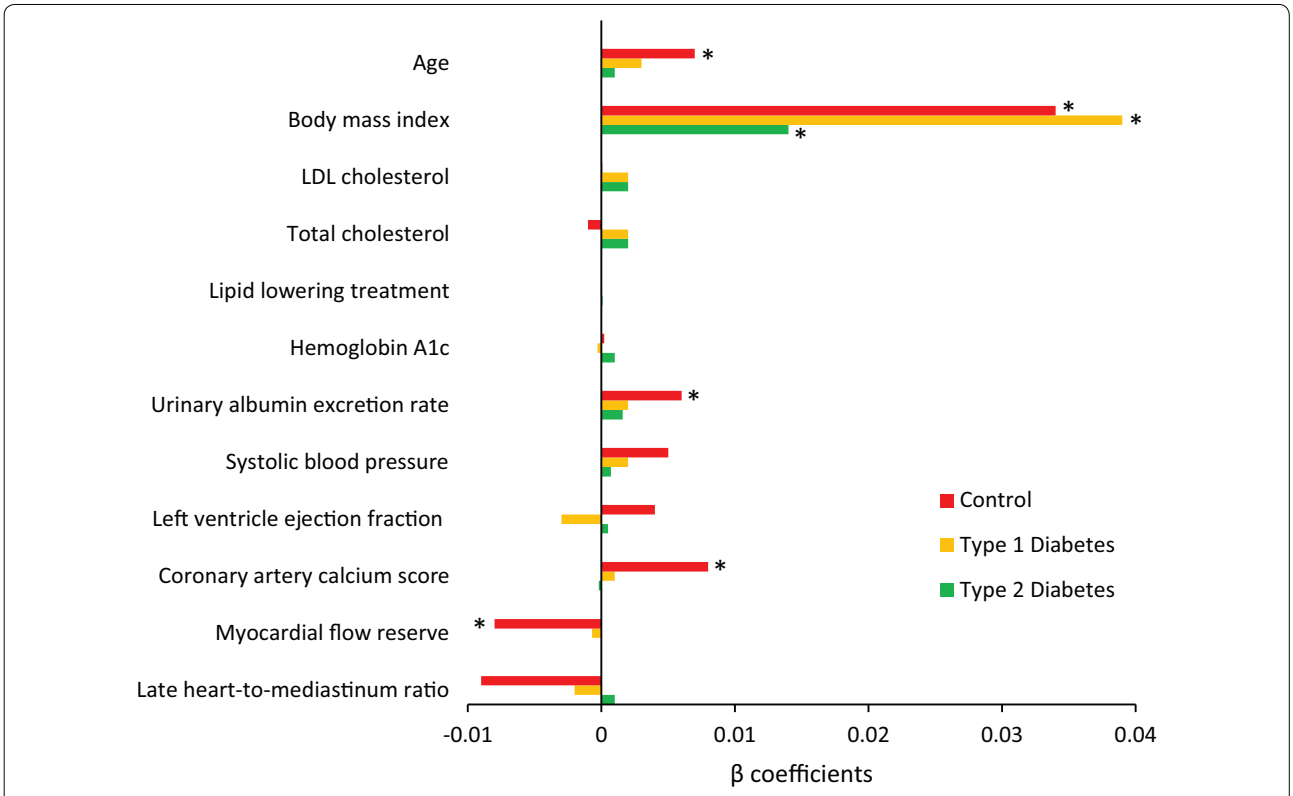

Fig. 1 Associations between cardiac adipose tissue, clinical characteristics and measures of cardiac function. The three groups were analyzed separately using linear regression models and the β coefficients represent standardized effects within the groups. For lipid lowering treatment the β coefficients represent with versus without treatment. *$p < 0.05$

Fig. 1). Cardiac adipose tissue was positively associated with CAC score ($p=0.008$) and negatively associated with myocardial flow reserve in the controls ($p=0.005$). No significant associations were found for the persons with type 1 or type 2 diabetes. Cardiac adipose tissue was not associated with left ventricular ejection fraction or ^{123}I-MIBG uptake in the three groups ($p \geq 0.08$).

Discussion

The main finding of our study was that total cardiac adipose tissue was not associated with advanced measures of cardiac micro- and macrovascular function including CAC score, myocardial flow reserve, and cardiac sympathetic integrity in persons with type 1 or type 2 diabetes. In contrast we found that cardiac adipose tissue was positively associated with CAC score and negatively associated with myocardial flow reserve in the controls.

Cardiac adipose tissue and coronary atherosclerosis

The hypothesis that cardiac adipose tissue influences the development of coronary atherosclerosis through endocrine and paracrine activities has provoked considerable interest in the association between cardiac adipose tissue and coronary atherosclerosis. Large studies including the Framingham Heart Study have reported distinct associations between perivascular fat and CAC score in the general population [22]. We extended this association to apply for total cardiac adipose tissue in our control group. Evidence from persons with type 1 and type 2 diabetes is still sparse and conflicting, and mostly address epicardial and not total cardiac adipose tissue [13]. Our finding that cardiac adipose tissue and CAC score did not correlate in persons with type 1 diabetes confirms findings from a recent study using CT angiography to evaluate epicardial adipose tissue and CAC in 88 persons with type 1 diabetes [6]. Results from studies in type 2 diabetes and mixed populations (diabetes and non-diabetes) are conflicting. In the studies reporting an association between epicardial adipose tissue and coronary artery disease, including high risk plaque characteristics and CAC [13–15], it remains uncertain if the associations demonstrated are truly independent from traditional risk factors. We report a lack of association between cardiac adipose tissue and CAC score in persons with type 2 diabetes free of cardiovascular disease in line with results from a study in a mixed population (34% had diabetes) with suspected or known coronary artery disease (referred for clinically indicated invasive coronary angiography) [16].

Cardiac adipose tissue and myocardial microcirculation

Due to the proximity of cardiac adipose tissue and the epicardial arteries and underlying myocardium, it is possible that cardiac adipose tissue has local effects on myocardial microcirculation and thereby influences the development of myocardial ischemia. Major advances in non-invasive imaging enable the investigation of new aspects of the microcirculation and with ^{82}Rb cardiac PET/CT it is possible to measure myocardial flow reserve, reflecting both the function of the large epicardial arteries and the microcirculation. We report an association between cardiac adipose tissue and myocardial flow reserve in non-diabetic controls, but not in persons with type 1 or type 2 diabetes. The few published reports on myocardial flow reserve and cardiac adipose tissue are in line with our results with divergent findings in non-diabetes and diabetes cohorts. A retrospective study, including 46 persons with and 153 persons without diabetes all evaluated for coronary artery disease, observed an association between myocardial flow reserve and epicardial adipose tissue only in the group of persons without diabetes [12]. In a mixed population of 85 persons (28 with diabetes) referred for ^{82}Rb PET/CT imaging for clinical indications, retrospective analysis revealed an association between higher epicardial adipose tissue and presence of impaired myocardial flow reserve (myocardial flow reserve ≤ 2) in the total cohort [11].

Why do we find associations between cardiac adipose tissue and CAC and myocardial flow reserve in the controls and not in the persons with diabetes? We speculate that (1) pathophysiological processes promoting coronary atherosclerosis and microvascular dysfunction other than paracrine effects of cardiac adipose tissue are dominant in diabetes; or (2) medical treatment may counteract the adverse effects of the cardiac adipose tissue. Differences in medication between controls and persons with diabetes could then explain the divergent findings.

We report no relationship between cardiac adipose tissue and cardiac sympathetic nerve integrity. To the best of our knowledge, this has not been investigated in persons with diabetes before.

How might our findings impact on clinical practice? Our findings indicate that total cardiac adipose tissue is not a parameter for use in the clinical setting as a marker of microvascular (myocardial flow reserve) or macrovascular (CAC) cardiovascular disease in persons with diabetes.

Cardiac adipose tissue as treatment target

As the epicardial adipose tissue is a transducer of the harmful effects of systemic inflammation and metabolic disorders on the heart it may represent an important treatment target [10, 23]. A recent study analyzed the adipose tissue surrounding coronary arteries during coronary artery bypass grafting interventions; Two important findings were demonstrated (1) there was increased inflammation of the adipose tissue surrounding the

coronary arteries during acute myocardial infarction; and (2) treatment with metformin had an ameliorative effect on the inflammation in the peri-coronary fat and reduced the risk of major cardiovascular events at 12-month follow-up in persons with prediabetes and acute myocardial infarction [24]. Along these lines, a recent study performed in a clinical setting during coronary angiography in non-obstructive coronary stenosis, demonstrated favourable effects of metformin on coronary endothelial dysfunction as well as cardiovascular event-rate in persons with prediabetes [25].

Study limitations

We applied robust methodology: CT acquisition and assessment followed standard methods and cardiac adipose tissue was automatically quantified using the cardiac software, Syngo.via Frontier [26, 27]. The volumes of cardiac adipose tissue observed in this study were consistent with those previously reported [6, 28–30]. Standardised and validated methodology was used to assess myocardial flow reserve and CAC score. Cardiac ^{123}I-MIBG scintigraphy was used for direct assessment of cardiac sympathetic integrity. However, we acknowledge that our study has limitations. The limited size of our study population was only powered to detect stronger correlations and accordingly we may have overlooked smaller correlations. Nevertheless, as the control population was smaller than the diabetic populations an even lower power was present for the controls where we did find the anticipated associations. Also, our sample size is large compared to clinical studies applying similar advanced imaging modalities in persons with diabetes [11, 12]. Estimates of cardiac adipose tissue are known to be subject to inter and intra observer variability. We applied validated methods and automatic analysis to ensure consistency of the volumes measured within our cohort. Nevertheless, we did not differentiate between epi- and pericardial fat, which is a limitation as previous studies found different associations between measures of systolic and diastolic function with epicardial and pericardial adipose tissue [28]. Finally, the only information on systolic function was left ventricle ejection fraction.

Conclusion

In contrast to what was found in healthy controls, we could not establish a relation between cardiac adipose tissue and coronary calcification or myocardial microvascular function in person with type 1 or type 2 diabetes. The role of cardiac adipose tissue in cardiovascular disease in diabetes remains unclear.

Abbreviations

CAC: Coronary artery calcium; CT: Computed tomography; eGFR: Estimated glomerular filtration rate; IQR: Interquartile range; MIBG: Metaiodobenzylguanidine; PET: Positron emission tomography; UACR: Urinary albumin creatinine rate; UAER: Urinary albumin excretion rate.

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Authors' contributions

EHZ, RHC, TWH and PR conceived and designed the research. EHZ, RHC, SAW, TWH and PH acquired the data. EHZ, RHC and TWH performed the statistical analysis. EHZ and RHC drafted the manuscript. SAW, CS, BJvS, PH, LH, AK, PR and TWH contributed to analysis, interpretation of data and revised the article critically for important intellectual content. EHZ is responsible for the integrity of the work as a whole. All authors read and approved the final manuscript.

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Availability of data and materials

Data for the present analysis can be provided by the first author on reasonable request.

Ethics approval and consent to participate

Both studies were conducted in accordance with the Helsinki declaration. The protocols were approved by the local Ethics Committee and all participants gave informed written consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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ARTICLE

Toe–brachial index as a predictor of cardiovascular disease and all-cause mortality in people with type 2 diabetes and microalbuminuria

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Abstract
Aims/hypothesis The study aimed to evaluate toe–brachial index (TBI) and ankle–brachial index (ABI) as determinants of incident cardiovascular disease (CVD) and all-cause mortality in people with type 2 diabetes and microalbuminuria.
Methods This was a prospective study including 200 participants. Unadjusted and adjusted (traditional risk factors and additional inclusion of N-terminal pro-brain natriuretic peptide [NT-proBNP] and coronary artery calcification) Cox regression models were performed. C statistics and relative integrated discrimination improvement (rIDI) evaluated risk prediction improvement.
Results Median follow-up was 6.1 years; 40 CVD events and 26 deaths were recorded. Lower TBI was associated with increased risk of CVD (HR per 1 SD decrease: 1.55 [95% CI 1.38, 1.68]) and all-cause mortality (1.41 [1.22, 1.60]) unadjusted and after adjustment for traditional risk factors (CVD 1.50 [1.27, 1.65] and all-cause mortality 1.37 [1.01, 1.60]). Lower ABI was a determinant of CVD (1.49 [1.32, 1.61]) and all-cause mortality (1.37 [1.09, 1.57]) unadjusted and after adjustment for traditional risk factors (CVD 1.44 [1.23, 1.59] and all-cause mortality 1.39 [1.07, 1.60]). After additional adjustment for NT-proBNP and coronary artery calcification, lower TBI remained a determinant of CVD ($p = 0.023$). When TBI was added to traditional risk factors, the AUC increased significantly for CVD, by 0.063 (95% CI 0.012, 0.115) from 0.743 ($p = 0.016$), but not for all-cause mortality; adding ABI did not improve the AUC significantly. The rIDI for TBI was 46.7% ($p < 0.001$) for CVD and 46.0% ($p = 0.002$) for all-cause mortality; for ABI, the rIDI was 51.8% ($p = 0.004$) for CVD and 53.6% ($p = 0.031$) for all-cause mortality.
Conclusions/interpretation Reduced TBI and ABI were associated with increased risk of CVD and all-cause mortality, independent of traditional risk factors in type 2 diabetes, and improved prognostic accuracy.

Keywords Ankle–brachial index · Cardiovascular disease · Carotid intima–media thickness · Microalbuminuria · Peripheral arterial disease · Toe–brachial index · Type 2 diabetes

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Abbreviations

ABI	Ankle–brachial index
CVD	Cardiovascular disease
IDI	Integrated discrimination improvement
IQR	Interquartile range
NT-proBNP	N-terminal pro-brain natriuretic peptide
rIDI	Relative integrated discrimination improvement
ROC	Receiver operating characteristic

TBI	Toe–brachial index
UAER	Urinary AER

Introduction

Cardiovascular disease (CVD) is among the leading causes of morbidity and mortality in type 2 diabetes, and accurate risk stratification of these high-risk individuals is important. Currently, risk prediction is often based on established cardiovascular risk factors. However, there is increasing recognition of the imprecision in relying only on these risk factors, and cardiovascular risk prediction may be improved by adding a measure of atherosclerosis.

Assessment of toe–brachial index (TBI) and ankle–brachial index (ABI) is non-invasive, inexpensive and easily performed in the clinical setting [1, 2]. TBI and ABI are diagnostic tests for peripheral arterial disease and prognostic for healing of foot ulcers; they are also markers of systemic atherosclerosis and thus could improve the identification of high-risk individuals.

Current evidence of the value of TBI in risk stratification in type 2 diabetes is sparse. Two recent studies investigated the predictive role of TBI in people with type 2 diabetes; in 81 participants included after acute coronary syndrome, low TBI (<0.6) was associated with increased risk of recurrent CVD [3]. A study with 17.2 years of follow-up, including a subgroup of 155 type 2 diabetic individuals, found no association between TBI and cardiovascular or all-cause mortality after adjustment [4].

ABI has been shown to predict cardiovascular morbidity and mortality in the general population [5] and in type 2 diabetes [6]. However, the validity of ABI in diabetes is still debated as people with diabetes often have medial arterial calcification, which might cause false elevated or false normal ankle pressure [7, 8]. The vessels in the toe are less susceptible to calcification; thus, TBI may only be slightly affected by calcification and therefore perform as a better marker of atherosclerosis compared with ABI [9].

Measurement of carotid intima–media thickness is also a non-invasive examination of atherosclerosis, but needs to be performed by a trained technician in designated clinical settings. Conflicting results have been published on the added value of carotid intima–media thickness in cardiovascular risk prediction in the general population [10] and in type 2 diabetes [11].

The aim of this prospective study was to determine the added value of TBI and ABI as well as carotid intima–media thickness in risk prediction for CVD and all-cause mortality in individuals with type 2 diabetes and microalbuminuria, but without the clinical features of coronary artery disease.

As has been recently published, N-terminal pro-brain natriuretic peptide (NT-proBNP) level and coronary artery calcification score are powerful predictors of outcome, but are also complicated and expensive to assess [12]. We investigated the added value of TBI, ABI and carotid intima–media thickness to the traditional risk factors with and without NT-proBNP level and coronary artery calcification score.

Methods

Participants and study procedures

In 2007–2008, we identified a cohort of 200 people with type 2 diabetes treated in a secondary care setting at the Steno Diabetes Center Copenhagen, Gentofte, Denmark. The selection process has previously been described in detail [13]. This follow-up was described in the original study protocol and members of the original study group are authors of this paper. Participants had type 2 diabetes according to WHO criteria without history or symptoms suggestive of coronary artery disease or other cardiac disease (assessed from medical records, interviews and questionnaires). Participants had persistent (two out of three consecutive measurements) urinary AER (UAER) >30 mg/24 h. Participants received multifactorial intervention (glycaemic, lipid and blood pressure control, anti-thrombotic therapy and lifestyle modification) in line with the findings from the Steno-2 Study [14].

The study complied with the Declaration of Helsinki. The research protocol was approved by the local ethics committee and all participants gave written informed consent.

Investigations of the peripheral and coronary arteries

Systolic blood pressure in the ankle and first toe were measured on both legs by the strain gauge technique and the lowest pressures were used for calculation of TBI and ABI, respectively [15]. Measurements were taken in a temperature-controlled room after participants had rested for 10 min in the supine position. The reliability of TBI and ABI has been evaluated in previous studies. The reproducibility of ABI is between 0.10 and 0.15. For TBI, the variability is greater, ranging from the same to about twice that of ABI [16]. Brachial blood pressure was measured in the dominant arm with an appropriately sized cuff after 10 min rest in the supine position. Two measurements were obtained and averaged. Baseline measures of TBI and ABI were available for 191 and 178 participants, respectively.

Carotid intima–media thickness was measured by an experienced technician at the posterior wall 20 mm proximal to the bifurcation of the common carotid artery bilaterally, taken as the distance between the luminal intima interface and the adventitial media interface (Acuson Cypress ultrasound scanner with a linear probe 7–10 MHz 7L3, Siemens, OH, USA). Carotid intima–media thickness was measured on both sides

and averaged [17]. Thickness measurements were available for 182 participants.

Coronary artery calcification scanning was performed as previously described [13]. For each participant, the total Agatston coronary artery calcification score was determined, including intimal and medial calcification of the left main, left anterior descending artery, circumflex artery and right coronary artery.

Biochemical and other analyses

UAER was measured in 24 h urine samples using an enzyme immunoassay (Vitros, Raritan, NJ, USA). Plasma NT-proBNP was analysed by immunoassay, as previously described [18]. Current smoking was defined as one or more cigarettes, cigars or pipes per day.

Follow-up

All participants were traced on 1 January 2014 through the Danish National Death Register and the Danish National Health Register to obtain information on cause of death and hospital admissions as previously described in detail [12]. Unless an unequivocal non-cardiovascular cause was established, all deaths were classified as cardiovascular. No participants were lost to follow-up.

The primary endpoint consisted of cardiovascular mortality, non-fatal myocardial infarction (ICD-10 codes I20–I25); (www.who.int/classifications/icd/en/), stroke (ICD-10 codes I61 or I63), ischaemic CVD (ICD-10 code I70) and heart failure (ICD-10 code I50). The analysis included only the first endpoint for participants who experienced multiple endpoints. The secondary endpoint was all-cause mortality.

Statistical analyses

Non-normally distributed variables (UAER, NT-proBNP, coronary artery calcification score) are reported as median with interquartile range (IQR), other continuous variables as means ± SD; categorical variables are summarised as total numbers with corresponding percentages.

The correlations between measures of peripheral arterial disease, coronary artery calcification score and carotid intima–media thickness were calculated from unadjusted and adjusted linear regression models and presented as standardised β coefficients. Adjustment included sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and UAER.

We applied Cox regression models to estimate the HRs with 95% CIs for the CVD endpoint and all-cause mortality per 1 SD decrease in TBI, ABI and carotid intima–media thickness. TBI and ABI were also analysed as categorical variables using established cut-off points [19–21]. In contrast to the well-defined and evidence-based limits of the ABI, the diagnostic criterion for a pathologic TBI remains ambiguous and is not strictly evidence based [1].

Carotid intima–media thickness was not dichotomised as no cut-off point is established.

First, we investigated whether any association existed between TBI, ABI and carotid intima–media thickness and our two pre-defined endpoints in the unadjusted model (Model 1). Subsequent adjustment included traditional cardiovascular risk factors based on existing evidence: sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and UAER (Model 2). A third model included these traditional cardiovascular risk factors plus NT-proBNP level and coronary artery calcification score (Model 3). Kaplan–Meier failure function was applied to compare the risks of the CVD endpoint and all-cause mortality according to TBI (above or below 0.64) and ABI (above or below 0.9) at baseline.

Next, we calculated the potential additional predictive ability of TBI and ABI over traditional cardiovascular risk factors using receiver operating characteristic (ROC) curves based on logistic regression models. The AUC was calculated in a model including only the traditional cardiovascular risk factors, and in the same model with TBI or ABI added.

We calculated the integrated discrimination improvement (IDI) statistics between the model including traditional cardiovascular risk factors vs the new model including traditional cardiovascular risk factors plus TBI or ABI. IDI assesses the ability of a new marker to improve the average sensitivity without sacrificing average specificity. Compared with AUC, the IDI may be a more powerful method to demonstrate improved diagnostic performance [22]. Relative IDI (rIDI) is provided, reported as a percentage, for ease of interpretation of the contribution of TBI and ABI. rIDI is defined as the increase in discrimination slope divided by the slope of the model including only the traditional cardiovascular risk factors [22].

Only participants with TBI (n = 193) and ABI (n = 179) baseline measurements available were included in the statistical analyses. A two-tailed p value <0.05 was considered significant. Statistical analyses were performed using SPSS for Windows (version 23.0, Chicago, IL, USA), SAS software (version 9.3, SAS Institute, Cary, NC, USA) and Stata/IC version 14.1 for Windows (StataCorp, College Station, TX, USA).

Results

Participant characteristics

A total of 200 participants were included in the study. Of these, 151 (76%) were men, mean ± SD age was 59 ± 9 years

and mean diabetes duration was 13 ± 7 years. Most participants were treated with oral glucose-lowering agents, insulin and cardiovascular medication (anti-hypertensive drugs [99%], renin–angiotensin–aldosterone system-blocking treatment [98%], statins [95%] and aspirin [90%]). Peripheral arterial disease, defined as ABI < 90 and/or TBI < 0.64 and/or prior surgery for peripheral arterial disease, was demonstrated in 20% of the participants at baseline. A higher detection rate for peripheral arterial disease was found applying TBI < 0.64 compared with ABI < 0.9 (15% vs 11%).

Table 1 lists the baseline characteristics of participants, categorised according to TBI below or above 0.64 and ABI below or above 0.90.

The unadjusted and adjusted correlations between measures of peripheral arterial disease, coronary artery calcification score and carotid intima–media thickness are shown in Table 2.

Incidence of the CVD endpoint and mortality

During the follow-up period (median [IQR] 6.1 [5.9–6.6 years]) 40 participants experienced at least one event from the combined cardiovascular endpoint: 11 cardiovascular events were fatal (two cases of acute myocardial infarction, one case of ischaemic CVD, six sudden and otherwise unexplained deaths and two cases of heart failure) and 29 cardiovascular events were non-fatal events leading to hospital admission (three cases of acute myocardial infarction, three strokes, 19 cases of ischaemic CVD and four cases of heart failure); 26 participants died (11 deaths were classified as CVD related, nine as cancer related and six as related to other causes).

TBI as a risk marker

Table 3 shows the associations for TBI with the composite cardiovascular endpoint and all-cause mortality with TBI modelled as a continuous and a binary variable.

Determinant modelled as a continuous variable In the crude analysis (Model 1), lower TBI was associated with higher risk of both the composite cardiovascular endpoint and all-cause mortality (Table 3). In Model 2, TBI remained associated with both endpoints (Table 3).

In Model 3 (Model 2 plus NT-proBNP and coronary artery calcification), TBI remained associated with the composite cardiovascular endpoint, but not with all-cause mortality (Table 3).

As shown in Table 4, in relation to the composite cardiovascular endpoint, the AUC increased from 0.743 (95% CI 0.661, 0.825) to 0.806 (0.730, 0.882) after adding TBI to the model including traditional risk factors. The improvement in AUC was significant (0.063 [0.012, 0.115]). In relation to all-cause mortality, the AUC increased from 0.736 (0.632, 0.840) to 0.752 (0.639, 0.865) after adding TBI to the model including traditional risk factors. The improvement in AUC was 0.016 (−0.055, 0.088).

The rIDI for TBI was 46.7% ($p < 0.001$) for the cardiovascular endpoint, and 46.0% ($p = 0.002$) in relation to all-cause mortality.

Determinant modelled as a binary variable The cumulative incidence of the composite cardiovascular endpoint and all-cause mortality was higher in participants with TBI < 0.64 (Table 3 and Fig. 1). In Model 2, TBI < 0.64 remained associated with the composite cardiovascular endpoint and all-cause mortality (Table 3). After further adjustment (Model 3), TBI was no longer significant for the cardiovascular endpoint, but the risk of all-cause mortality remained higher in participants with TBI < 0.64 (Table 3).

ABI as a risk marker

Table 3 shows the associations for ABI with the composite cardiovascular endpoint and all-cause mortality with ABI modelled as a continuous and a binary variable.

Determinant modelled as a continuous variable In the crude analysis (Model 1), lower ABI was associated with higher risk of both the composite cardiovascular endpoint and all-cause mortality (Table 3). In Model 2, ABI remained associated with both endpoints. However, in Model 3 (Model 2 plus NT-proBNP and coronary artery calcification), ABI was no longer significant for the two endpoints (Table 3).

As shown in Table 4, in relation to the composite cardiovascular endpoint, the AUC increased from 0.761 (95% CI 0.670, 0.852) to 0.816 (0.725, 0.908) after adding ABI to the model including traditional risk factors. The improvement in AUC was 0.055 (−0.003, 0.113). In relation to all-cause mortality, the AUC increased from 0.746 (0.628, 0.865) to 0.766 (0.639, 0.894) after adding ABI to the model including traditional risk factors. The improvement in AUC was 0.020 (−0.059, 0.099).

The rIDI for ABI was 51.8% ($p = 0.004$) in relation to the cardiovascular endpoint and 53.6% ($p = 0.031$) for all-cause mortality.

Determinant modelled as a binary variable The cumulative incidence of the composite cardiovascular endpoint and all-cause mortality was higher in participants with ABI < 0.90 (Table 3 and Fig. 1). In Model 2, ABI < 0.90 remained associated with both endpoints (Table 3). However, in Model 3, after comprehensive adjustment, ABI < 0.90 was not associated with the risk of CVD or all-cause mortality (Table 3).

Table 1 Clinical characteristics of the study population at baseline categorised according to TBI below or above 0.64 and ABI below or above 0.90

Characteristic	TBI		ABI	
	(<0.64; *n* = 30)	(≥0.64; *n* = 163)	(<0.90; *n* = 22)	(≥0.90; *n* = 157)
Male, *n* (%)	25 (83)	119 (74)	19 (86)	115 (74)
Age (years)	63 ± 6	58 ± 9	63 ± 4	58 ± 9
Known duration of diabetes (years)	16 ± 7	12 ± 7	15 ± 7	12 ± 7
BMI (kg/m^2)	31 ± 5	33 ± 6	30 ± 5	32 ± 6
HbA_{1c} (%)	7.9 ± 1.3	7.9 ± 1.4	8.0 ± 1.1	7.8 ± 1.4
HbA_{1c} (mmol/mol)	63 ± 14.2	63 ± 15.3	64 ± 12.0	62 ± 15.3
UAER (mg/24 h)	123 (46–214)	93 (38–230)	153 (75–541)	95 (38–223)
P-creatinine (μmol/l)	84 ± 16	75 ± 19	76 ± 16	75 ± 18
eGFR (ml min^{-1} 1.73 m^{-2})	81 ± 14	91 ± 18	88 ± 15	91 ± 18
Systolic blood pressure (mmHg)	131 ± 16	129 ± 16	131 ± 19	129 ± 16
LDL-cholesterol (mmol/l)	2.0 ± 0.7	1.8 ± 0.8	2.2 ± 0.9	1.8 ± 0.8
Current smoker, *n* (%)	9 (30)	47 (29)	7 (32)	45 (29)
NT-proBNP (ng/l)	59.4 (26.3–165.2)	45.8 (16.9–88.8)	59.4 (28.3–179.6)	42.4 (15.3–88.5)
Coronary artery calcium score (Agatston units)	545 (199–1207)	130 (2–478)	679 (224–1971)	94 (1–433)
Carotid intima–media thickness (mm)	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.2
Treatment with:				
Oral glucose-lowering agents, *n* (%)	23 (77)	140 (87)	18 (82)	132 (85)
Insulin, *n* (%)	21 (70)	98 (61)	16 (73)	91 (58)
Anti-hypertensive drugs, *n* (%)	30 (100)	161 (100)	22 (100)	155 (99)
Statin, *n* (%)	28 (93)	155 (96)	19 (86)	149 (96)
Aspirin, *n* (%)	27 (90)	148 (92)	21 (95)	141 (90)

Non-normally distributed variables (UAER, NT-proBNP, coronary artery calcification score) are reported as median with IQR; other continuous variables as means ± SD

Categorical variables are shown as total numbers with corresponding percentages

Sensitivity analyses

The conclusions were unaffected after exclusion of people with ABI > 1.3 when applying 0.70 as cut-off for TBI and in analyses omitting adjustment for systolic blood pressure (electronic supplementary material [ESM] Table 1).

Table 2 Correlations between measures of peripheral arterial disease, coronary artery calcification score and carotid intima–media thickness

Measurement	TBI	ABI
Coronary artery calcification		
Unadjusted	−0.39 (−0.25, −0.52)***	−0.36 (−0.21, −0.50)***
Adjusted	−0.27 (−0.10, −0.43)**	−0.33 (−0.15, −0.49)***
Carotid intima–media thickness		
Unadjusted	−0.22 (−0.07, −0.37)**	−0.26 (−0.11, −0.42)**
Adjusted	−0.12 (−0.27, 0.03)	−0.21 (−0.05, −0.37)*

Values represent standardised β coefficients with 95% CIs

Adjustment included sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and urinary albumin excretion rate

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for correlations between TBI and coronary artery calcification and carotid intima–media thickness, respectively; and correlation between ABI and coronary artery calcification and carotid intima–media thickness, respectively.

Carotid intima–media thickness as a risk marker

Table 3 shows the associations between carotid intima–media thickness and the composite cardiovascular endpoint and all-cause mortality with determinant modelled as a continuous variable.

In the unadjusted analysis (Model 1), lower carotid intima–media thickness was associated with lower risk of the composite cardiovascular endpoint and all-cause mortality (Table 3). In Model 2, carotid intima–media thickness was not associated with the composite cardiovascular endpoint or all-cause mortality (Table 3).

Discussion

The current prospective study, including 200 individuals with type 2 diabetes, microalbuminuria and no clinical features of coronary artery disease followed for 6 years, demonstrates TBI

Table 3 TBI and ABI in relation to risk of fatal and non-fatal cardiovascular events and all-cause mortality

Measurement/model	Cardiovascular events (n = 40)		All-cause mortality (n = 26)	
	HR (95% CI)	p value	HR (95% CI)	p value
TBI (n = 193; SD = 0.23)				
1	1.55 (1.38, 1.68)	<0.0001	1.41 (1.22, 1.60)	0.010
2	1.50 (1.27, 1.65)	<0.0001	1.37 (1.01, 1.60)	0.049
3	1.36 (1.06, 1.56)	0.023	1.24 (0.78, 1.53)	0.25
ABI (n = 179; SD = 0.17)				
1	1.49 (1.32, 1.61)	<0.0001	1.37 (1.09, 1.57)	0.015
2	1.44 (1.23, 1.59)	<0.0001	1.39 (1.07, 1.60)	0.021
3	1.27 (0.95, 1.48)	0.087	1.27 (0.81, 1.55)	0.20
Carotid intima–media thickness (n = 182; SD = 0.15 mm)				
1	0.63 (0.19, 0.96)	0.028	0.48 (0.01, 0.88)	0.008
2	0.93 (0.53, 1.22)	0.68	0.64 (0.06, 1.05)	0.096
3	0.85 (0.46, 1.19)	0.43	0.60 (0.01, 1.06)	0.097
TBI (<0.64; n = 30)				
1	3.4 (1.7, 6.9)	<0.0001	3.8 (1.6, 8.8)	0.002
2	2.5 (1.2, 5.2)	0.016	3.5 (1.4, 8.6)	0.008
3	1.8 (0.8, 4.0)	0.18	3.2 (1.2, 8.5)	0.017
ABI (<0.90; n = 22)				
1	4.9 (2.2, 10.5)	<0.0001	3.9 (1.5, 10.3)	0.007
2	3.2 (1.4, 7.5)	0.007	3.7 (1.3, 10.6)	0.002
3	2.1 (0.9, 5.1)	0.10	3.2 (1.0, 10.8)	0.058

HRs for the determinant modelled as a continuous variable represents risk related to 1 SD decrease

HRs for the determinant modelled as a binary variable represents risk compared with TBI ≥ 0.64 (n = 163) or ABI ≥ 0.90 (n = 157) group

Model 1 is unadjusted; Model 2 is adjusted for sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and UAER

Model 3 is as Model 2, but with coronary artery calcium score and NT-proBNP added

and ABI as determinants of CVD and all-cause mortality after adjustment for traditional cardiovascular risk factors. After additional adjustment for NT-proBNP level and coronary artery calcification score, lower TBI remained associated with CVD.

Analyses of the added discriminatory ability of TBI and ABI demonstrated a significant increase in the AUC for the cardiovascular endpoint when adding TBI to a model including traditional cardiovascular risk factors. The IDI statistics

Table 4 C statistics for TBI and ABI in relation to risk of fatal and non-fatal cardiovascular events and all-cause mortality

Panel/model	AUC (95% CI)	Improvement AUC (95% CI)	p value
Panel a: composite CVD endpoint			
Base model	0.743 (0.661, 0.825)	0.0632 (0.0116, 0.1147)	0.016
Base model + TBI	0.806 (0.730, 0.882)		
Panel b: all-cause mortality			
Base model	0.736 (0.632, 0.840)	0.0162 (−0.0553, 0.0876)	0.66
Base model + TBI	0.752 (0.639, 0.865)		
Panel c: composite CVD endpoint			
Base model	0.761 (0.670, 0.852)	0.0553 (−0.00270, 0.113)	0.061
Base model + ABI	0.816 (0.725, 0.908)		
Panel d: all-cause mortality			
Base model	0.746 (0.628, 0.865)	0.0201 (−0.0592, 0.0993)	0.62
Base model + ABI	0.766 (0.639, 0.894)		

Base model includes sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and UAER

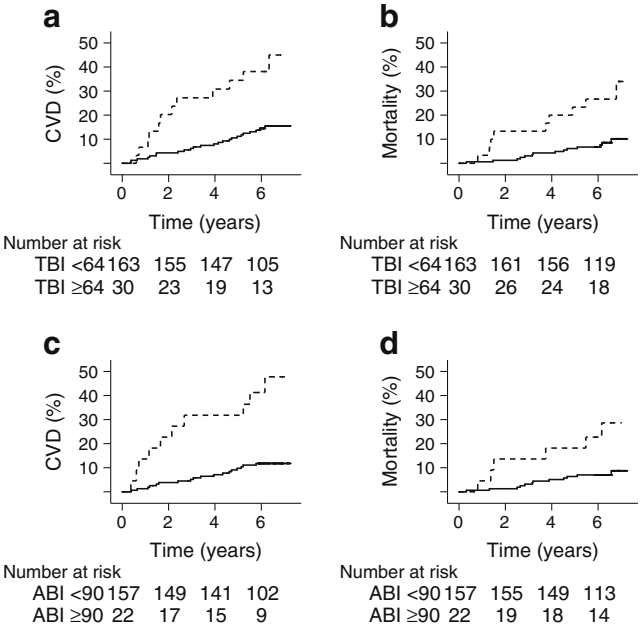

Fig. 1 Kaplan–Meier failure function estimates with two categories of TBI, above or below a cut-off of 0.64, for (**a**) the composite cardiovascular endpoint (HR 3.4 [95% CI 1.7, 6.9], $p < 0.001$) and (**b**) all-cause mortality (3.8 [1.6, 8.8], $p = 0.002$), and with two categories of ABI, above or below cut-off of 0.9, for (**c**) the composite cardiovascular endpoint (4.9 [2.2, 10.5], $p < 0.001$) and (**d**) all-cause mortality (3.9 [1.5, 10.3], $p = 0.007$). Numbers refer to participants in each category at risk at the beginning of each 2 year interval. Dashed line, below cut-off; solid line, above cut-off

show that adding TBI or ABI to traditional cardiovascular risk factors significantly improves the predictive capability for both CVD and all-cause mortality.

Recently, a study in 81 individuals with type 2 diabetes included after acute myocardial infarction and followed for 3.7 years demonstrated that low TBI (<0.6) and low toe blood pressure (<50 mmHg) predicted major cardiovascular events defined as a new episode of myocardial infarction, ischaemic cerebrovascular disease or peripheral arterial disease in appropriately adjusted models (low TBI, HR [95% CI] 2.92 [1.04, 8.19]; low toe blood pressure 3.83 [1.45, 10.1]) [3]. A study including 155 type 2 diabetic and 469 non-diabetic participants demonstrated low ABI (<0.90) to be associated with cardiovascular (HR [95% CI] 2.57 [1.50, 4.40]) and all-cause (2.02 [1.47, 2.76]) mortality after adjustment for risk factors included in the Framingham risk score. However, these associations were no longer significant (HR [95% CI] 1.74 [0.97, 3.14] and 1.30 [0.46, 3.62]) in analyses restricted to the diabetic subgroup [4]. The authors reported lower TBI to be associated with cardiovascular and all-cause mortality after adjustment for age, sex and glucose metabolism status, but the association was no longer significant after adjustment for the Framingham risk factors. Results for the predictive value of TBI restricted to the diabetic subgroup were not presented. In contrast to our study, individuals with prior CVD were included and follow-up was longer (median 17.2 years). A study by Hyun et al demonstrated TBI < 0.40 (TBI categories <0.40, 0.40–0.61, 0.62–1.08 and >1.08, with 0.62–1.08 as the reference category) to predict high risk of cardiovascular mortality in analysis adjusted for traditional cardiovascular risk factors (HR [95% CI] 2.25 [1.47, 3.43]). The study included 469 participants with clinically suspected atherosclerotic peripheral arterial disease, of whom 168 had diabetes; similar associations were demonstrated in the diabetic subgroup (2.28 [1.15, 4.55]). Median follow-up was 7.0 years [23].

Our present study provides support for the use of TBI as a tool refining risk prediction in type 2 diabetes. The addition of TBI to traditional cardiovascular risk factors improved risk prediction for CVD applying C statistics, and for CVD and all-cause mortality calculated as rIDI.

Recently, we demonstrated that a simple screening algorithm combining the NT-proBNP level and coronary artery calcification score added substantially to the risk information provided by traditional cardiovascular risk factors in the current cohort [12]. TBI remained associated with the composite cardiovascular endpoint even after adjustment for these powerful risk markers.

Our study expands on the existing literature by demonstrating that ABI – when modelled as a continuous and as a binary variable – was associated with incident CVD and all-cause mortality in asymptomatic people with type 2 diabetes and microalbuminuria independently of traditional risk factors. Continuous analysis has the greatest statistical power. However, cut-offs have greater applicability in the clinic.

The addition of ABI to traditional cardiovascular risk factors improved risk prediction for CVD and all-cause mortality evaluated by rIDI. The Fremantle Diabetes Study followed 1294 individuals with type 2 diabetes and previous CVD and demonstrated that low (≤0.90) compared with normal (0.91–1.40) ABI was associated with cardiovascular mortality [6]. In 2368 people with type 2 diabetes and coronary artery disease included in the Bypass Angioplasty Revascularization Investigation 2 Diabetes Trial, low ABI (<1.0) conferred an increased risk of mortality and major cardiovascular events during follow-up of a median 5.2 years [24].

Despite the existing literature, ABI has not been broadly implemented in daily practice for risk assessment in type 2 diabetes. This may be because of the reliability of ABI in people with diabetes in whom medial arterial calcification is frequently observed [7, 9, 25]. In a study including 83 people with diabetes (type 2, 83%) defined to be at high risk of peripheral arterial disease, examination with colour-flow duplex ultrasound demonstrated the presence of peripheral arterial occlusive disease in 57% of those with ABI between 0.9 and 1.3. This study confirmed that ABI often is incorrectly normal because of vessel stiffness in diabetes [7]. The vessels in the toe are less susceptible to medial calcification [9], therefore TBI may be more reliable for risk assessment in diabetes.

However, the association was similar in a sub-analysis excluding participants with ABI > 1.3.

Measurement of carotid intima–media thickness has been proposed to improve risk assessment beyond established cardiovascular risk factors. However, a meta-analysis including 14 population-based cohorts (45,828 individuals) demonstrated that adding carotid intima–media thickness to the Framingham risk score was associated with only a small improvement in 10 year risk prediction of first-time myocardial infarction or stroke (increase in AUC from 0.757 to 0.759) [10]. Similarly, a retrospective study including 783 people with type 2 diabetes, but without a history of CVD, demonstrated that adding carotid intima–media thickness to Framingham risk score resulted in a small increase in AUC from 0.645 to 0.656 [11]. In our analysis, carotid intima–media thickness was associated with the development of the combined cardiovascular endpoint and all-cause mortality in the crude analysis, but these associations were no longer significant after adjustment for traditional cardiovascular risk factors. However, our study was much smaller and thus had less power. Our results suggest no added value of carotid intima–media thickness measurements in cardiovascular risk prediction in type 2 diabetes. However, carotid intima–media thickness remains of interest in pathophysiological studies and as an endpoint in clinical trials.

The finding that both TBI and ABI were correlated with carotid intima–media thickness as well as coronary artery calcification illustrates that atherosclerosis is often a universal disease affecting different arterial beds. Despite this correlation, TBI was able to predict CVD in addition to the risk information provided by coronary artery calcification, further highlighting the strength of TBI.

Clinical implications

TBI is a relatively simple test easily performed in the clinical setting. Our findings of the added predictive value on top of traditional risk factors for both CVD and all-cause mortality highlight the great potential impact of implementing TBI measurement in type 2 diabetes in the clinical setting. Identifying individuals with the highest risk can enhance guidance of intensive management of vascular risk factors, including tighter goal setting for blood pressure, LDL-cholesterol and other cardiovascular risk factors.

Strengths and limitations

The strengths of our study include the prospective design, which included careful assessment of cardiovascular risk factors. This allowed extensive adjustment for these factors, including levels of NT-proBNP and coronary artery calcification score. Moreover, no participants were lost to follow-up. The significant results of the continuous models of ABI and TBI, without applying arbitrary thresholds, are other strong points.

Limitations include the relatively small sample size and that the study only included people with type 2 diabetes and microalbuminuria recruited from a single centre, limiting the generalisation of our findings. Individuals with microalbuminuria can be considered to be at a higher CVD risk and the lack of people with normoalbuminuria may have influenced the results.

Moreover, the inter- and intra-rater reliability of the ABI and TBI were not evaluated in our study. The larger variability of TBI might have impacted on the performance of TBI on the AUC models.

Conclusion

In people with type 2 diabetes and microalbuminuria but without clinical features of coronary artery disease, lower levels of TBI were associated with increased risk of CVD and all-cause mortality independently of traditional risk factors. After further adjustment for NT-proBNP level and coronary artery calcification score, lower TBI remained a significant predictor of CVD. The addition of TBI to traditional cardiovascular risk factors improved risk prediction for CVD and all-cause mortality. Lower ABI levels were associated with increased risk of CVD and all-cause mortality after adjustment for traditional cardiovascular risk factors, and improved risk prediction.

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Data availability The datasets generated during and/or analysed during the current study are not publicly available because of data protection issues. An anonymised form is available from the corresponding author on reasonable request.

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authors approved the final version of the manuscript. EHZ is responsible for the integrity of the work as a whole.

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Cardiovascular Diabetology

ORIGINAL INVESTIGATION

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Symmetric and asymmetric dimethylarginine as risk markers of cardiovascular disease, all-cause mortality and deterioration in kidney function in persons with type 2 diabetes and microalbuminuria

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Abstract

Background: To evaluate symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) as risk markers of cardiovascular disease, all-cause mortality and deterioration in renal function in a well characterised type 2 diabetic population with microalbuminuria and without symptoms of coronary artery disease.

Methods: 200 participants followed for 6.1 years. SDMA and ADMA were measured at baseline. Endpoints included (1) composite cardiovascular endpoint (n = 40); (2) all-cause mortality (n = 26); and (3) decline in eGFR of >30% (n = 42). Cox models were unadjusted and adjusted for traditional risk factors (sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, creatinine and urinary albumin excretion rate). To assess if SDMA or ADMA improved risk prediction beyond traditional risk factors we calculated c statistics and relative integrated discrimination improvement (rIDI). C statistic (area under the curve) quantifies the model's improved ability to discriminate events from non-events. rIDI quantifies the increase in separation of events and non-events on a relative scale.

Results: Higher SDMA was associated with increased risk of all three endpoints (unadjusted: $p \leq 0.001$; adjusted: $p \leq 0.02$). Higher ADMA was associated with all-cause mortality (unadjusted: $p = 0.002$; adjusted: $p = 0.006$), but not cardiovascular disease or decline in eGFR ($p \geq 0.29$).The c statistic was not significant for any of the endpoints for either SDMA or ADMA ($p \geq 0.10$). The rIDI for SDMA was 15.0% ($p = 0.081$) for the cardiovascular endpoint, 52.5% ($p = 0.025$) for all-cause mortality and 48.8% ($p = 0.007$) for decline in eGFR; for ADMA the rIDI was 49.1% ($p = 0.017$) for all-cause mortality.

Conclusion: In persons with type 2 diabetes and microalbuminuria higher SDMA was associated with incident cardiovascular disease, all-cause mortality and deterioration in renal function. Higher ADMA was associated with all-cause mortality. SDMA and ADMA significantly improved risk prediction for all-cause mortality, and SDMA for deterioration in renal function beyond traditional risk factors.

Keywords: Microalbuminuria, Type 2 diabetes, Cardiovascular disease, Macrovascular disease, Symmetric dimethylarginine, Asymmetric dimethylarginine

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Background

Cardiovascular disease is a major complication in type 2 diabetes despite multifactorial intervention [1, 2]. There is an ongoing search for biomarkers that can improve risk prediction in type 2 diabetes. Symmetric and asymmetric dimethylarginine (SDMA and ADMA, respectively) are dimethylarginines structurally related to L-arginine. Due to their biological functions, both markers have been explored as cardiovascular biomarkers. ADMA is considered an independent risk factor for cardiovascular disease and mortality in a range of populations at different levels of cardiovascular risk [3, 4] and in type 1 diabetes [5, 6]. Although elevated levels of ADMA in persons with type 2 diabetes and macrovascular disease have been reported in cross sectional studies [7, 8], there are conflicting results concerning the prognostic value [9, 10]. It is unknown whether associations seen for ADMA extend to the structural isomer SDMA. A recent meta-analysis demonstrated that higher SDMA is a risk factor for cardiovascular disease and mortality in different populations, with the strongest associations observed in the general population [3]. This meta-analysis did not report results for any diabetic cohorts. Studies of SDMA in type 2 diabetes are few; however a cross sectional study demonstrated higher SDMA in persons with cardiovascular disease [8].

The use of metabolomics has been instrumental in identifying new biomarkers of chronic kidney disease such as the dimethylarginines [11]. ADMA and SDMA were originally reported to accumulate in renal failure [12] and may also be risk factors for deterioration in renal function in type 2 diabetes. ADMA is an outcome predictor of acute kidney injurie [13] and has been reported to predict an increased rate of decline in GFR and development of end stage renal disease in type 1 diabetes [5] and has been related to presence of renal dysfunction in type 2 diabetes [8, 14].

We evaluated SDMA and ADMA as risk markers of cardiovascular disease, all-cause mortality and decline in renal function in a well characterised type 2 diabetic population with microalbuminuria and without symptoms suggestive of coronary artery disease. Moreover, we assessed whether SDMA and ADMA improved risk prediction beyond traditional risk factors using c statistics and integrated discrimination improvement (IDI).

Methods

Participants

200 participants were recruited from the outpatient clinic at Steno Diabetes Center Copenhagen. Inclusion criteria were (1) type 2 diabetes according to WHO criteria; (2) no history of coronary artery disease or symptoms suggestive of cardiac disease (assessed from interviews with the patient and patient records); and (3) persistent urinary albumin excretion rate (UAER) >30 mg/24 h (in two out of three consecutive measurements). 613 consecutive patients were invited by letter to participate in the study. 72 patients declined the invitation. 341 patients were excluded. Exclusion criteria were (1) normal or non-persistent elevated UAER (n = 52); (2) symptoms/signs or a history of cardiac disease, including Q waves in 12-lead ECGs (n = 180); (3) relative contraindications to computed tomography angiography or coronary angiography, including abnormal plasma creatinine levels (n = 86); (4) physical or mental disability (n = 10); or (5) malignancy (n = 13) [15].

At the time the study was designed, in the absence of data from other prospective studies using the biomarkers in a type 2 diabetic population, the study was conducted as an exploratory study using the sample size available.

Biochemical and other measures

Plasma concentrations of SDMA and ADMA were determined simultaneously by high-performance liquid chromatography with fluorescence detection as previously described [16], using modified chromatographic separation conditions [17]. For both SDMA and ADMA, the intra- and interassay coefficients of variation were <2 and <4%, respectively. Blood was centrifuged immediately after collection, and plasma was frozen at −80 °C and stored in a research biobank for analysis immediately after the last participant was examined. Thus the maximal storage time of the samples prior to analysis of both biomarkers was 13 months. Quantification of SDMA and ADMA was available for all participants. UAER was measured in 24-h collected urine samples by enzyme immunoassay (Vitros, Raritan, NJ, USA). Current smoking was defined as one or more cigarettes, cigars or pipes per day. Brachial blood pressure was measured twice after 10 min rest using an appropriate cuff size, and averaged.

Follow up

All participants were traced through the Danish National Death Register and the Danish National Health Register on the 1st of January 2014. No participants were lost to follow-up. Definitions of the three predefined endpoints have previously been described [18, 19]. The combined cardiovascular endpoint was defined as cardiovascular mortality, stroke, ischaemic cardiovascular disease and heart failure. For participants with multiple events, only the first was included. Moreover, we followed 177 out of the 200 (88.5%) participants originally included with yearly measurements of plasma creatinine used for calculations of eGFR applying the Chronic Kidney Disease

Epidemiology Collaboration (CKD-epi) equation [20]. The renal endpoint was defined as decline in eGFR >30% (evaluated as change from baseline to the last available measurement), as proposed by Coresh et al. [21].

Statistical analyses

Symmetric dimethylarginine, ADMA and UAER were non-normally distributed and are summarized as median with interquartile range (IQR), and log10 transformed in all analyses. All other continuous variables are given as mean ± standard deviation (SD) and categorical variables as total numbers with corresponding percentages.

We used *t*-test for continuous variables and X^2 test for categorical variables to test for differences in potential confounders in the population categorized according to SDMA and ADMA above or below the median.

First, we applied Cox proportional hazards analysis to compute the unadjusted hazard ratios (HR)'s per 1 SD increment of SDMA and ADMA with 95% confidence interval (CI) for the three endpoints, next we calculated HR's adjusted for traditional cardiovascular risk factors based on existing evidence: sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, plasma creatinine and UAER.

To quantify the added predictive value of SDMA and ADMA, we calculated receiver operating characteristic (ROC) curves based on logistic regression models and applied c statistics to compare the area under the curve (AUC) for the model including traditional cardiovascular risk factors and the AUC for the model including traditional cardiovascular risk factors plus SDMA and ADMA, respectively. For the significant associations in the adjusted models, we further illustrated the risk information with ROC-curves. We then calculated the IDI, a measure suggested by Pencina et al. [22] as a more powerful method to demonstrate improved diagnostic performance of a biomarker. To ease the interpretation, relative IDI (rIDI) is provided and reported as a percentage. rIDI is defined as the increase in discrimination slope when adding SDMA or ADMA respectively to traditional risk factors divided by the slope of the model including only the traditional risk factors [22].

Finally, we applied the Kaplan–Meier failure function to compare the risks of the combined cardiovascular endpoint, all-cause mortality and deterioration in renal function according to the median level of SDMA, and to compare the risks of all-cause mortality according to the median level of ADMA.

A two-tailed *p* value of <0.05 was considered significant. Statistical analyses were performed using SPSS for Windows (version 23.0, Chicago, IL, USA) and SAS software (version 9.4, SAS Institute, Cary, NC, USA).

Results

Patient characteristics

Baseline characteristics of all the 200 participants and categorized according to the median level of SDMA and ADMA respectively are listed in Table 1. Participants with high SDMA were older, had higher plasma creatinine, lower eGFR and systolic blood pressure compared to participants with low SDMA. Participants with high ADMA levels were older, with higher UAER and plasma creatinine and lower eGFR compared to participants with low ADMA. All participants were receiving multifactorial treatment on top of oral antidiabetic medication or insulin including antihypertensive drugs (99%), renin–angiotensin–aldosterone system-blocking treatment (98%), statins (95%) and aspirin (92%). Figure 1 illustrates the positive correlation between SDMA and ADMA ($R^2 = 0.20$; $p < 0.001$).

Incidence of cardiovascular disease, all-cause mortality and decline in eGFR

Median (IQR) follow up for the combined cardiovascular endpoint and mortality was 6.1 (5.9–6.6) years.

Combined cardiovascular endpoint (n = 40)

11 fatal cardiovascular events (two cases of acute myocardial infarction, one case of ischaemic cardiovascular disease, six sudden and otherwise unexplained deaths and two cases of heart failure) and 29 non-fatal cardiovascular events (three cases of acute myocardial infarction, three strokes, 19 cases of ischaemic cardiovascular disease, four cases of heart failure all leading to hospital admission).

All-cause mortality (n = 26)

11 related to cardiovascular disease, 9 cancer-related and 6 related to other causes.

Decline in eGFR >30% (n = 42)

Median (IQR) follow up was 4.9 (3.8–5.4) years. In 23 participants the decline was confirmed in two or more measurements. In 17 participants the decline was seen at the final study visit and could therefore not be confirmed. No participants progressed to dialysis or end-stage renal disease during follow up.

The distribution of time to event for the combined cardiovascular endpoint, all-cause mortality and deterioration in renal function is shown in Additional file 1: Figure S1.

Symmetric dimethylarginine as a risk marker

Table 2 shows the association between SDMA and cardiovascular disease, all-cause mortality, and decline in eGFR. Higher SDMA was associated with all endpoints

Table 1 Clinical characteristics of the study population at baseline categorized according to SDMA and ADMA values below or above the median

Characteristics	All participants (n = 200)	Symmetric dimethylarginine		p value	Asymmetric dimethylarginine		p value
		<0.4525 µmol/l (n = 100)	≥0.4525 µmol/l (n = 100)		<0.4625 µmol/l (n = 100)	≥0.4625 µmol/l (n = 100)	
Male, *n* (%)	*152 (76)*	76 (76)	75 (75)	0.87	77 (23)	74 (74)	0.62
Age (years)	*59 ± 9*	56.4 ± 9.2	60.9 ± 7.6	*<0.001*	57.2 ± 9.8	60.1 ± 7.3	*0.015*
Known duration of diabetes (years)	*13 ± 7*	10.8 ± 6.6	14.8 ± 7.6	*<0.001*	11.7 ± 7.3	13.9 ± 7.3	*0.033*
Body mass index (kg/m^2)	*32.6 ± 5.8*	33.0 ± 5.3	32.1 ± 6.2	0.24	32.3 ± 4.8	32.8 ± 6.6	0.48
HbA_{1c} (%)	*7.9 ± 1.3*	7.93 ± 1.36	7.80 ± 1.34	0.49	7.92 ± 1.41	7.81 ± 1.29	0.55
HbA_{1c} (mmol/mol)	*63 ± 14*	63 ± 14.9	62 ± 14.6	0.49	63 ± 15.4	62 ± 14.1	0.55
Urinary albumin excretion rate (mg/24-h)	*103 (39–230)*	104.5 (47.9–219.8)	98.0 (38.0–241.0)	0.86	80.5 (33.0–176.9)	133.0 (56.0–303.0)	*0.011*
P-creatinine (µmol/L)	*76 ± 18*	67.0 ± 13.8	85.9 ± 17.4	*<0.001*	72.0 ± 17.4	80.9 ± 18.2	*<0.001*
eGFR (ml/min/1.73 m^2)	*130 ± 17*	99.3 ± 13.0	79.7 ± 15.6	*<0.001*	94.6 ± 16.9	84.4 ± 16.3	*<0.001*
Systolic blood pressure (mmHg)	*152 ± 76*	133 ± 18	127 ± 16	*0.012*	130 ± 17	130 ± 17	0.60
Diastolic blood pressure (mmHg)	*75 ± 11*	77 ± 11	73 ± 11	*0.016*	76 ± 11	73 ± 11	*0.051*
LDL cholesterol (mmol/L)	*1.85 ± 0.78*	1.90 ± 0.88	1.81 ± 0.67	0.39	1.81 ± 0.78	1.90 ± 0.78	0.45
Current smoker, *n* (%)	*59 (30)*	34 (34)	25 (25)	0.16	31 (31)	28 (28)	0.64
Treatment with							
Oral antidiabetic, *n* (%)	*170 (85)*	87 (87)	83 (83)	0.43	89 (89)	81 (81)	0.11
Insulin, *n* (%)	*124 (62)*	58 (58)	66 (66)	0.24	59 (59)	65 (65)	0.38
Antihypertensive drugs, *n* (%)	*198 (99)*	100 (100)	98 (98)	0.16	99 (99)	99 (99)	1.00
RAAS blockade, *n* (%)	*196 (98)*	96 (96)	100 (100)	*0.043*	98 (98)	98 (98)	1.00
Beta-blocker, *n* (%)	*27 (14)*	9 (9)	18 (18)	*0.063*	11 (11)	16 (16)	0.31
Calcium channel blockers, *n* (%)	*80 (40)*	43 (43)	37 (37)	0.39	36 (36)	44 (44)	0.25
Diuretics, *n* (%)	*128 (64)*	57 (57)	41 (41)	*0.039*	63 (63)	65 (65)	0.77
Statin, *n* (%)	*189 (95)*	94 (94)	95 (95)	0.76	97 (97)	92 (92)	0.12
Aspirin, *n* (%)	*183 (92)*	92 (92)	91 (91)	0.80	90 (90)	93 (93)	0.45

p values for differences between participants with symmetric and asymmetric dimethylarginine below or above the median

Italic values indicate significance of p value ($p < 0.05$)

eGFR estimated glomerular filtration rate, *RAAS* renin-angiotensin-aldosterone system

in both unadjusted ($p \leq 0.001$) and adjusted ($p \leq 0.02$) analyses.

As shown in Fig. 2a, b in relation to the composite cardiovascular endpoint, the AUC increased from 0.745 (95% CI 0.668–0.822) to 0.768 (0.686–0.850), and in relation to all-cause mortality, the AUC increased from 0.743 (0.644–0.843) to 0.803 (0.713–0.893) after adding SDMA to the model including traditional risk factors. As shown in Fig. 2c for decline in eGFR >30% the AUC increased from 0.722 (0.631–0.813) to 0.752 (0.664–0.840). Increase in AUC (c statistic) quantifies the model's improved ability to discriminate events from non-events. In simpler terms, if the model including SDMA on top of traditional risk factors is more likely to assign higher risk to persons with events. These changes in the AUC were all non-significant ($p \geq 0.10$). Pencina et al. [22] have described how AUC does not change materially even for powerful predictors and the increase in AUC is directly

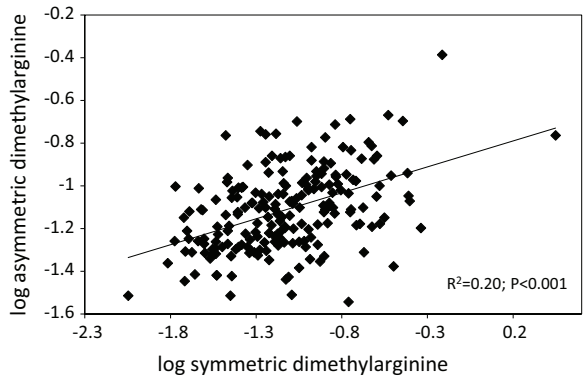


Fig. 1 Correlation between symmetric and asymmetric dimethylarginine

affected by the robustness of the baseline model. IDI statistics have been suggested by Pencina to further evaluate the incremental contribution of a new biomarker. rIDI quantifies the increase in separation of events and non-events on a relative scale. The rIDI was 15.0% (p = 0.081) for the cardiovascular endpoint, 52.5% (p = 0.025) for all-cause mortality and 48.8% (p = 0.007) for decline in eGFR >30%.

The cumulative incidence of decline in eGFR was higher in participants with SDMA level above the median (p = 0.0003; Additional file 1: Figure S2 panel c). However, the cumulative incidence of cardiovascular disease and all-cause mortality was similar in participants with SDMA level above or below the median (p ≥ 0.098; Additional file 1: Figure S2 panel a and b).

Asymmetric dimethylarginine as a risk marker

Table 2 shows the association between ADMA and cardiovascular disease, all-cause mortality, and decline in eGFR. In unadjusted analysis, higher ADMA was associated with all-cause mortality (p = 0.002), but not with cardiovascular disease (p = 0.38) or decline in eGFR >30% (p = 0.29). The association between ADMA and all-cause mortality persisted after adjustment for traditional risk factors (p = 0.006). As shown in Fig. 2d when adding ADMA to traditional risk factors the AUC increased from 0.743 (95% CI 0.644–0.843) to 0.794 (0.699–0.889) in relation to all-cause mortality. The change was non-significant (p = 0.11). The rIDI for ADMA was 49.1% (p = 0.017) for all-cause mortality.

The cumulative incidence of all-cause mortality was similar in participants with ADMA level above or below the median (p = 0.74; Additional file 1: Figure S2 panel d).

Discussion

In our cohort of 200 persons with type 2 diabetes and microalbuminuria, we demonstrated higher SDMA to be an independent determinant of incident cardiovascular disease, all-cause mortality and decline in renal function. We further demonstrated higher ADMA to be associated with all-cause mortality after adjustment for traditional risk factors, while ADMA was not associated with incident cardiovascular disease and decline in renal function. When applying c statistics, SDMA and ADMA respectively added to traditional risk factors did not significantly increase the risk prediction. IDI statistics has been suggested by Pencina as a necessary and more powerful method going beyond statistical significance and c statistic to evaluate the incremental contribution of a new biomarker [22]. We demonstrated added predictive value using IDI statistics for all-cause mortality after addition of SDMA and ADMA, respectively; and for decline in renal function adding SDMA to traditional risk factors.

Symmetric dimethylarginine is without direct inhibitory effect on nitric oxide synthesis and has therefore been given little attention compared to ADMA [12]. We are only aware of two studies that have investigated the association of SDMA to incident cardiovascular disease in persons with type 2 diabetes. In 270 persons with type 2 diabetes Hsu et al. [23] reported elevated

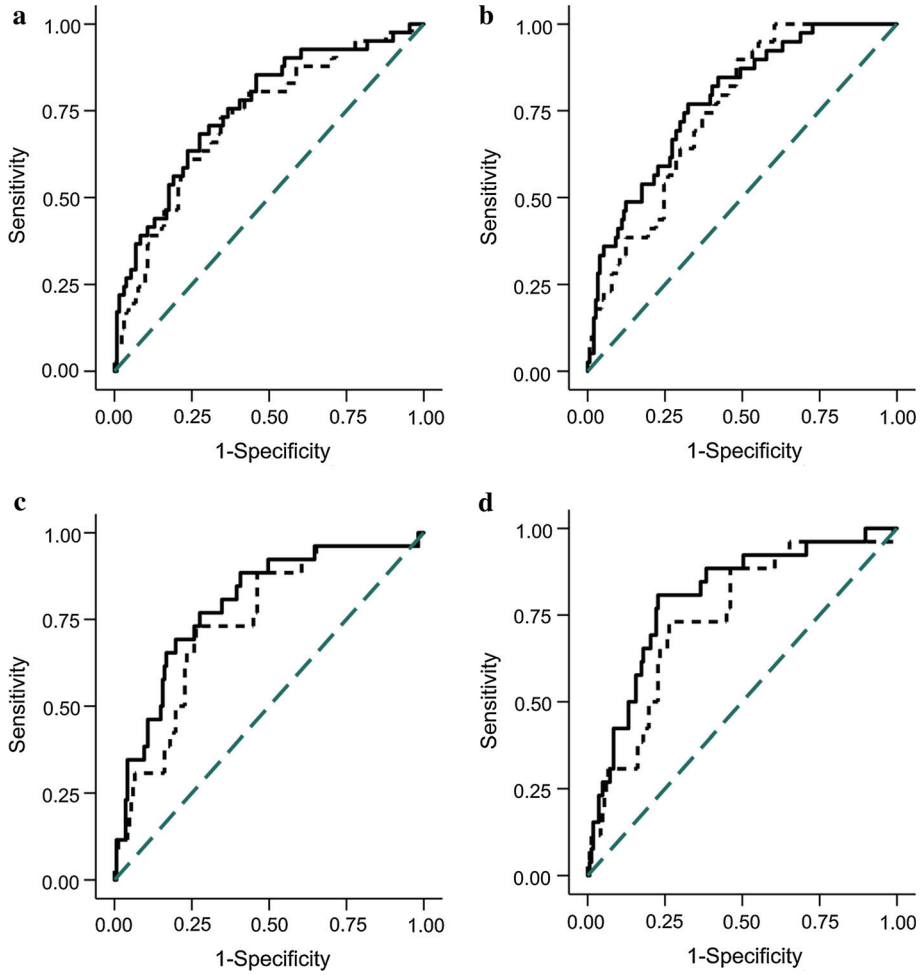
Table 2 Biomarkers in relation to risk of fatal and nonfatal cardiovascular events, all-cause mortality and decline in eGFR >30%

Biomarker	Model	Cardiovascular events (n = 40)	p	All-cause mortality (n = 26)	p	Decline in eGFR > 30% (n = 42)	p
Symmetric dimethylarginine log scale (1 SD = 0.06)	Unadjusted	1.5 (1.2–1.9)	*0.001*	1.6 (1.2–2.1)	*0.001*	1.9 (1.3–2.6)	*<0.001*
	Adjusted	1.7 (1.1–2.6)	*0.019*	2.3 (1.4–3.9)	*0.001*	2.2 (1.4–3.7)	*0.002*
Asymmetric dimethylarginine log scale (1 SD = 0.13)	Unadjusted	1.2 (0.8–1.6)	0.38	1.7 (1.2–2.5)	*0.002*	1.2 (0.8–1.6)	0.29
	Adjusted	1.0 (0.7–1.5)	0.93	1.8 (1.2–2.7)	*0.006*	1.0 (0.7–1.4)	0.85

Values are hazard ratios with 95% confidence intervals, and represent 1 SD increment of log-transformed values of the biomarkers. Adjustment included sex, age, systolic blood pressure, LDL cholesterol, smoking, HbA_{1c}, plasma creatinine, and urinary albumin excretion rate

Italic values indicate significance of p value (p < 0.05)

eGFR estimated glomerular filtration rate



	AUC (95% CI)	p-value
Panel a: composite cardiovascular disease endpoint		
Base model	0.745 (0.668, 0.822)	0.24
Base model + symmetric dimethylarginine	0.768 (0.686, 0.850)	
Panel b: all-cause mortality		
Base model	0.743 (0.644, 0.843)	0.10
Base model + symmetric dimethylarginine	0.803 (0.713, 0.893)	
Panel c: deterioration in kidney function		
Base model	0.722 (0.631, 0.813)	0.16
Base model + symmetric dimethylarginine	0.752 (0.664, 0.840)	
Panel d: all-cause mortality		
Base model	0.743 (0.644, 0.843)	0.11
Base model + asymmetric dimethylarginine	0.794 (0.699, 0.889)	

Fig. 2 Receiver operating characteristic (ROC) curves. Base model includes sex, age, systolic blood pressure, LDL-cholesterol, smoking, HbA_{1c}, creatinine and urinary albumin excretion rate. *Dashed line* reference; *Dotted line* Base model; *Full line* Base model + symmetric dimethylarginine/asymmetric dimethylarginine

SDMA to predict risk of cardiovascular events (cardiovascular death, non-fatal myocardial infarction and stroke) in univariate analysis, but significance was lost after adjustment. Median follow-up was 5.7 years. A recent study by Anderssohn et al. reported an association of SDMA with prevalent cardiovascular disease in 783 older persons with type 2 diabetes. This association lost significance after adjusting for age, sex and renal

function. After 4 years of follow-up SDMA was not significantly associated with risk of incident coronary artery or cerebrovascular disease [9]. In our cohort we showed an association between higher SDMA and incident cardiovascular disease, all-cause mortality as well as deterioration in kidney function. SDMA has emerged as a marker of renal disease and in contrast to ADMA which is cleared mainly through enzymatic action [24], SDMA is almost completely excreted by the kidneys [25]. Levels of SDMA have previously been shown to be closely related to glomerular filtration rate in a cross-sectional study in persons with coronary artery disease [26]. This relation was confirmed in the present study, where high levels of SDMA were associated with lower levels of eGFR at baseline. Importantly, in our cohort SDMA was associated with deterioration in kidney function after adjustment for kidney function at baseline. Besides the strong correlation with renal function, the underlying mechanism of SDMA as a marker of risk in diabetes could potentially be explained by a pro-inflammatory effect. Where ADMA directly inhibits the production of nitric oxide by interfering with the nitric oxide synthase, SDMA acts as a competitor of L-arginine, the substrate for nitric oxide synthase. This ultimately leads to an increased endothelial production of reactive oxygen species [26, 27] and this pro-inflammatory effect of SDMA may trigger vascular pathology. We demonstrated a moderate correlation between SDMA and ADMA. However, the relationship between SDMA and ADMA may not be straightforward and they may reflect different aspects of pathophysiology. We suggest SDMA as a promising new marker of endothelial dysfunction and inflammation with the potential to improve risk prediction in persons with type 2 diabetes and microalbuminuria.

The lack of association between ADMA and incident cardiovascular disease is in contrast to a number of previous studies. However, the prospective studies of ADMA in type 2 diabetes have mainly included subjects at high cardiovascular risk including manifest cardiovascular disease [23, 28–30]. Krzyzanowska et al. demonstrated an independent association between high ADMA and manifest cardiovascular disease (myocardial infarction, percutaneous coronary intervention, coronary-artery bypass graft, stroke, carotid revascularization and all-cause mortality) in 125 participants with type 2 diabetes after a median follow-up of 21 months [tertile III vs tertile I: HR 2.37 (95% CI 1.05–5.35), $p = 0.038$] [29]. Of the 125 participants, 40% had a history of macrovascular disease. Hsu et al. reported elevated ADMA to predict risk of cardiovascular events when analyzed both as categorical [tertile III vs tertile I: HR 2.3 (95% CI 1.1–4.8), $p = 0.026$] and as continuous variable [per 1 SD (0.09 μmol/l) increase HR: 1.30 (95% CI 1.01–1.68), $p = 0.04$] after appropriate adjustment [23]. 78% of the participants had concomitant coronary artery disease at baseline. Cavusoglu et al. reported elevated levels of ADMA to be independently associated with an increased risk of cardiovascular outcomes at 2-years of follow-up in high-risk type 2 diabetic males with known or suspected coronary artery disease referred for coronary angiography [tertile III vs tertile I–II: composite outcome of all-cause mortality, myocardial infarction or stroke HR 2.00 (1.10–3.62), $p = 0.02$] [30]. In contrast, two larger studies, the Framingham Offspring study enrolling 3320 participants (372 with diabetes) and a study in 997 individuals (359 with diabetes) referred for coronary angiography demonstrated higher ADMA to be associated with cardiovascular events and all-cause mortality only in the participants without diabetes at baseline [10, 31]. In the study including 783 older persons with type 2 diabetes, the association between higher ADMA and prevalent cardiovascular disease lost significance after adjustment for age, sex and renal function, and in the prospective analysis ADMA was not associated with incident coronary artery or cerebrovascular disease [9].

There is some evidence that both glycaemic control and nephropathy status may directly affect ADMA levels [23, 32, 33]. Therefore, ADMA levels may change with progression of disease and complications status and the prognostic value of ADMA in persons with diabetes could therefore be highly dependent on the study population investigated. This could explain the conflicting results concerning the prognostic value of ADMA for cardiovascular complications in type 2 diabetes. Our study population differs from previous studies as we excluded participants with a history of or symptoms suggestive of coronary artery disease. In contrast we did find a relation to all-cause mortality. This is in accordance with findings in the aforementioned study by Cavusoglu et al. [tertile III vs tertile I–II: all-cause mortality HR 2.63 (1.13–6.11), $p = 0.03$] [30]. Other authors have included all-cause mortality in a combined cardiovascular endpoint and reported positive associations [29].

The ratio of SDMA/ADMA has been suggested as a predictive biomarker for decline in renal function in persons with type 2 diabetes [14]. In our study the SDMA/ADMA ratio was associated with all-cause mortality ($p = 0.039$), but not with cardiovascular disease or deterioration in kidney function ($p \geq 0.12$) in analysis adjusted for traditional risk factors.

Clinical implications

We demonstrate that the addition of SDMA to traditional risk factors improved risk prediction for all-cause mortality and decline in renal function and our findings illustrate that SDMA may be useful in the risk assessment

of persons with type 2 diabetes. However, evidence suggests that the role of both SDMA and ADMA in diabetes is complex and not completely understood. Therefore, further research is needed in type 2 diabetic subjects at different risk of cardiovascular and renal disease to further elucidate the role of the two dimethylarginines.

Study limitations

The main limitation is the relatively small sample size. Moreover, it is important to consider that we included persons with type 2 diabetes and microalbuminuria recruited from a single center, limiting the generalisation of our results. For both SDMA and ADMA we report non-significant improvements in risk prediction evaluated with c statistics. However, we demonstrated added predictive value using IDI statistics. It remains a challenge to establish the ranges of meaningful improvement for IDI and the clinical value of ADMA and SDMA as risk markers remains to be evaluated in interventions studies.

Conclusion

In a well-characterized population with type 2 diabetes, microalbuminuria and without a history or symptoms suggestive of coronary artery disease, higher SDMA was associated with incident cardiovascular disease, all-cause mortality and deterioration in renal function, and improved risk prediction for all-cause mortality and renal decline beyond traditional risk factors. Higher ADMA improved risk prediction for all-cause mortality beyond traditional risk factors.

Additional file

Additional file 1: Figure S1. Distribution of time to event for the combined cardiovascular endpoint, all-cause mortality and deterioration in renal function. **Figure S2.** Kaplan–Meier failure function estimates.

Abbreviations

ADMA: asymmetric dimethylarginine; AUC: area under the curve; eGFR: estimated glomerular filtration rate; IDI: integrated discrimination improvement; rIDI: relative integrated discrimination improvement; ROC: receiver operating characteristic; SDMA: symmetric dimethylarginine; UAER: urinary albumin excretion rate.

Authors' contributions

EHZ, BJvS, HR, HHP, PKJ and PR conceived and designed the research; TT measured ADMA and SDMA and wrote the method description for these analysis; EHZ, BJvS, FP, HR, PR and TWH analysed and interpreted the data; BJvS and TWH performed the statistical analysis; EHZ wrote the manuscript; FP, TT, HR, HHP, PKJ and TWH critically revised the manuscript for key intellectual content; and PKJ and PR obtained funding and supervised the study. EHZ is responsible for the integrity of the work as a whole. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analysed during the current study are not publicly available due to data protection aspects but are available in an anonymized form from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study complied with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all participants gave written informed consent.

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Paper 7

Cuff inflations do not affect night-time blood pressure: comparison of 24 h ambulatory blood pressure measured by a cuff and a tonometric device in type 2 diabetes

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Discomfort related to cuff inflation may bias 24 h ambulatory blood pressure (BP) measurements, especially during night-time. We accessed the impact of cuff inflations by comparing 24 h BP recorded with a cuff-less tonometric wrist device and an upper-arm oscillometric cuff device. Fifty-three participants with type 2 diabetes were assigned randomly to four 24-h BP recordings with a cuff (TM2430: visit 1 or 2, and 4) and a tonometric device (BPro: visit 1 or 2, 3, and 4). The mean 24 h systolic BP was significantly higher when measured with the cuff versus the tonometric device (141.6 ± 14.6 vs. 128.3 ± 14.6 mmHg, $P \leq 0.01$), as was nocturnal BP (6.7 ± 5.3 vs. 10.3 ± 7.6%, $P = 0.002$). In conclusion, nocturnal BP decline was higher when measured with the cuff device, suggesting that cuff inflations did not increase night-time BP. Further evaluation of the tonometric device using the updated European Society of Hypertension International Protocol revision 2010 is recommended before applying it in daily clinical practice. *Blood Press Monit* **20:369–372 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.**

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Keywords: blood pressure measurements, night-time blood pressure, reproducibility, sleep quality

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Introduction

Traditionally, 24-h blood pressure (BP) devices are upper-arm type self-inflating cuff monitors. Some patients find the frequent arm compressions by the cuff uncomfortable [1]. Especially, the night-time BP might be affected because of impaired sleep quality and it has been claimed that disturbed sleep caused by the monitor may result in overestimation of the night-time BP. However, the influence of cuff inflations on the level of night-time BP/diurnal BP profile under ambulatory settings has not yet been clarified. A more convenient cuff-less wrist device based on tonometry has become commercially available. This device records 24 h brachial BP, derived from radial pulse waves that are calibrated to brachial BP.

We investigate the impact of cuff inflations on subjective and objective sleep quality and level of night-time BP by comparing a cuff-less wrist device (BPro) with a standard self-inflating arm-cuff device (TM2430).

Methods

In a crossover study, we recruited 53 type 2 diabetes patients followed at Steno Diabetes Center, Denmark. Participants were randomized to four 24-h BP measurements in sequence A (tonometric, cuff, tonometric, tonometric + cuff) or B (cuff, tonometric, tonometric, tonometric + cuff). The cuff device was positioned on the left upper arm, the tonometric device around the left wrist, except at visit 4, when both devices were worn at the same time, which is why the tonometric device was positioned on the right wrist. Three participants were excluded from visit 4 because of inter-arm systolic blood pressure (SBP) difference more than or equal to 10 mmHg and two participants refused the second cuff-device measurement.

The cuff device is validated according to the British Society of Hypertension and Association for Advancement of Medical Instrumental [2] and recommended by the DABL Educational Trust. The tonometric device is not recommended by the DABL Educational Trust [3], and has not been validated according to current protocols. However, the device has been validated according to the modified 2002 European Society of Hypertension protocol [4], is listed on the British Hypertension Society's website as validated according to 'international protocol', and has been compared with a cuff device with moderate agreement [5].

Discrimination between daytime and night-time was based on self-reported sleep time.

At visits 1, 2, and 3, the participants wore a validated [6] accelerometer, estimating sleep time and sleep quality.

Following each BP recording, participants evaluated device-related discomfort and influence on night-time sleep using a Visual Analog Scale from 0 to 10.

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The study conformed to the Declaration of Helsinki. The study protocol was approved by the regional ethics committee and all participants provided written informed consent.

Statistical analysis

Paired Student's *t*-test compared the interdevice and intradevice differences for 24 h, daytime, and night-time BP, and nocturnal SBP decline. Inter-SBP and intra-SBP device agreement was visualized by Bland–Altman plots. A two-tailed *P*-value less than 0.05 was considered statistically significant.

Results

The study included 42 men and 11 (20.8%) women, aged 64.1 ± 9.6 years, and 96.2% were on antihypertensive treatment (maintained during the study). At visit 1, the mean office BP was 134.1 ± 15.2 mmHg systolic and 75.6 ± 10.3 mmHg diastolic. Table 1 summarizes 24 h, night-time, and daytime systolic and diastolic BP at all visits.

Comparison of the tonometric and cuff device

The first tonometric recording of 24 h and night-time SBP was significantly lower than the first cuff device recording (difference in 24 h SBP: 13.3 ± 15.8 mmHg, $P < 0.001$; night-time SBP: 6.9 ± 17.3 mmHg, $P = 0.01$, Fig. 1a). The second tonometric recording was also significantly lower than the second cuff recording (24 h SBP: 13.8 ± 15.6 mmHg, $P < 0.001$; night-time SBP 6.4 ± 16.6 mmHg, $P = 0.01$). Also, when both devices were worn concurrently during the same 24-h period, the 24 h SBP was lower for the tonometric device (9.6 ± 15.2 mmHg, $P < 0.001$; Fig. 1b). The cuff device was evaluated as significantly more uncomfortable, to have a higher influence on night-time sleep, and to be more painful than the tonometric device ($P \leq 0.023$; Table 1). The difference in 24 h SBP between the two devices was not related to the evaluation of daytime discomfort between the two devices ($P = 0.23$). Also, the difference in night-time SBP between the two devices was not related to accelerometer-accessed sleep quality ($P = 0.27$).

Nocturnal SBP decline

Nocturnal SBP decline was significantly lower when measured with the tonometric than the cuff device ($P = 0.002$; Table 1). Accelerometer-accessed sleep quality did not differ between nights carrying the tonometric or cuff device ($P = 0.26$) nor was it associated with nocturnal SBP decline recorded with the tonometric or the cuff device at the two first visits. Furthermore, intraindividual comparison of sleep quality assessed with the accelerometer on the first night with the tonometric versus the first night with the cuff device showed no difference ($P = 0.26$).

Reproducibility of the tonometric and cuff device

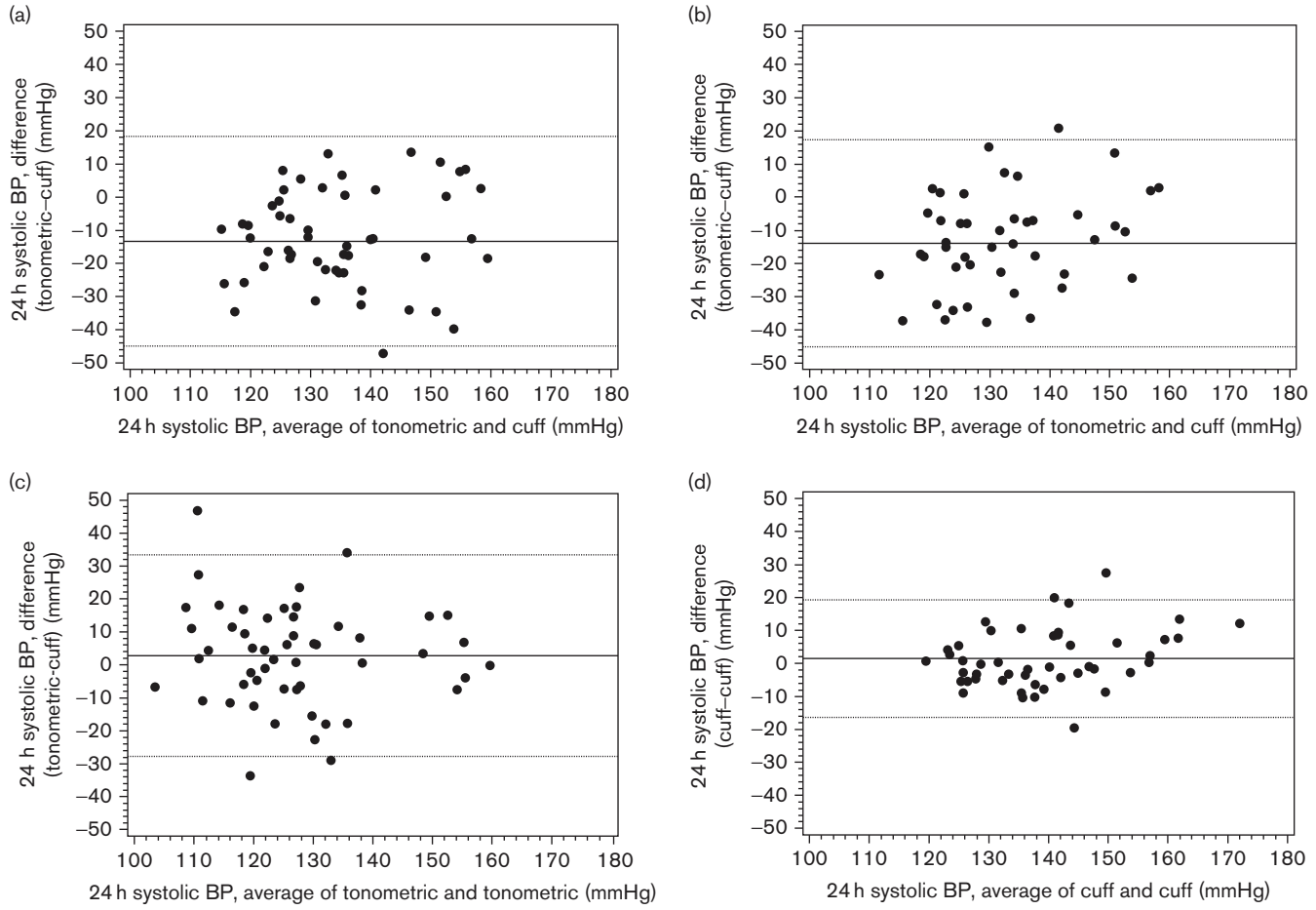
The Bland–Altman plots (Fig. 1c and d) show agreement between the first and second recording with the two devices. There was no significant difference in 24 h or night-time SBP ($P \geq 0.19$) between the first and second recording with the tonometric device, or between the first and second recording with the cuff device ($P \geq 0.09$).

Table 1 Ambulatory blood pressure, evaluation of device-related discomfort, and related parameters at visits 1–4

Visit	Visit 1 and 2 ($n = 53$)			Visit 3 ($n = 53$)	Visit 4 ($n = 48$)		
BP device	Tonometric device	Cuff device	*P*-value	Tonometric device	Tonometric device	Cuff device	*P*-value
24 h BP (mmHg)							
Systolic	128.3 ± 14.6	141.6 ± 14.6	< 0.001	125.5 ± 15.9	129.1 ± 15.9	138.6 ± 11.5	< 0.001
Diastolic	75.0 ± 11.9	78.1 ± 8.8	0.018	73.1 ± 10.0	73.2 ± 9.8	77.5 ± 7.3	0.001
Daytime BP (mmHg)							
Systolic	131.9 ± 14.2	145.1 ± 15.0	< 0.001	129.0 ± 15.9	133.0 ± 16.9	142.4 ± 12.5	< 0.001
Diastolic	77.0 ± 11.7	80.3 ± 9.1	0.019	75.2 ± 10.2	75.4 ± 10.7	79.9 ± 7.9	0.003
Night-time BP (mmHg)							
Systolic	123.1 ± 15.5	130.0 ± 16.6	0.006	120.2 ± 16.7	124.3 ± 16.5	126.0 ± 13.7	0.46
Diastolic	72.1 ± 12.5	70.9 ± 9.9	0.39	70.0 ± 10.1	70.6 ± 9.6	69.0 ± 8.9	0.21
Daytime discomfort	1 (0–2) $n = 52$	5 (2–7) $n = 53$	< 0.001	1 (0–1) $n = 52$	1 (0–1) $n = 48$	4 (2–7) $n = 48$	< 0.001
Night-time discomfort	0 (0–1) $n = 51$	5 (2–8.5) $n = 52$	< 0.001	0 (0–1) $n = 52$	0 (0–1) $n = 48$	5 (1–7) $n = 47$	< 0.001
Experience of pain	0 (0–2) $n = 50$	2 (0–5) $n = 53$	0.023	0 (0–1) $n = 52$	0 (0–0.5) $n = 48$	1 (0–4) $n = 47$	< 0.001
Influence on night-time sleep	0 (0–0) $n = 48$	5 (2–8) $n = 50$	< 0.001	0 (0–1) $n = 50$	0 (0–0) $n = 45$	4 (0–8) $n = 43$	< 0.001
Nocturnal systolic BP decline (%)	6.7 ± 5.3	10.3 ± 7.6	0.002	6.9 ± 4.3	6.4 ± 5.0	11.3 ± 8.7	< 0.001
Estimated sleeping time (h)	7.0 ± 1.8	6.8 ± 2.1	0.41	6.8 ± 1.8	–	–	–
Self-reported sleeping time (h)	8.1 ± 1.6	8.0 ± 1.1	0.76	–	–	–	–
Lying down time (h)	8.6 ± 1.8	8.1 ± 2.1	0.11	8.3 ± 1.9	–	–	–

Values are represented as mean ± SD and median (interquartile range).
Evaluation of daytime and night-time discomfort, experience of pain, and influence on night-time sleep with a Visual Analog Scale range from 0 to 10, where 0 indicates no discomfort and 10 indicates maximal discomfort. The Wilcoxon signed-rank test was used to compare the visual analog scale score for the tonometric and the cuff device. Student's paired *t*-test was used to compare BP, nocturnal systolic BP decline, sleeping time, and lying down time.
BP, blood pressure.

Fig. 1



Bland–Altman plots of 24 h systolic blood pressure (BP) comparing (a) tonometric device versus cuff device at different visits (b) tonometric device versus cuff device at the same visit (visit 4) (c) first versus second recording with the tonometric device (d) first versus second recording with the cuff device.

However, the SDs for the tonometric measurements exceeded the SDs from the cuff measurements.

Discussion

This study compared an upper-arm type, self-inflating cuff device (Takeda; A & D Medical, Tokyo, Japan) against a tonometric cuff-less wrist device (BPro; HealthSTATS International, Singapore) for ambulatory 24-h BP measurements in participants with type 2 diabetes.

Evaluation of device discomfort clearly showed that participants rated the cuff device significantly more uncomfortable than the tonometric device both during daytime and night-time. However, there was no association between nocturnal SBP decline and subjective evaluation of the BP devices influence on night-time sleep for either device. This was predictable for the tonometric device, which in general did not bother participants, but unexpected for the cuff device as participants subjectively were disturbed by wearing this device. Actually, we found a higher nocturnal SBP decline during nights among participants wearing the cuff device.

The accelerometer-accessed objective sleep quality did not correlate with 24 h or night-time SBP, nor was there an intraindividual difference in sleep quality between the first night with the tonometric device and the first night with the cuff device. Thus, subjectively, participants found the cuff inflations uncomfortable and disturbing during sleep, but objectively, cuff inflations did not impair sleep quality. Brigden *et al.* [7] performed 24 h intra-arterial BP monitoring, followed by 24 h combined intra-arterial and arm-cuff BP monitoring in 13 hospitalized patients, showing that brachial cuff inflations did not induce pressor responses or influence daytime or night-time BP, consistent with our results based on measurement during normal everyday activity.

The high reproducibility suggests that both devices may be suitable for repeated BP measurement in the individual participant. However, the SD of the mean difference

was high for both devices, especially for the tonometric device.

Twenty-four hour and night-time SBP was significantly higher when measured with the cuff device compared with the tonometric device at the two first visits. The difference was not explained by variance in either subjective or objective sleep quality. We wonder whether the tonometric device selectively fails to measure high BP values associated with movement. A good correlation between BP measured with the tonometric BPro device and oscillometric and auscultatoric office BP measured with a cuff device has been found by our group in 25 diabetic patients [8]. This is consistent with the fact that under office conditions, movements are few.

Conclusion

Participants graded the cuff device more uncomfortable and disturbing during sleep, but nocturnal BP decline was higher measured with the cuff device. Furthermore, subjective and objective sleep quality did not correlate with night-time BP – all indicating that cuff inflation did not affect BP. The BP measurements with both devices were reproducible, but were significantly higher for the cuff device than the tonometric device.

Further evaluation of the tonometric device using the updated European Society of Hypertension International Protocol revision 2010 is recommended before applying it in daily clinical practice.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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Paper 8

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Pleiotropic effects of liraglutide treatment on renal risk factors in type 2 diabetes: Individual effects of treatment

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ABSTRACT

Aims/hypothesis: Management of diabetic nephropathy includes reduction of albuminuria, blood pressure and weight. The GLP-1 receptor agonist liraglutide may possess these pleiotropic effects in addition to the glucose lowering effect. We aimed to elucidate the individual liraglutide treatment response by determining if high responders (highest reduction) in each risk factor also had high response in other renal risk factors (cross-dependency).

Methods: Open-label study: 31 type 2 diabetics treated with liraglutide for 7 weeks. After 3 weeks washout 23 re-started treatment and were followed for 1 year.

HbA_{1c}, weight, systolic blood pressure (SBP), urinary albumin excretion rate (UAER) and mGFR (^{51}Cr-EDTA) were evaluated. Changes in high (Q4) vs. low responders (Q1–Q3) were compared for each renal risk factor. The effects of treatment/off treatment/re-treatment (off–on/off–on effect) were evaluated to account for random effects.

Results: After 7 weeks HbA_{1c} was reduced 6(95% CI: 3;9) mmol/mol, weight 2.5(1.8;3.2) kg, SBP 4(−1;9) mmHg, UAER 30(12;44)% and mGFR 11(7;14) ml/min per 1.73 m^2. mGFR high responders had a significant reduction in weight compared to low responders (4.3 vs. 1.9 kg; $p = 0.002$). SBP high responders had a tendency of a higher reduction in UAER compared to low responders (47 vs. 23%, $p = 0.14$). No cross-dependency was observed in any of the other renal risk factors ($p \geq 0.16$). Treatment response did not differ after 7 weeks and 1 year ($p \geq 0.12$).

Conclusions/interpretation: Liraglutide possesses pleiotropic effects on renal risk factors. On patient level, effect on the individual risk factor cannot be anticipated based on response in other risk factors. Response when re-starting treatment did not differ, indicating that our primary findings were not random.

1. Introduction

Ultimately 20–40% of persons with type 2 diabetes will develop diabetic nephropathy, and in parallel with the global rise in type 2 diabetes, diabetic nephropathy has become the leading cause of end stage renal disease (ESRD) (American Diabetes Association, 2016).

Treatment of elevated glucose and hypertension in particular with the use of renin angiotensin system (RAS) inhibiting drugs has reduced the risk of diabetic nephropathy (UKPDS group, 1998a; UKPDS group, 1998b; Patel et al., 2007; Lewis, Hunsicker, Bain, & Rohde, 1993; Rossing, Hommel, Smidt, & Parving, 1994). Targeting other renal risk factors such as obesity has been suggested to further reduce the renal risk (Afshinnia, Wilt, Duval, Esmaeili, & Ibrahim, 2010). Despite optimal treatment, persons with nephropathy and residual albuminuria, still progress to ESRD and suffer from cardiovascular morbidity and mortality (Heerspink & de Zeeuw, 2011). For more than a decade, there has been an unmet need of further treatment options in prevention of hard renal and cardiovascular outcomes in persons with type 2 diabetes and nephropathy. Liraglutide is a glucagon-like peptide-1 (GLP-1) receptor agonist, sharing 97% of the amino acid sequence of human GLP-1. Liraglutide treatment has a well-established glucose-lowering and weight reducing effect in persons with type 2 diabetes (Nauck et al., 2009; Vilsboll, Christensen, Junker, Knop, & Gluud, 2012). Furthermore, liraglutide

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treatment has moderate blood pressure lowering potential, and has been associated with reductions in albuminuria and cardiovascular disease and mortality (Fonseca et al., 2014; Marso et al., 2016). Exploitation of the pleiotropic effects of liraglutide treatment could potentially add clinical value in further prevention of diabetic nephropathy, and be translated into a new renoprotective treatment option.

Despite evidence suggesting pleiotropic effects of liraglutide treatment in persons with type 2 diabetes, the expected effect on the individual person level is currently unknown. The concept of cross-dependency, meaning that the effect of liraglutide treatment on one renal risk factor is also affecting other renal risk factors, is a possibility. We explored liraglutide effect on renal risk factors including HbA_{1c}, weight, systolic blood pressure (SBP) and urinary albumin excretion rate (UAER). We included renal function (mGFR) as a renal risk factor because an acute reduction in mGFR could reflect a potential beneficial effect similar to what is seen when initiating treatment with RAS blocking agents (Holtkamp et al., 2011). Furthermore, hyperfiltration has been considered a risk factor for kidney disease and hyperfiltration may be caused by low sodium delivery in the distal tubular system which could be improved by agents increasing distal sodium delivery such as SGLT2inhibitors (Cherney et al., 2014) or liraglutide (Skov et al., 2016; Tonneijck et al., 2016).

We hypothesized that some individuals would respond to liraglutide treatment on all renal risk factors, whereas other would not respond on any of the renal risk factors. Moreover, we hypothesized that there was concordance between individual responses on the renal risk factors. As an example, clinicians would assume that in persons with a pronounced weight reduction following liraglutide treatment, an overt effect on HbA_{1c} would be present, or that a pronounced glucose-lowering effect would lead to a larger reduction in albuminuria.

The objective of this study was to elucidate the individual liraglutide treatment response on different renal risk factors in order to enhance our understanding of the pleiotropic effects of liraglutide treatment. The aim was to determine if the "high" responders (highest reduction) on an individual risk factor also had the highest response on other risk factors (cross-dependency). Moreover, we explored whether any baseline variables could predict a high response to liraglutide treatment. We also analyzed the effect of treatment after re-starting (the off–on/off–on effect: treatment/off treatment/re-treatment) to see if the participants overall had the same response to treatment, in order to account for potential random effects.

2. Methods

2.1. Study design and participants

This study is a secondary analysis of a previously conducted clinical trial. Original study design and participants have previously been described in details (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015a; von Scholten, Hansen, Goetze, Persson, & Rossing, 2015b). In brief, we included persons with type 2 diabetes, HbA_{1c} ≥48 mmol/mol (6.5%), known hypertension and an estimated GFR (eGFR) ≥60 ml/min per 1.73 m^2. Moreover, participants had to be on stable anti-hypertensive treatment for 4 weeks and stable glucose-lowering treatment for 2 weeks, including prescription of metformin, at time of inclusion. Exclusion criteria included insulin therapy, clinical heart failure and blood pressure ≥170 mmHg systolic or 105 mmHg diastolic. All participants were recruited from the outpatient clinic at Steno Diabetes Center. Participants were treated for 7 weeks with liraglutide in an open-label study. After a 3-week washout period liraglutide treatment was re-started in a subgroup of 23 participants and continued for 1 year. Participants re-starting liraglutide treatment ($n = 23$) did not differ from the total population ($n = 31$) in relation to age ($p = 0.10$), HbA_{1c} ($p = 0.10$), weight ($p = 0.23$), SBP ($p = 0.46$), UAER ($p = 0.13$) or mGFR ($p = 0.70$) at baseline.

The study complies with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all participants gave written informed consent. Trial registration: ClinicalTrials.gov identifier: NCT01499108.

2.2. Outcome measures

HbA_{1c}, weight, SBP, UAER and mGFR were measured at baseline, after 7 weeks of liraglutide treatment, after 3 weeks of washout, and after 1 year of liraglutide treatment. These five outcome measures are subsequently referred to as renal risk factors. HbA_{1c} was measured by high-performance liquid chromatography calibrated against the IFCC standard. Office BP was measured three times with two minutes apart in a sitting position after ten minutes rest with appropriate cuff-size. Twenty-four-hour urine collections were performed to measure UAER. mGFR was assessed during 4 hour measurement of plasma ^{51}Cr-EDTA by standard methods (Brochner-Mortensen, 1972).

2.3. Endpoints

For each of the five renal risk factors (HbA_{1c}, weight, SBP, UAER and mGFR) we defined high responders as the upper quartile (Q4) with the largest reduction in the renal risk factor. We defined low responders as the three quartiles (Q1–3) with the lowest reduction in that same renal risk factor. As primary endpoint we compared changes in high responders (Q4) vs. low responders (Q1–Q3) for each renal risk factor to investigate whether high responders in one renal risk factor also were high responders in the other renal risk factors. As secondary endpoints 1) we evaluated the linear correlations between changes in the renal risk factors, to investigate the treatment response without arbitrary categorization; 2) in the subgroup of participants re-starting liraglutide treatment, we evaluated whether the participant responded similarly during the second treatment period compared to the first 7 weeks of treatment. We evaluated this off–on/off–on effect to examine for random effects.

2.4. Statistical analysis

UAER is as the only non-normally distributed variable summarized as geometric mean (interquartile range (IQR)) and analyzed after log10-transformation. All other continuous variables are summarized as means ± SD or median (range), and categorical variables are reported as total numbers with corresponding percentages.

For the primary endpoint, unpaired t-test was used to study cross-dependency in a high treatment response between the 5 renal risk factors. Unpaired t-test was also applied when evaluating prediction of a high treatment response by comparing differences in baseline variables for high vs. low responders for each renal risk factor. For the secondary endpoint, linear regression was used to evaluate linear correlation in the treatment response between the 5 renal risk factors. Finally, paired samples t-test was used to study the off–on/off–on effect of liraglutide treatment by comparing the overall treatment response in each renal risk factor in the two separate treatment periods. Two sided values of $p < 0.05$ were considered significant. Statistical analysis was performed using IBM SPSS 23.0 (IBM, Amonk, NY, USA).

3. Results

3.1. Baseline Characteristics

Thirty-one participants completed the first 7 weeks of liraglutide treatment and were included in the analysis. Eight were female (26%), and at baseline the mean age (SD) was 62 (10) years, mean diabetes duration

Table 1
Baseline characteristics.

Demographics	Study participants ($n = 31$)
Age (years)	64 (10)
Women, *n* (%)	8 (26)
Known diabetes duration (years)	6 (5)
Weight (kg)	96.1 (14.2)
Body mass index (kg/m^2)	31.9 (4.4)
HbA_{1c} (mmol/mol)	61 (14)
HbA_{1c} (%)	7.7 (1.3)
Plasma creatinine (μmol/L)	73.8 (17.4)
mGFR (mL/min per 1.73 m^2)	99.5 (24.8)
UAER (mg/24-hour)*	32.7 (10.4–61.4)
Systolic blood pressure (mmHg)	141 (17)
Diastolic blood pressure (mmHg)	85 (10)
RAAS-blocking treatment, *n* (%)	27 (87)

Data are percentage (%) and mean (SD) or median (range).
Abbreviations: RAAS: renin-angiotensin-aldosterone-system; UAER: urinary albumin excretion rate.
* Geometric mean (IQR).

was 7 (5) years and HbA_{1c} was 61 (14) mmol/mol (7.7 (1.3)%). Thirteen (42%) participants had micro- or macroalbuminuria (UAER ≥30 mg/24-hour). All participants were on antihypertensive drug treatment, unchanged during the study (See Table 1). Twenty-three participants continued liraglutide treatment for a year and were included in the analysis of the off–on/off–on effect of liraglutide treatment.

3.2. Treatment response

As previously reported after 7 weeks of treatment, HbA_{1c} was reduced by mean 6 (95% CI: 3 to 9) mmol/mol, weight by 2.5 (1.8 to 3.2) kg, SBP by 4 (−1 to 9) mmHg and UAER by 30 (12 to 44)%. mGFR was reduced by 11 (7 to 14) ml/min per 1.73 m^2 (von Scholten, Lajer, et al., 2015a).

The treatment response varied substantially among the participants: with 5–95% percentile of −4 to 25 mmol/mol for HbA_{1c}, −1.8 to 6.0 kg for weight, −23 to 34 mmHg for SBP, −49 to 80% for UAER and −6 to 31 ml/min per 1.73 m^2 for mGFR.

3.3. Cross-dependency in treatment response after 7 weeks of liraglutide treatment

We compared high HbA_{1c} responders to low HbA_{1c} responders. HbA_{1c} was reduced by mean 17.0 (95% CI: 12.1 to 21.8) mmol/mol in high responders compared to a reduction of 2.3 (0.7 to 4.0) mmol/mol in low responders. No differences in the other renal risk factors were observed when comparing high HbA_{1c} responders to low HbA_{1c} responders ($p \geq 0.39$), meaning that high HbA_{1c} responders were not high responders in UAER or in any of the other renal risk factors (Table 2).

High weight responders had a weight reduction of 4.8 (4.0 to 5.5) kg compared to 1.8 (1.1 to 2.4) kg in low weight responders after 7 weeks of liraglutide treatment. For individuals with high weight response, changes in the other renal risk factors were similar to changes in the low weight responders ($p \geq 0.13$). Thus, high weight responders were not high HbA_{1c} responders, however in individuals with a high weight-response, there was a tendency of a higher reduction in UAER compared to low weight responders 47 (9 to 69) vs. 22 (1 to 39)%, ($p = 0.13$) (Table 2).

SBP was reduced 20 (8 to 32) mmHg in high responders vs. 1 (−5 to 2) mmHg increase in low responders with no other significant differences in renal risk factors observed between high and low SBP

Table 2
High vs. low responders in renal risk factors.

Variable	Overall change ($n = 31$)	Q4 ($n = 8$)	Q1–Q3 ($n = 23$)	Difference between change (Q4 vs. Q1–Q3 (95% CI))	*P* value
a) HbA_{1c} high vs. low-responders (Q4 vs. Q1–3)					
HbA_{1c} (mmol/mol)	**6.1 (3.2; 9.0)**	**17.0 (12.1; 21.8)**	**2.3 (0.7; 4.0)**	**−14.7 (−18.3; −11.0)**	–
Weight (kg)	2.5 (1.8; 3.2)	2.0 (0.2; 3.8)	2.7 (1.9; 3.5)	0.7 (−0.9; 2.3)	0.39
SBP (mmHg)	4 (−1; 9)	4 (−6; 14)	4 (−2; 11)	0 (−12; 12)	0.97
UAER (mg/24-hour)*	30 (12; 44)	29 (0; 50)	30 (7; 47)	1 (−40; 41)	0.97
mGFR (mL/min per 1.73 m^2)	11 (7; 14)	10 (4; 16)	11 (7; 16)	1 (−7; 10)	0.76
b) Weight high vs. low responders (Q4 vs. Q1–3)					
HbA_{1c} (mmol/mol)	6.1 (3.2; 9.0)	7.3 (0.4; 14.1)	5.7 (2.4; 9.1)	−1.5 (−8.2; 5.1)	0.65
Weight (kg)	**2.5 (1.8; 3.2)**	**4.8 (4.0; 5.5)**	**1.8 (1.1; 2.4)**	**−3.0 (−4.2; −1.8)**	–
SBP (mmHg)	4 (−1; 9)	4 (−8; 16)	4 (−2; 10)	0 (−12; 12)	0.99
UAER (mg/24-hour)*	30 (12; 44)	47 (9; 69)	22 (1; 39)	−25 (−59; 11)	0.13
mGFR (mL/min per 1.73 m^2)	11 (7; 14)	13 (4; 22)	10 (6; 14)	−3 (−12; 5)	0.42
c) SBP high vs. low responders (Q4 vs. Q1–3)					
HbA_{1c} (mmol/mol)	6.1 (3.2; 9.0)	9.5 (0.9; 18.0)	5.0 (2.1; 7.8)	−4.5 (−11.0; 1.9)	0.16
Weight (kg)	2.5 (1.8; 3.2)	3.5 (2.3; 4.8)	2.2 (1.3; 3.0)	−1.3 (−2.9; 0.2)	0.089
SBP (mmHg)	**4 (−1; 9)**	**20 (8; 32)**	**−1 (−5; 2)**	**−21 (−30; −12)**	
UAER (mg/24-hour)*	30 (12; 44)	47 (26; 61)	23 (−2; 41)	24 (−58; 12)	0.14
mGFR (mL/min per 1.73 m^2)	11 (7; 14)	12 (4; 19)	11 (6; 15)	−1 (−9; 7)	0.79
d) UAER high vs. low responders (Q4 vs. Q1–3)					
HbA_{1c} (mmol/mol)	6.1 (3.2; 9.0)	4.1 (−0.9; 9.1)	6.8 (3.2; 10.4)	2.7 (−3.9; 9.3)	0.41
Weight (kg)	2.5 (1.8; 3.2)	3.2 (1.8; 4.6)	2.3 (1.4; 3.2)	−0.9 (−2.5; 0.7)	0.23
SBP (mmHg)	4 (−1; 9)	7 (−13; 27)	3 (−1; 7)	−4 (−16; 8)	0.65
UAER (mg/24-hour)*	**30 (12; 44)**	**66 (50; 77)**	**9 (−8; 24)**	**−57 (−74; −40)**	–
mGFR (mL/min per 1.73 m^2)	11 (7; 14)	11 (8; 14)	11 (6; 16)	−1 (−9; 8)	0.83
e) mGFR high vs. low responders (Q4 vs. Q1–3)					
HbA_{1c} (%)	6.1 (3.2; 9.0)	7.5 (−1.0; 21.5)	5.7 (2.3; 9.0)	−1.8 (−8.5; 4.8)	0.57
Weight (kg)	2.5 (1.8; 3.2)	4.3 (2.6; 6.1)	1.9 (1.2; 2.7)	−2.3 (−3.7; −0.9)	0.002
SBP (mmHg)	4 (−1; 9)	7 (0; 15)	3 (−4; 10)	−4 (−15; 8)	0.53
UAER (mg/24-hour)*	30 (12; 44)	23 (4; 39)	32 (8; 49)	10 (−33; 46)	0.65
mGFR (mL/min per 1.73 m^2)	**11 (7; 14)**	**23 (17; 32)**	**7 (4; 10)**	**−16 (−22; −11)**	–

High responders defined as the upper quartile (Q4) with the highest reduction in the renal risk factor, compared to low responders defined as the three quartiles (Q1–3) with the lowest reduction in the same renal risk factor. Data are mean change from baseline (95% confidence interval) and *percentage change from baseline (95% confidence interval). P-value from the unpaired t-test.

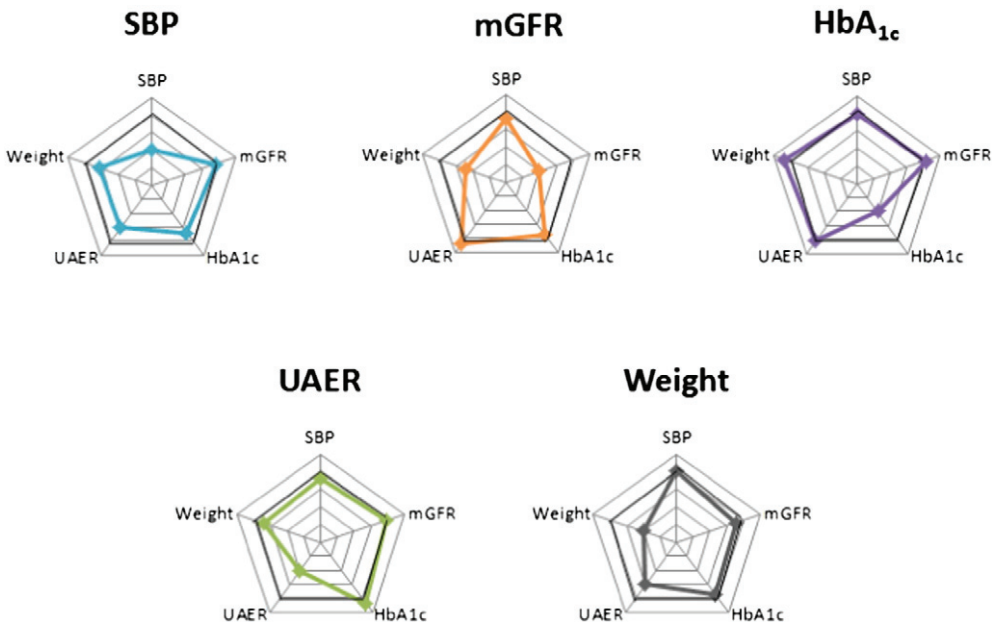

Fig. 1. The radar-charts reveal how high responders for one risk factor are responding in terms of the other risk factors. A point close to the middle reflects a large reduction in the renal risk factor. The black line represents no differences between high and low responders in treatment response, and a point outside the black line represents an increase or a lesser reduction in the renal risk factor for high compared to low responders.

responders, ($p \geq 0.14$). However although not significant UAER was reduced 47 (26 to 61)% in high SBP responders compared to 23 (−2 to 41)% in low SBP responders, ($p = 0.14$) (Table 2).

UAER was reduced by 66 (50 to 77)% in high vs. 9 (−8 to 24)% in low responders, with no other significant differences in renal risk factors observed between groups ($p \geq 0.23$) (Table 2).

A reduction in mGFR of 23.0 (17 to 32) ml/min per 1.73 m^2 was observed in high vs. 7 (4 to 10) ml/min per 1.73 m^2 in low responders. In individuals with a high response in mGFR, a significant reduction in weight was observed compared to low responders, 4.3 (2.6 to 6.1) vs. 1.9 (1.2 to 2.7) kg; ($p = 0.002$) Table 2.

Fig. 1 gives an overview over the cross-dependencies in the treatment responses. The radar-charts reveal no clinical relevant cross-dependencies in the individual person response.

3.4. Linear correlations between changes in renal risk factors

We examined linear correlations between changes in renal risk factors after 7 weeks of treatment. Reduction in HbA_{1c} was not correlated with reduction in SBP ($p = 0.67$), UAER ($p = 0.65$), weight ($p = 0.93$) or mGFR ($p = 0.97$). There was no correlation between reduction in weight and SBP ($p = 0.55$), or between reduction in SBP and mGFR ($p = 0.31$) or UAER ($p = 0.76$). Reduction in weight had a tendency to correlate with reduction in UAER ($p = 0.07$) and mGFR ($p = 0.06$).

3.5. Prediction of high response to liraglutide treatment

For each renal risk factor we tested if a high response in the risk factor was associated with a high baseline value of the same risk factor. Higher HbA_{1c} at baseline predicted high HbA_{1c} response ($R^2 = 0.38$; $p < 0.001$).

In an explorative analysis, we tested if age, diabetes duration, sex, weight, HbA_{1c}, SBP, UAER or mGFR at baseline were associated with a high response to liraglutide treatment in one or more of the 5 renal risk factors. No other baseline variables than HbA_{1c} predicted high response in HbA_{1c} ($p \geq 0.13$). No baseline variables predicted reduction in weight ($p \geq 0.18$) or SBP ($p \geq 0.15$). However, low mGFR had a tendency to predict ($p = 0.07$) high response in SBP. Lower mGFR at baseline predicted high response in UAER ($R^2 = 0.14$; $p = 0.036$), while lower HbA_{1c} had a tendency to predict a high response in UAER ($p = 0.052$). None of the baseline variables predicted high response in mGFR ($p \geq 0.10$).

3.6. The "off–on/off–on" effect of liraglutide treatment

Twenty-three participants restarted liraglutide treatment and therefore had two treatment periods: 7 weeks and 42 weeks separated by three weeks of washout. The overall response on each renal risk factor did not differ when comparing the response during the second treatment period to the response during the first 7 weeks of treatment. HbA_{1c} was reduced 7.1 (95% CI: 3.8 to 10.5) mmol/mol in the first treatment period compared to 2.0 (−3.1 to 7.2) mmol/mol in the second treatment period, $p = 0.12$. Weight was reduced 2.9 (2.1 to 3.7) kg in the first compared to 1.6 (−0.2 to 3.4) kg in the second treatment period, $p = 0.14$. SBP was reduced 5 (−1 to 12) mmHg in the first treatment period vs. 5 (−2 to 13) mmHg in the second treatment period, $p = 0.98$. UAER was reduced 28 (11 to 41) vs. 17 (−8 to 37) ($p = 0.44$)% in the second treatment period, and mGFR 12 (8 to 17) vs. 10 (5 to 14) ml/min per 1.73 m^2, ($p = 0.34$).

Fig. 2 shows the decline in mGFR during the first 7 weeks of treatment, the increase in mGFR during the 3 weeks of washout, and the reduction in mGFR when treatment is reintroduced. The figure illustrates how for the majority of the participants of the same magnitude of treatment response was seen during the two treatment periods.

4. Discussion

In this open-label study including 31 participants with type 2 diabetes, reductions in HbA_{1c}, weight, SBP, UAER and mGFR were observed after 7 weeks of liraglutide treatment, although with high individual variation in the treatment response. To elucidate the individual liraglutide treatment response for different renal risk factors, we defined the upper quartile with the highest reduction in each of these five renal risk factors as high responders and assessed the response in the other renal risk factors. We demonstrated that high responders in one renal risk factor were not the same as high

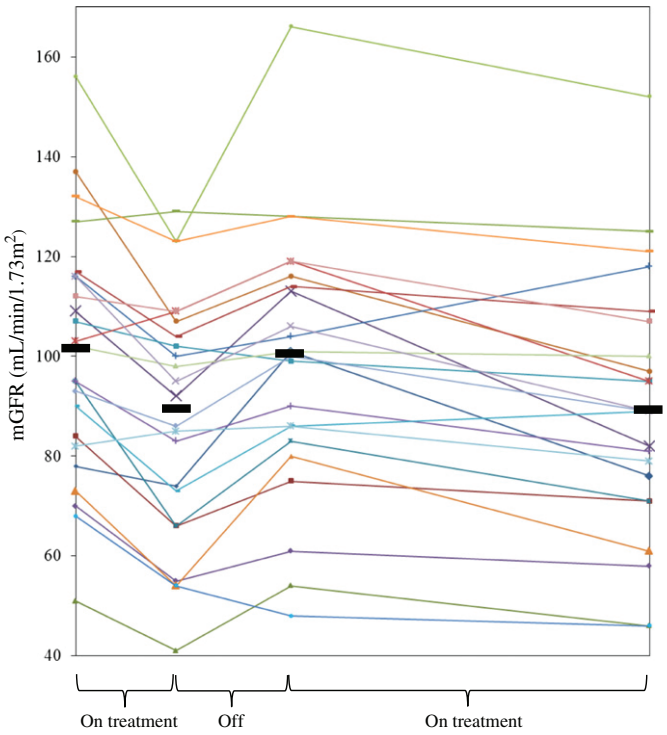

Fig. 2. The off–on/off–on effect of liraglutide treatment. Each line represents one person. The figure illustrates the decline in mGFR during the first 7 weeks of treatment, the increase in mGFR during the 3 weeks of washout, and the reduction in mGFR when treatment is reintroduced. For the majority of the participants the same magnitude of treatment response was seen during the two treatment periods. The horisontal black lines represent mean mGFR.

responders in the other renal risk factors. Of particular interest 1) persons with high weight response did not have high HbA_{1c} response; and 2) high responders in HbA_{1c} or SBP were not high responders in albuminuria. There were no linear correlations between the responses in the different renal risk factors. Furthermore, we explored if any of the baseline variables predicted high response to liraglutide treatment. Higher baseline HbA_{1c} predicted high response in HbA_{1c}. Overall, we found a consistency between the reductions in the five renal risk factors seen in the first and in the second treatment period. This indicated that the individual response to liraglutide treatment was not random.

Liraglutide treatment is known to have glucose-, weight- and BP lowering effects, which was also demonstrated in this study. The effect of liraglutide treatment on albuminuria and mGFR is less well established. The albuminuria reducing effect of liraglutide is an area of growing interest. We have recently showed that liraglutide treatment, in the present study, was associated with a significant reduction in albuminuria (von Scholten, Lajer, et al., 2015a). We concluded that liraglutide treatment might possess renoprotective potential, which was further demonstrated in the LEADER trial (Marso et al., 2016). The prespecified primary microvascular outcome in the LEADER trial was a composite of nephropathy and retinopathy outcomes. The benefit with liraglutide was driven by lower rates of renal outcomes, such as new-onset persistent macroalbuminuria in particular. However, it was not tested if the reduction in albuminuria is independent of improvement in glycemic control and therefore is drug-specific.

In the current study, liraglutide treatment was associated with reductions in mGFR. Since the reduction in mGFR was fully reversible after 3 weeks of washout, we considered the effect to be haemodynamic; potentially caused by changes in the intraglomerular pressure, and not structural changes in the kidneys (von Scholten, Lajer, et al., 2015a). A recent patient-level pooled analysis (3173 persons with type 2 diabetes) and the LIRA-RENAL study (279 persons with inadequately controlled type 2 diabetes and moderate renal impairment) demonstrated no significant changes in eGFR after 26 weeks of liraglutide treatment, however the LIRA-RENAL revealed an acute reduction in eGFR (von Scholten, Orsted, Svendsen, Persson, & Rossing, 2015c; Davies et al., 2016). In previous papers, the potential beneficial effects of acute reductions in mGFR or eGFR have been demonstrated and discussed (Holtkamp et al., 2011). In particular intervention studies with RAAS-inhibitors as well as the recent kidney data from the EMPA-REG OUTCOME trial have demonstrated that an acute fall in mGFR/eGFR was followed by a slower rate of chronic GFR decline, and the acute fall has been shown to predict long-term kidney stability (Apperloo, de Zeeuw, & de Jong, 1997; Holtkamp et al., 2011; Wanner et al., 2016). It is possible that the acute reduction in mGFR observed with liraglutide treatment is predictive of long-term kidney stability; however investigations in larger renal outcome studies are warranted.

We examined whether "high" responders (highest reduction) on each renal risk factor also had the highest response on the other renal risk factors. Cross-dependency in the treatment response between some of the renal risk factors could be explained by internal dependency. For example, we expected 1) high responders in weight to be high responders in HbA_{1c}; 2) high responders in SBP to be high responders in UAER; and 3) high responders in HbA_{1c} to be high responders in UAER. None of these anticipations were verified. There was a tendency that persons with high response in SBP had a higher reduction in UAER compared to low responders; it may be an issue of power that this trend was not significant. Moreover, there were no linear correlations between reductions in any of the five renal risk factors, although reduction in weight had a tendency to correlate with reduction in UAER and mGFR. These findings suggest that the pleotropic effects of liraglutide treatment on renal risk factors are not merely explained by internal dependency. The fact that no relevant cross-dependencies in the individual treatment response to liraglutide treatment was identified, underline the need for careful evaluation of the individual effects of liraglutide when starting treatment.

It has been difficult to predict the response to treatment with a GLP-1 receptor agonist in relation to glycemic control and weight

reduction. A recent study by Jones et al. showed that clinical markers of low β-cell function were associated with a reduced glycemic response to treatment with a GLP-1 receptor agonist in patients with type 2 diabetes. The patients with fasting C-peptide <0.25 nmol/l had markedly lower glycemic response, with a mean HbA_{1c} reduction of 5.2 mmol/mol compared to 15.2 mmol/mol in patients with preserved β-cell function. In contrast, baseline measurements associated with glycemic response were not associated with change in weight (Jones et al., 2016). A phase two trial showed that treatment with exenatide in dose of both 0.8 mg and 2.0 mg reduced mean HbA_{1c} compared with placebo in patients with type 2 diabetes. A reduction in body weight was also seen in the group treated with 2.0 mg exenatide, whereas body weight was unchanged in the group treated with 0.8 mg or placebo. This indicates that higher exenatide concentrations are required for effects on weight than on HbA_{1c} (Kim et al., 2007). In our study, higher baseline HbA_{1c} predicted a high response in HbA_{1c}. It is well described that baseline HbA_{1c} is a strong predictor for HbA_{1c} response to pharmacological treatment with other glucose-lowering agents (Bloomgarden, Dodis, Viscoli, Holmboe, & Inzucchi, 2006). Except for HbA_{1c}, a high baseline value for one renal risk factor did not predict a high response in the same renal risk factor, indicating that the treatment response only partly is related to regression towards the mean. In an explorative analysis low mGFR at baseline predicted a high reduction in UAER. This might be explained by the fact that participants with the lowest mGFR also had higher levels of albuminuria.

Our findings emphasize the necessity of carefully evaluating the effect of liraglutide treatment on all the renal risk factors when initiating treatment. Both because a high treatment response is not easily predicted and because a high treatment response in one renal risk factor is not necessarily followed by a high response in any of the other risk factors liraglutide treatment is known to affect. It has recently been discussed that the ultimate drug effect is better evaluated when the multiple effects of a drug are integrated instead of evaluating the drug effect on a single target risk marker (Schievink, Mol, & Lambers Heerspink, 2015). It is a novel approach to evaluate multiple risk markers when estimating the ultimate drug effect. Our data show that a high response in all the renal risk factors liraglutide is known to affect cannot be anticipated in the same person.

4.1. Strengths and limitations

Strengths of our study include the novel approach to investigate the individualized response to liraglutide treatment and the possibility to investigate the “off–on/off–on” effect of liraglutide treatment in a clinical study. This has, to the best of our knowledge, never been investigated before. Furthermore, renal function was measured with robust methods.

However, our current study must be interpreted within the context of some potential limitations: 1) the study was relatively small, and the subdivision in a small group of high responders (the upper quartile) and a larger group of patients with low response (three other quartiles) is practical but a limitation. 2) The limited sample size implies a higher sampling variability increasing the risk of a type II error. 3) The open-label study design might account for part of the large variation in treatment-effect on the renal risk factors. 4) 42% of the participants had micro- or macroalbuminuria, however subdivision based on albuminuria status was not performed due to the small number of participants. It is unknown whether the responses of the risk factors to liraglutide treatment differ in relation to albuminuria status. 5) The participants represent a subgroup of type 2 diabetic patients primarily treated with metformin, even after many years duration of diabetes, limiting the generalization of our results. 6) Only a sub-group of 23 participants were included in the 1-year re-challenge period, however, these participants had clinical characteristics similar to the total population reducing the risk of selection bias. 7) The lack of statistical difference in relation to off–on/off–on effect in HbA1c and weight might be explained by a type II error. 8) Information on other risk markers such as lipids and inflammatory markers was not available. 9) No control persons were included; however these were not required in order to answer our research question.

4.2. Clinical implications

Our findings stress the importance of evaluating the individual effect of liraglutide when starting treatment. The effect of liraglutide treatment on different renal risk factors is individual and a high response in all risk factors cannot be anticipated by a high response in one risk factor. Intuitively the clinician would expect a high response in HbA_{1c} when the individual is experiencing a pronounced weight reduction, however our data suggest, that this and other cross-dependencies cannot be anticipated.

Renal outcome studies are needed to investigate whether the pleiotropic effects of liraglutide treatment on multiple renal risk factors have clinical impact for postponing or even preventing development of chronic kidney disease.

5. Conclusion

Liraglutide treatment possesses pleiotropic effects on renal risk factors. On the individual person-level there are no relevant cross-dependencies; thus the effect on the individual renal risk factor cannot be anticipated based on response in other risk factors. Overall the participants experienced same response when re-starting treatment, indicating that our primary findings are not caused by random effects.

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BRIEF REPORT

Pleiotropic effects of liraglutide in patients with type 2 diabetes and moderate renal impairment: Individual effects of treatment

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Liraglutide has pleiotropic effects favouring cardiovascular and renal risks. We investigated individual responses to liraglutide in six cardio-renal risk factors to examine whether responses in one risk factor are associated with changes in other risk factors (cross-dependency). We performed secondary analysis of the LIRA-RENAL trial (n = 279) in type 2 diabetes. HbA1c, body weight, systolic blood pressure (SBP), low density lipoprotein (LDL)-cholesterol, urine albumin-to-creatinine ratio (UACR) and estimated glomerular filtration rate (eGFR) were measured at baseline and after 26 weeks of liraglutide/placebo treatment: "Good responders" had a change within the best quartile. In the liraglutide-treated group, good HbA1c responders showed similar changes in other risk factors analysed to low responders ($P \geq 0.17$). Good body weight responders had a larger reduction in HbA1c than low body weight responders (−1.6 ± 0.94 vs. −1.0 ± 0.82%; $P = 0.003$), but similar changes in the other risk factors ($P \geq 0.11$). Good and low responders in SBP, UACR, LDL-cholesterol or eGFR showed similar changes in other risk factors ($P \geq 0.07$). Treatment response to liraglutide is largely individual; aside from an association between body weight and HbA1c reduction, there are no obvious cross-dependencies in risk factor response.

KEYWORDS

diabetic nephropathy, liraglutide, type 2 diabetes.

1 | INTRODUCTION

Several newer antihyperglycaemic drugs have pleiotropic effects[1] favouring cardiovascular and renal risk in type 2 diabetes.

Liraglutide, a once-daily human glucagon-like peptide-1 (GLP-1) analogue, lowers glucose, body weight, blood pressure, lipids and albuminuria.[2–4] The long-term Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) clinical trial established that liraglutide reduces cardiovascular events and offers long-term renoprotection in patients with type 2 diabetes and established cardiovascular disease.[5]

We explored the pleiotropic effects of liraglutide on an individual level in the Efficacy and Safety of Liraglutide versus Placebo as Add-on to Glucose-Lowering Therapy in Patients with Type 2 Diabetes and Moderate Renal Impairment (LIRA-RENAL) study. We investigated individual responses to liraglutide in six cardio-renal risk factors (HbA1c, body weight, systolic blood pressure [SBP], low density lipoprotein [LDL]-cholesterol, urine albumin-to-creatinine ratio [UACR] and estimated glomerular filtration rate [eGFR]) to examine whether beneficial responses in one risk factor are associated with changes in other risk factors (cross-dependency).

2 | MATERIALS AND METHODS

2.1 | Study design and participants

A secondary analysis of LIRA-RENAL, which was a 26-week, randomized, double-blind, placebo-controlled, parallel group trial, aimed to assess superiority of liraglutide 1.8 mg versus placebo as an add-on to

existing glucose-lowering treatment (oral glucose-lowering agents and/or insulin therapy) in participants with type 2 diabetes and moderate renal impairment (stage 3 chronic kidney disease, defined as eGFR of 30-59 mL/min/1.73m^2). Patients were to maintain their background diabetes medication throughout the trial but the dose of insulin or sulphonylurea could be reduced in case of hypoglycaemic episodes.[6]

The study was conducted according to the Declaration of Helsinki and was approved by the relevant authorities. Trial registration: ClinicalTrials.gov identifier NCT01620489.

2.2 | Measurement of risk factors

HbA1c, body weight, SBP, LDL-cholesterol and UACR were measured and eGFR was calculated (based on the Modification of Diet in Renal Disease [MDRD] formula) at baseline, at regular intervals during the trial, and after 26 weeks of treatment, using routine methods.[6] UACR was calculated as the mean of the morning urine samples from the day before the visit and the day of the visit.

2.3 | Statistical analyses

Good response was defined as an observed change from baseline (ratio to baseline for UACR and LDL-cholesterol) at week 26 at or below the first quartile of the distribution in the liraglutide group (ie, greatest reduction). For eGFR, good response was defined as an observed ratio to baseline at or above the third quartile of the distribution in the liraglutide group (ie, increase or smallest reduction).

We evaluated separately in the liraglutide and placebo groups the association of good response among the six risk factors using Fisher's exact test.

Furthermore, we evaluated (a) linear correlations among changes in the six risk factors to investigate treatment response on a continuous scale, and (b) associations between baseline values and responder status for each of the six risk factors in the liraglutide-treated group.

Within each treatment group, a two-sample t-test using Satterthwaite's approximation was used to compare good and low responders for each risk factor in terms of both baseline values and change from baseline in the other risk factors. Pairwise associations in change from baseline among the six risk factors were also evaluated using scatterplots, and Pearson's correlation coefficients were calculated.

For all analyses, LDL-cholesterol, UACR and eGFR values were log-transformed. Because all analyses are exploratory, no correction for multiplicity was applied.

3 | RESULTS

Of 279 participants exposed to study medication, 220 who had at least one of the six variables measured at baseline and after 26 weeks of liraglutide (n = 109) or placebo (n = 111) treatment were included in the present analysis.

Participants had a mean ± standard deviation (SD) age of 66.7 (±8.5) years, diabetes duration of 15.0 (±8.3) years, and 48.6% were female. At baseline, mean (±SD) HbA1c was 8.0 (±0.8)%, body weight 94.6 (±17.7) kg and SBP 136 (±15) mm Hg. Participants had a geometric mean (coefficient of variation) UACR of 7.1 (6.6) mg/mmol, LDL-cholesterol of 2.3 (0.5) mmol/L and eGFR of 47.2 (0.2) mL/min/1.73m^2. Baseline characteristics (Supporting Information Table S1) were generally well balanced by treatment group.

3.1 | Cross-dependency for response in risk factors

Changes in risk factors for good versus low responders, for the liraglutide-treated participants, are presented in Table 1. Good (reduction ≥1.7%) and low HbA1c responders showed similar changes from baseline to end of trial in other risk factors analysed ($P \geq 0.17$; Figure 1A). Good body weight responders (reduction ≥4.6 kg) had a significantly larger reduction in HbA1c than low body weight responders ($P = 0.003$), but similar changes in the other risk factors (Figure 1B). No significant difference was seen between good and low responders in SBP (reduction ≥10 mm Hg), UACR (≤54% of baseline value), LDL-cholesterol (≤85% of baseline value) or eGFR (≥107% of baseline value) from baseline to end of trial in any of the other risk factors ($P \geq 0.07$).

Results for good versus low responders in the six risk factors, for placebo-treated participants, are presented in Supporting Information Table S2. Overall, findings were similar to the liraglutide-treated group, with few associations between changes in the six risk factors.

We further investigated whether a good response in one risk factor was associated with good response in each of the other risk factors, using the cut-offs stated for a good response. Results of these analyses of binary response variables were largely consistent with the analyses of continuous change from baseline (Supporting Information Table S3A and B).

3.2 | Linear correlations between changes in the six risk factors

We analysed linear correlation between observed changes in the six risk factors after 26 weeks of treatment with liraglutide. HbA1c reduction was associated with body weight reduction ($r = 0.24$; $P = 0.01$) but changes in the other risk factors were not significantly correlated ($P \geq 0.11$).

3.3 | Association between baseline value and response for each risk factor

For each risk factor, we analysed the association between the baseline value and response to liraglutide treatment. This association was significant for HbA1c ($P < 0.001$), SBP ($P < 0.001$) and LDL-cholesterol ($P = 0.002$), but not for body weight, eGFR or UACR ($P \geq 0.051$).

4 | DISCUSSION

Liraglutide treatment has pleiotropic effects that favourably change both cardiovascular and renal risk variables. We observed no obvious cross-dependency in the risk factor response, except for

TABLE 1 Good versus low responders in risk factors for the liraglutide-treated group

Variable	Q4	Q1–Q3	*P* value
(A) HbA1c good versus low responders (Q4 [n = 29] vs. Q1–Q3 [n = 76])			
HbA1c (%)	−2.2 (0.48)	−0.77 (0.67)	—
Body weight (kg)	−3.3 (3.4)	−2.2 (3.8)	0.17
SBP (mm Hg)	1 (17)	−3 (15)	0.28
UACR (%)[a]	0.81 [1.6]	0.91 [1.2]	0.67
eGFR (%)[a]	0.97 [0.17]	0.98 [0.18]	0.75
LDL-cholesterol (%)[a]	1.1 [0.29]	1.0 [0.31]	0.22
(B) Body weight good versus low responders (Q4 [n = 29] vs. Q1–Q3 [n = 77])			
HbA1c (%)	−1.6 (0.94)	−1.0 (0.82)	0.003
Body weight (kg)	−7.0 (2.3)	−0.8 (2.5)	—
SBP (mm Hg)	−3 (15)	−1 (15)	0.51
UACR (%)[a]	0.67 [1.1]	0.97 [1.3]	0.12
eGFR (%)[a]	0.98 [0.14]	0.98 [0.19]	0.99
LDL-cholesterol (%)[a]	1.0 [0.34]	1.0 [0.29]	0.89
(C) SBP good versus low responders (Q4 [n = 33] vs. Q1–Q3 [n = 73])			
HbA1c (%)	−1.1 (0.71)	−1.2 (0.97)	0.69
Body weight (kg)	−3.3 (3.8)	−2.1 (3.7)	0.13
SBP (mm Hg)	−18 (10)	6 (11)	—
UACR (%)[a]	0.69 [1.1]	0.98 [1.4]	0.13
eGFR (%)[a]	0.97 [0.13]	0.98 [0.20]	0.94
LDL-cholesterol (%)[a]	1.0 [0.32]	1.0 [0.30]	0.81
(D) UACR good versus low responders (Q4 [n = 21] vs. Q1–Q3 [n = 61])			
HbA1c (%)	−1.4 (0.80)	−1.0 (0.96)	0.17
Body weight (kg)	−2.9 (3.8)	−2.4 (3.9)	0.63
SBP (mm Hg)	−3 (18)	−0.3 (16)	0.57
UACR (%)[a]	0.29 [0.69]	1.3 [0.88]	—
eGFR (%)[a]	0.98 [0.28]	0.96 [0.13]	0.79
LDL-cholesterol (%)[a]	1.0 [0.23]	1.0 [0.30]	0.69
(E) eGFR good versus low responders (Q4 [n = 27] vs. Q1–Q3 [n = 78])			
HbA1c (%)	−1.3 (0.89)	−1.1 (0.89)	0.40
Body weight (kg)	−2.4 (3.3)	−2.6 (3.8)	0.82
SBP (mm Hg)	0 (16)	−3 (15)	0.46
UACR (%)[a]	1.2 [2.5]	0.76 [0.87]	0.18
eGFR (%)[a]	1.20 [0.17]	0.91 [0.11]	—
LDL-cholesterol (%)[a]	1.0 [0.29]	1.0 [0.31]	0.72
(F) LDL-cholesterol good versus low responders (Q4 [n = 24] vs. Q1–Q3 [n = 71])			
HbA1c (%)	−0.92 (0.77)	−1.3 (0.87)	0.07
Body weight (kg)	−2.9 (4.7)	−2.7 (3.1)	0.88
SBP (mm Hg)	−5 (13)	−0.8 (16)	0.26
UACR (%)[a]	0.75 [1.4]	0.89 [1.3]	0.56
eGFR (%)[a]	0.95 [0.3]	0.99 [0.14]	0.56
LDL-cholesterol (%)[a]	0.71 [0.15]	1.16 [0.23]	—

Abbreviations: eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; SBP, systolic blood pressure; UACR, urine albumin-to-creatinine ratio.

[a] Relative change of log-transformed values. Values are mean (standard deviation) or geometric mean [coefficient of variation]. Two-sample t-test using the Sattertwhaite approximation.

association of a good response in body weight with a greater reduction in HbA1c. The same association was not seen among placebo-treated patients.

We have recently published data from a small study (n = 31) examining the pleiotropic effects of liraglutide. Surprisingly, a pronounced body weight loss was not associated with a greater reduction in HbA1c, and a pronounced reduction in HbA1c was not associated with a greater reduction in urinary albumin excretion rate. We had expected a cross-dependency between reduction in SBP and urinary albumin excretion rate, but this was also not seen.[7] With the obvious limitations of a small open-label study, we wished to confirm our findings.

The present analysis of LIRA-RENAL confirms the highly individual treatment response to liraglutide. Aside from a good response in body weight being associated with a greater reduction in HbA1c, there was no obvious cross-dependency in risk factor response in the liraglutide-treated group. Overall changes in the placebo group were smaller (vs. the liraglutide group) but, interestingly, there were several cross-dependencies in risk factor response in this group, suggesting that the population size and length of study were sufficient to detect possible associations. An HbA1c reduction following body weight reduction may be more evident over time, and therefore we speculate that the longer study duration in LIRA-RENAL (26 weeks vs. 7 weeks for the open-label study) may partly explain this observed difference. The association was not seen in the placebo-treated group.

We had originally hypothesized that in some individuals liraglutide treatment would lead to a response in all risk factors, whereas other individuals would not respond in any risk factor. Our findings, from analysing cross-dependency in liraglutide response in two different populations with type 2 diabetes, do not support this hypothesis.

Individual characteristics may influence the magnitude of the treatment response to liraglutide.[8] We showed that a high baseline level of HbA1c, SBP and LDL-cholesterol was associated with a greater response in the corresponding variable. We acknowledge that regression towards the mean can contribute to these associations. Interestingly, a high baseline body weight was not linked to a good body weight response. In accordance with our finding, Berkovic et al. showed that higher baseline HbA1c was related to a greater reduction in HbA1c after liraglutide treatment for 6 months in 207 participants with type 2 diabetes.[9]

Studies showing the beneficial effect of GLP-1 receptor agonists on hard outcomes are available, and future analysis of the link between individual patient risk factor responses and occurrence of renal and cardiovascular events could ultimately enhance the opportunity to personalize treatment. Other mechanisms such as inflammation that are not directly reflected in the cardio-renal risk factors may also be involved.

4.1 | Clinical implications

We found substantial heterogeneity in the individual risk factor response to liraglutide treatment. This may help guide clinicians to not just take into account treatment response in one variable (ie, HbA1c or body weight), but to also consider a number of other variables when assessing the effect of liraglutide, in anticipation of a beneficial clinical outcome. Furthermore, a good response in one risk factor does not appear to predict response in other variables, except for an association between body weight and glycaemic control.

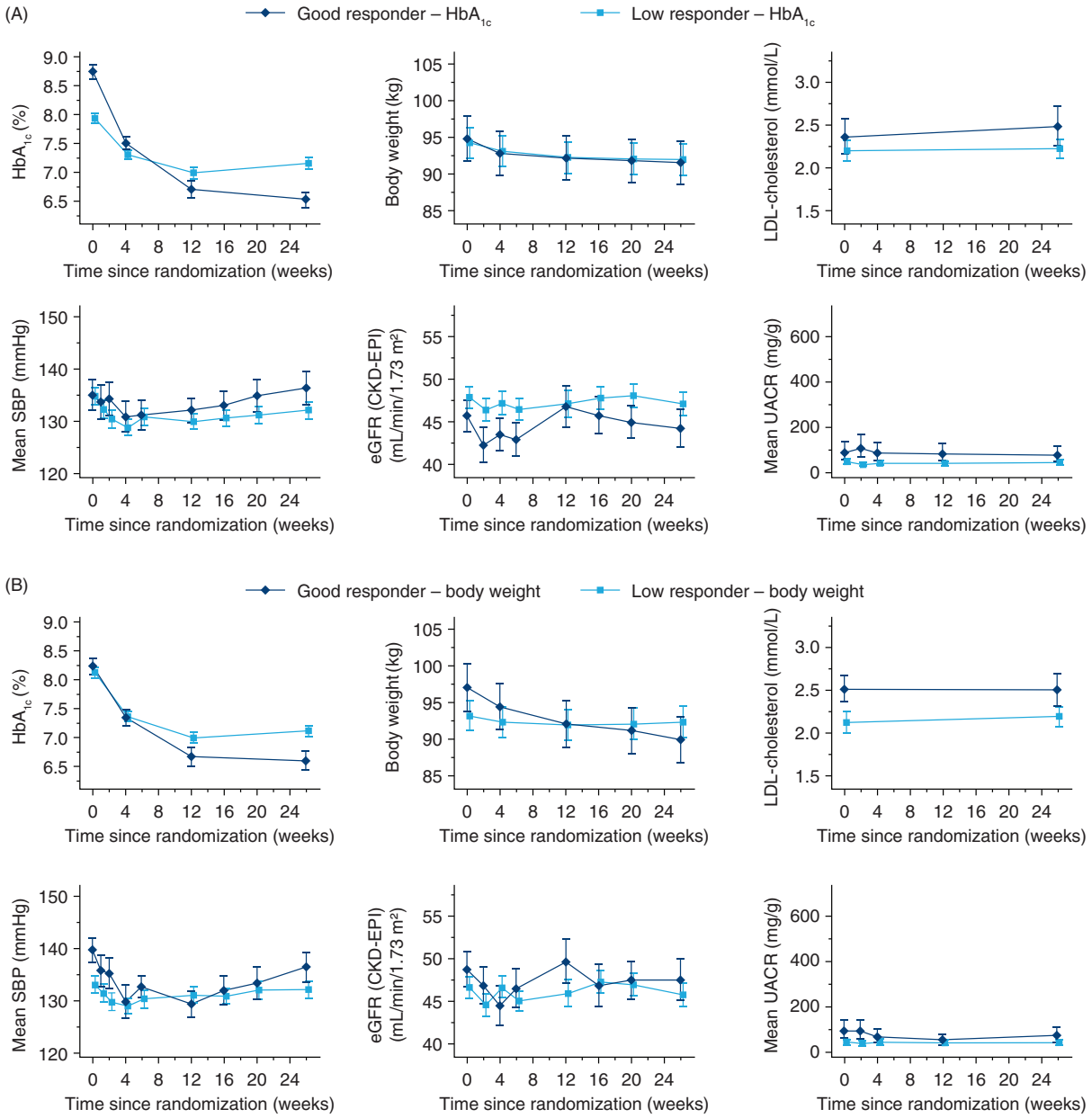

FIGURE 1 Cross-dependency in risk factor response. A, Changes in risk factors for good versus low HbA1c responders for liraglutide-treated participants. B, Changes in risk factors for good versus low body weight responders for liraglutide-treated participants. Good responders were defined as having a change from baseline to week 26 within the best quartile in the liraglutide-treated group. Good responders in HbA1c (reduction ≥1.7%) had similar changes in the other risk factors compared with low responders in HbA1c ($P \geq 0.17$). Good body weight responders had a significantly greater reduction in HbA1c (−1.6 ± 0.94 vs. −1.0 ± 0.82%) compared with low body weight responders ($P = 0.003$), but no difference in change of other risk factors between responder groups ($P \geq 0.11$). Observed mean ± standard error (SE). For log-transformed data of low density lipoprotein (LDL)-cholesterol, urine albumin-to-creatinine ratio (UACR) and estimated glomerular filtration rate (eGFR), observed means and SE were calculated on the log-transformed values and then back-transformed to the original scale

4.2 | Strengths and limitations

The strength of this study is that we confirmed our previous findings from a small open-label study[7] in a secondary analysis of a large, randomized, controlled trial. As this is still an exploratory analysis, however, we cannot exclude the possibility that significant associations between risk factor responses may exist. An important limitation is the day-to-day variation in the cardio-renal risk factors, which could obscure a true correlation among the risk factors. In particular, UACR, SBP and serum creatinine vary from day to day and this variation may hamper detection of a true correlation. The aim of the present analysis was to determine cross-dependency in risk factor response. Future analyses of a dedicated liraglutide outcome trial are required to assess if the changes in risk factors translate into renal and cardiovascular events.

In conclusion, liraglutide treatment has pleiotropic effects that favourably change cardiovascular and renal risk. We show that treatment response to liraglutide is largely individual and, aside from an association between body weight reduction and HbA1c reduction, there are no obvious cross-dependencies in the risk factor response. Future analysis of the link between risk factor responses and occurrence of renal and cardiovascular events could ultimately lead to

personalized treatment and help elucidate which effects are most important for optimal outcomes.

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CONFLICT OF INTEREST

E. H. Z. declares no duality of interest associated with this manuscript. B. J. v. S. and B. G. are Novo Nordisk employees and B. J. v. S. has equity interest in Novo Nordisk. F. P. reports research grants received from AstraZeneca, lecture fees from AstraZeneca, MSD, Janssen, Lily, Boehringer Ingelheim, Novo Nordisk and Novartis, and being consultant/advisory board member for AstraZeneca, Bayer, Amgen and MSD. T. W. H. has equity interest in Novo Nordisk. P. R. received lecture fees (to his institution) from Bayer, Novo Nordisk, AstraZeneca and Boehringer Ingelheim, research grants from AstraZeneca and Novo Nordisk, has served as a consultant for Bayer, AstraZeneca, Astellas, Boehringer Ingelheim, AbbVie and Novo Nordisk (all honoraria to his institution), and has equity interest in Novo Nordisk.

Author contributions

E. H. Z., F. P., T. W. H. and P. R. conceived and designed the research, analysed and interpreted the data; B. G. performed the statistical analysis; E. H. Z. and T. W. H. drafted the manuscript; E. H. Z., B. J. v. S., F. P. and P. R. critically revised the manuscript for key intellectual content; all authors approved the final version of the manuscript. E.H.Z. is responsible for the integrity of the work as a whole.

Data sharing

The subject level analysis datasets for the research presented in the publication are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Paper 10

Circulation: Cardiovascular Imaging

ORIGINAL ARTICLE

Effect of Liraglutide on Arterial Inflammation Assessed as [^{18}F]FDG Uptake in Patients With Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Trial

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BACKGROUND: The mechanism behind the cardiovascular protection observed with human GLP-1 RA (glucagon-like peptide-1 receptor agonists) in type 2 diabetes is unknown. We hypothesized that treatment with the GLP-1 RA liraglutide had a positive effect on vascular inflammation.

METHODS: LIRAFLAME (Effect of liraglutide on vascular inflammation in type-2 diabetes: A randomized, placebocontrolled, double-blind, parallel clinical PET/CT trial) was a double-blind, randomized controlled trial performed at a single university hospital clinic in Denmark. Patients with type 2 diabetes were via computer-generated randomization list assigned (1:1) liraglutide up to 1.8 mg or placebo once daily for 26 weeks. The primary end point was change in vascular inflammation over 26 weeks assessed by [^{18}F]-fluorodeoxyglucose positron emission tomography/computed tomography. Analyses were based on intention-to-treat. Key secondary outcomes included change in other indices of atherosclerosis.

RESULTS: Between October 26, 2017, and August 16, 2019, 147 patients were screened and 102 were randomly assigned to liraglutide (n=51) or placebo (n=51) and 99 (97%) completed the trial. Change in the [^{18}F]-fluorodeoxyglucose positron emission tomography measure of vascular inflammation (active-segment target-to-background ratio) did not differ between treatment groups: change from baseline to 26 weeks was −0.04 (95% CI, −0.17 to 0.08) in the liraglutide group compared with −0.09 (−0.19 to 0.01) in the placebo group (mean difference, 0.05 [95% CI, −0.11 to 0.21], P=0.53). Secondary analyses restricted to [^{18}F]-fluorodeoxyglucose positron emission tomography of the carotid arteries as well as other indices of atherosclerosis confirmed the primary result. We performed an explorative analysis of interaction between treatment group and history of cardiovascular disease (P=0.052).

CONCLUSIONS: In this low to moderate risk population with type 2 diabetes, liraglutide did not change vascular inflammation assessed as [^{18}F]-fluorodeoxyglucose uptake compared with placebo. An explorative analysis indicated a possible effect in persons with history of cardiovascular disease, in line with current guidelines where liraglutide is recommended to patients with history of cardiovascular disease.

REGISTRATION: URL: https://www.clinicaltrials.gov; Unique identifier: NCT03449654.

Key Words: atherosclerosis ■ cardiovascular diseases ■ carotid arteries ■ glucagon-like peptide 1 ■ inflammation ■ Type 2 Diabetes

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For Sources of Funding and Disclosures, see page 642.

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CLINICAL PERSPECTIVE

Treatment with the human glucagon-like peptide-1 receptor agonists liraglutide protects against cardiovascular disease by an unknown mechanism. We investigated in a double-blind, randomized controlled trial if liraglutide affects vascular inflammation in patients with type 2 diabetes. The primary end point was change in vascular inflammation over 26 weeks assessed by [18F]-fluorodeoxyglucose positron emission tomography/computed tomography. We found, in a type 2 diabetes population with an overall moderate risk of cardiovascular disease, that liraglutide did not change the [18F]-fluorodeoxyglucose uptake compared with placebo. An explorative analysis indicated a possible effect in persons with history of cardiovascular disease, in line with guidelines where liraglutide is recommended to these patients.

Nonstandard Abbreviations and Acronyms

CACS	coronary artery calcium score
CIMT	carotid intima-media thickness
CT	computed tomography
CVD	cardiovascular disease
GLP-1	glucagon-like peptide 1
HO	Harmony Outcomes
ICC	intraclass correlations coefficient
LDL	low-density lipoprotein
PET	positron emission tomography
TBR	target-to-background ratio

Large cardiovascular outcome trials have demonstrated that treatment with 4 different human GLP-1 (glucagon-like peptide 1) receptor agonists reduces the high risk of cardiovascular disease (CVD) in type 2 diabetes.[1–4] These trials included patients with established CVD (HO [Harmony Outcomes] testing albiglutide[3]) or with a high prevalence of CVD (81% in LEADER [Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results] testing liraglutide[1] and 83% in SUSTAIN-6 [Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes] testing semaglutide[2]). Recently, the positive outcome of the REWIND trial (Researching Cardiovascular Events With a Weekly Incretin in Diabetes) using dulaglutide (including 32% with CVD) extended the cardiovascular protection observed with GLP-1 receptor agonists beyond high risk to a broader population of patients with type 2 diabetes and cardiovascular risk factors.[4] Evidence from these cardiovascular outcome trials have changed clinical practice guidelines[5] and fueled a debate regarding the mechanism behind the cardiovascular protection observed with human GLP-1 receptor agonists.

GLP-1 receptor agonists have well-established beneficial effects on several cardio-renal risk markers, including reduction in body weight, HbA_{1c}, blood pressure, LDL (low-density lipoprotein)-cholesterol, and albuminuria.[6] An interesting observation from the HO trial was that the magnitude of the cardiovascular protection was comparable to what has been demonstrated for liraglutide, semaglutide, and dulaglutide; however, in the HO trial, there were no major differences between the placebo- and the albiglutide-treated group in the effect on blood pressure, body weight, or renal function over time and only a modest reduction in HbA_{1c} with albiglutide-treatment.[3] Thus, it appears unlikely that the cardiovascular protection observed with GLP-1 receptor agonists is mediated solely by an effect on the classic cardio-renal risk markers. Results from atherosclerotic mice models have suggested that GLP-1 receptor agonists reduce the aortic plaque areas and change gene expression in the aorta related to proteins representing inflammatory pathways associated with leucocyte recruitment, adhesion, and migration.[7] Inflammation plays a key role in all phases of atherosclerotic plaque development. Treatment with liraglutide reduces inflammatory markers in the blood in humans,[8] but the direct effect on vascular inflammation has not been investigated.

The LIRAFLAME trial (Effect of liraglutide on vascular inflammation in type-2 diabetes: A randomized, placebo-controlled, double-blind, parallel clinical PET/CT trial) was designed to investigate the effect of liraglutide on vascular inflammation in patients with type 2 diabetes. We used state-of-the art positron emission tomography/computed tomography (PET/CT) with the radiolabeled glucose analog fluorodeoxyglucose ([18F]FDG) for specific in vivo evaluation of vascular inflammation. We hypothesized that 26 weeks treatment with liraglutide would reduce vascular inflammation assessed as [18F]FDG uptake compared with placebo. Likewise, we investigated changes in coronary artery calcium score (CACS), carotid intima-media thickness (CIMT), endothelial function, circulating biomarkers, as well as collected information on adverse events.

METHODS

Data Sharing

Individual, deidentified participant data are not freely available due to the risk of patient reidentification.

Deidentified participant data or anonymized clinical study reports can be obtained from the first author upon reasonable request. Necessary data protection agency and ethical committee approvals must be provided in compliance with relevant legislation.

Study Design

We performed a randomized, double-blind, placebo-controlled, parallel-group trial in 102 patients with type 2 diabetes. Participants were recruited from the outpatient clinic at the Steno Diabetes Center Copenhagen in Denmark and through

newspaper advertisements. Patients were assigned in a 1:1 ratio to receive subcutaneous injections of liraglutide or placebo (with matched administration device, diluent and volume injected) once daily for 26 weeks. The protocol was approved by the local ethics committee (H-16044546) and the Danish Medicines Agency (2016110109). The study was performed in compliance with the principles of the Declaration of Helsinki and according to Good Clinical Practice guidelines. The statistical analysis plan is available in the Data Supplement.

Participants

Patients were eligible if they met the inclusion criteria: type 2 diabetes (World Health Organization criteria); age >50 years; HbA_{1c} ≥48 mmol/mol (6.5%); eGFR (estimated glomerular filtration rate) ≥30 mL/min/1.73 m^2 (estimated by CKD-EPI formula); stable glucose- and cholesterol-lowering treatment for a minimum of 4 weeks before the baseline PET/CT. Main exclusion criteria were type 1 diabetes; treatment (90 days before screening) with oral glucocorticoids, calcineurin inhibitors, dipeptidyl peptidase 4 inhibitors, GLP-1 receptor agonists and other agents which, in the investigator's opinion, could interfere with the effect of liraglutide; cancer or any other clinically significant disorder, except for conditions associated with type 2 diabetes history which, in the investigator's opinion, could interfere with the results of the trial (detailed in and exclusion criteria are in the Data Supplement). All the patients provided written informed consent before participation.

Randomization and Masking

Identical liraglutide and placebo pens as well as the random liraglutide/placebo allocation sequence were provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Two persons not otherwise involved in the study had access to the computer-generated random liraglutide/placebo allocation sequence. They assigned and verified study medication numbers to the participants before the randomization visit. All study medication was numbered sequentially. The investigators, participants, and treating physicians were blinded to treatment allocation.

Procedures

All patients received a starting dose of 0.6 mg/d. The dose was escalated, if tolerated, as follows: first week 0.6 mg/d; second week 1.2 mg/d; and third week 1.8 mg/d. Maintenance dose was 1.8 mg/d; however, we allowed a flexible dose-escalation procedure to reach the maximum tolerated dose for each patient.

The study had 6 visits: (1) screening; (2) baseline [18F]FDG-PET/CT; (3) baseline randomization; (4) 13 weeks follow-up; (5) 26 weeks follow-up [18F]FDG-PET/CT, and (6) 26 weeks end of study. The [18F]FDG-PET/CT imaging was performed at Rigshospitalet, Denmark at Department of Clinical Physiology, Nuclear Medicine & PET. All other visits took place at the Steno Diabetes Center Copenhagen.

Arterial inflammation was examined using [18F]FDG-PET/CT of the carotid arteries and the aorta. Details of the procedure and analyses are described in the Data Supplement. In brief, [18F]FDG-PET/CT imaging of the carotid arteries and aorta was undertaken according to recommended methods.[9]

Maximum standardized uptake value of [18F]FDG was measured along the carotids and ascending aorta in axial orientation. Target-to-background ratio (TBR) was calculated as the ratio of standardized uptake value of the artery compared with background venous activity (Figure 1). Three established methods of uptake quantification were employed[9]: mean of maximum TBR in (1) all active segments (TBR >1.6), (2) most diseased segments, and (3) the whole vessels. PET/CT images were analyzed by a masked, experienced reader.

Reproducibility of [18F]FDG TBR measurements was tested using N=10 scans selected at random with >30 days between the readings.

Coronary atherosclerosis was assessed using CT-based CACS, and vascular anatomy was further evaluated by ultrasound CIMT. The endothelial function was examined by reactive hyperemia index evaluated with EndoPat (Itamar Medical, Israel), and glycocalyx integrity evaluated with the GlycoCheck device (Maastricht, the Netherlands).

Physical examination including height, weight, and blood pressure as well as blood samples for quantification of glucose metabolism, and other laboratory measures were performed at visit 3, 4, and 6. Adverse events were recorded at each visit. Details of the imaging protocols and methods are described in the Data Supplement.

Outcomes

The prespecified primary end point was change in [18F]FDG uptake in both the carotid arteries and the aorta using active segments analysis.[9]

Secondary end points were (1) change in arterial [18F]FDG uptake in the carotid arteries and the aorta using most diseased segment and mean uptake in the whole vessels; (2) change in CACS; (3) change in CIMT; (4) change in endothelial function; and (5) change in circulating biomarkers.

Statistical Analysis

The trial was powered to detect a clinically relevant 10% reduction in [18F]FDG uptake. We assumed a baseline [18F]FDG value (TBR mean of max) of 2.8, an absolute difference of 0.28 between groups (corresponding to a 10% reduction) and a SD of the change of 0.43.[10] Using these assumptions in a 2-sample *t* test power calculation, we would require 40 participants in each treatment group, with 80% power and an α of 0.05 (2-sided). A difference of 0.28 between groups is feasible to detect using FDG-PET.[11] We aimed to recruit at least 100 participants to account for drop-outs and deviations from the expected baseline [18F]FDG value.

As per the original specification in the trial protocol, results are primarily presented for (1) a modified intent-to-treat efficacy population, that consist of all included participants, for whom any aspect of treatment was initiated, and who had follow-up PET data available. Subjects were used in the analysis as randomized, and (2) an optimal efficacy population, consisting of all treated patients who were compliant (assessed by investigator) and completed the trial on maximum protocol treatment (1.8 mg/d) without change in lipid-lowering intervention during the study. We also present blood glucose corrected values of the vascular [18F]FDG uptake.[9] Only observed data were part of the analyses, no imputation was made and if data was missing exclusion was case-wise.

Normal distributed data are presented as mean±SD. Non-normal distributed data were presented as median with interquartile range and were log2 transformed before analysis. A value of 0.1 was added to the CACS before log2 transformation, since

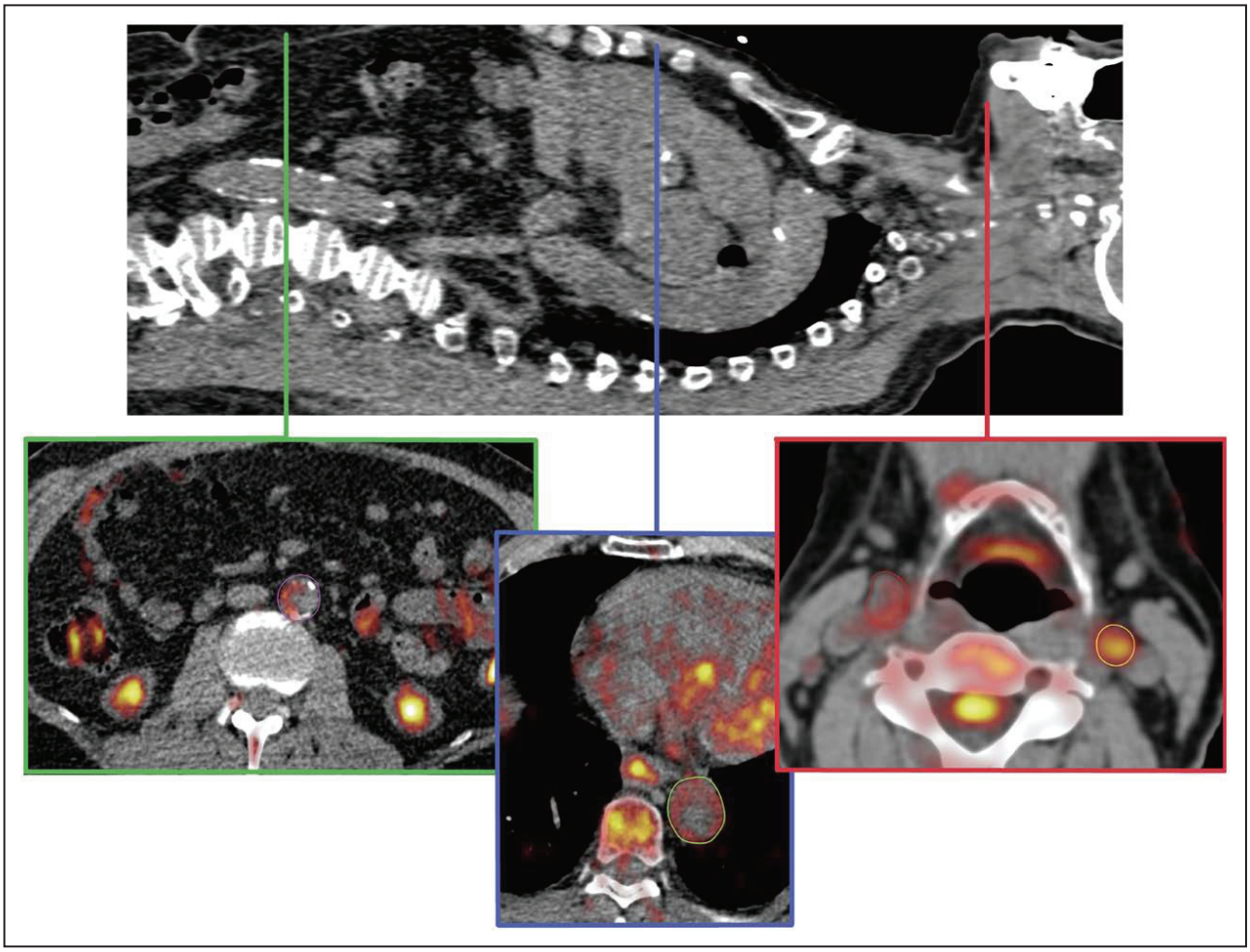

Figure 1. [18F]fluorodeoxyglucose (FDG)-positron emission tomography (PET)/computed tomography (CT) imaging approach. The abdominal aorta, the thoracic aorta, and the carotid arteries were identified and manually traced with free hand or ellipse regions of interest (ROI) on all axial CT images without use of the PET images. Afterwards the ROIs were copied onto the spatially aligned PET examination as shown in the figure. The FDG uptake was quantified in each ROI as the standardized uptake value (SUV) by measuring a maximum pixel activity value (SUVmax). Target-to-background ratio was finally calculated as a ratio of SUVmax and the average blood SUV estimated from venous blood in the superior cava vein or the jugular vein.

the unequal distribution included values of zero. Differences in baseline characteristics between the liraglutide and the placebo group were tested using unpaired *t* test, the χ^2 test, or Fisher exact test as appropriate. Differences between the liraglutide and the placebo group were tested using (1) paired *t* test for comparisons between baseline and end-of-treatment within groups (descriptive) and (2) unpaired *t* test for comparison of the change from baseline to end-of-treatment between the 2 groups (primary analysis). For TBR analysis, a mean value was used for each patient in all analysis, and multiple segments was not considered separately for the same patient. Adjustment for blood glucose and interaction analysis with CVD were analyzed in separate between-group regression models. Correlations were tested using Pearson correlation coefficient.

Intraclass correlations coefficients (ICC) with 95% CIs were calculated using a 2-way mixed model to test the intraobserver agreement of [18F]FDG TBR measurements.

Adverse events and serious adverse events are reported in tabulated form without significance testing.

All statistical analyses were performed using SAS software (version 9.4; SAS Institute, NC) and IBM SPSS statistics (version 25, IBM, NY). A 2-sided *P* value <0.05 was considered significant.

The trial is registered with the EU Clinical Trials Register, 2016-001523-31.

Role of Funding Source

Novo Nordisk A/S supplied the liraglutide and matching placebo and was the main funder the study by an unrestricted grant. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Between October 26, 2017, and August 16, 2019, 102 patients were randomly assigned to receive liraglutide (n=51) or placebo (n=51; Figure 2). Clinical characteristics at baseline are presented in Table 1. The population consisted mainly of overweight men above 60 years of age. The median diabetes duration was 10.9 years

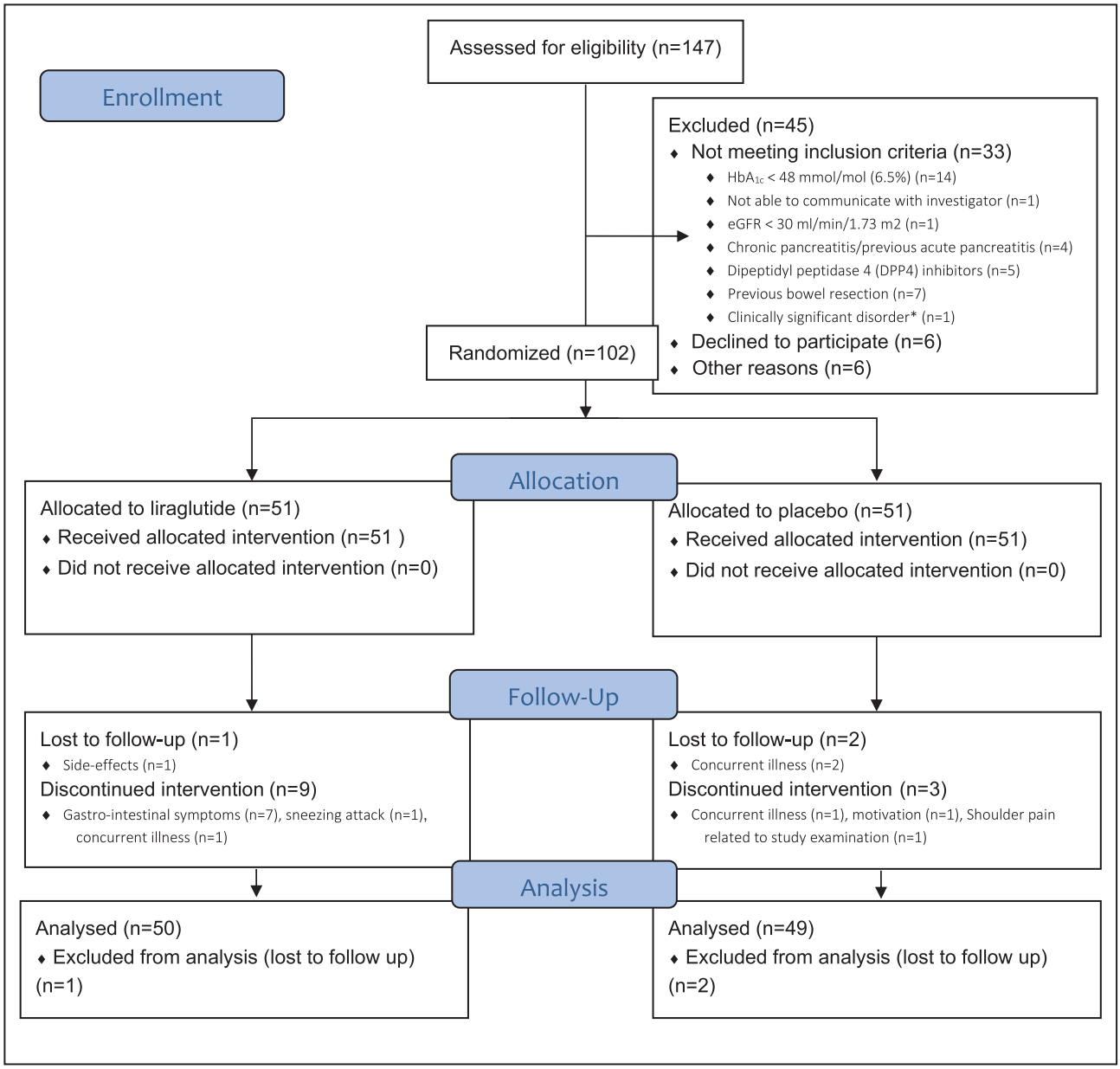

Figure 2. Trial profile.
eGFR indicates estimated glomerular filtration rate.

(interquartile range, 5.7–18.2), mean HbA_{1c} was 58.4 mmol/mol (SD, 10.1; 7.5% [0.92]), and eGFR was 83.2 mL/min/1.73 m^2 (SD, 16.3). At baseline 23 (22.6%) reported history of CVD (history of acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, stroke, peripheral arterial thrombosis [ie, arterial blot clot in the legs or arms], claudication, and nitroglycerin requiring angina pectoris).

Clinical characteristics at baseline were balanced between the liraglutide and placebo group, apart from a slightly higher concentration of triglycerides and more patients receiving beta-blockers in the group randomized to liraglutide (Table 1).

The intention-to-treat population (excluding those who did not complete the final [18F]FDG-PET/CT, n=3 [Figure 2]) consisted of 50 patients treated with liraglutide and 49 with placebo. The optimal efficacy population consisted of 63 patients; 24 treated with liraglutide and 39 with placebo, Table I in the Data Supplement.

For the group treated with liraglutide compared with placebo, HbA_{1c} and weight were reduced as follows: mean change in HbA_{1c} was −5.1 mmol/mol (95% CI, −8.1 to −2.0) [−0.47% (−0.74 to −0.18)] compared with −0.1 mmol/mol (−1.9 to 1.7) [−0.01% (−0.17 to 0.16)] in the placebo group (mean difference −5.0 mmol/mol [95% CI, −8.5 to −1.5] [−0.46% (−0.78 to −0.14)], P=0.006) and mean change in body weight was −3.7 kg (95% CI, −4.8 to −2.6) compared with −0.2 kg (−0.8 to 0.4) in the placebo group (mean difference −3.5 kg [95% CI, −4.8 to −2.3], P<0.001). The LDL-cholesterol concentrations and systolic blood pressure did not change significantly in any of the treatment groups (Table 2).

Table 1. Characteristics of the Patients at Baseline

	Total (n=102)	Liraglutide (n=51)	Placebo (n=51)	*P* value
Sex (woman)	16 (15.7%)	6 (11.8%)	10 (19.6%)	0.28
Age, y	66.4 (8.2)	65.9 (8.6)	66.9 (7.8)	0.56
Body mass index, kg/m^2	29.9 (4.6)	30.5 (5.3)	29.3 (3.8)	0.16
Type 2 diabetes				
Known duration, y	10.9 (5.7–18.2)	12.2 (5.4–18.2)	10.2 (5.7–19.2)	0.65
HbA_{1c}, mmol/mol	58.4 (10.1)	58.7 (9.6)	58.0 (10.6)	0.73
HbA_{1c}, %	7.5 (0.92)	7.5 (0.88)	7.5 (0.97)	0.73
Kidney function				
Estimated glomerular filtration rate, mL/(min·1.73 m^2)	83.2 (16.3)	82.7 (17.6)	83.7 (15.0)	0.75
Urinary albumin creatinine ratio, mg/g	6.0 (3.5–14.5)	6.0 (3.5–15.0)	6.0 (3.5–14.5)	0.65
Cardiovascular risk factors				
Systolic blood pressure, mm Hg	135.3 (17.3)	133.4 (14.5)	137.3 (19.7)	0.25
Diastolic blood pressure, mm Hg	79.3 (7.8)	79.8 (7.0)	78.9 (8.4)	0.57
Total cholesterol, mmol/L	4.1 (0.8)	4.1 (0.8)	4.1 (0.8)	0.85
LDL-cholesterol, mmol/L	2.1 (0.7)	2.1 (0.7)	2.1 (0.6)	0.48
HDL cholesterol, mmol/L	1.2 (0.4)	1.2 (0.4)	1.3 (0.3)	0.14
Triglycerides, mmol/L	1.8 (1.0)	2.1 (1.2)	1.6 (0.8)	0.01
Current smoker	14 (13.7%)	10 (19.6%)	4 (7.8%)	0.08
Hypertension	79 (77.5%)	43 (84.3%)	36 (70.6%)	0.10
History of cardiovascular event				
Myocardial infarction	13 (12.8%)	8 (15.7%)	5 (9.8%)	0.37
Stroke	6 (5.9%)	3 (5.9%)	3 (5.9%)	1.00
Peripheral arterial thrombosis	2 (2.0%)	2 (3.9%)	0 (0.0%)	0.50
History of cardiovascular symptoms				
Claudication	4 (3.9%)	3 (5.9%)	1 (2.0%)	0.62
Angina pectoris	5 (4.9%)	4 (7.8%)	1 (2.0%)	0.36
Any cardiovascular disease	23 (22.6%)	14 (27.5%)	9 (17.7%)	0.24
Glucose lowering medication				
Insulin use	39 (38.2%)	20 (39.2%)	19 (37.3%)	0.84
SGLT2 inhibitors	20 (19.6%)	8 (15.7%)	12 (23.5%)	0.32
Cardiovascular medication				
Angiotensin converting enzyme inhibitors	30 (29.4%)	15 (29.4%)	15 (29.4%)	1.00
Angiotensin II receptor blockers	45 (44.1%)	22 (43.1%)	23 (45.1%)	0.84
Mineralocorticoids	5 (4.9%)	2 (3.9%)	3 (5.9%)	1.00
Calcium channel antagonists	35 (34.3%)	17 (33.3%)	18 (35.3%)	0.83
β blockers	19 (18.6%)	14 (27.5%)	5 (9.8%)	0.02
Aspirin treatment	37 (36.3%)	16 (31.4%)	21 (41.2%)	0.30
Lipid-lowering treatment	88 (86.3%)	46 (90.2%)	42 (82.4%)	0.25

Data are n (%) or mean (SD) or median (IQR). Differences in baseline characteristics between the liraglutide and the placebo group were tested using unpaired *t* test, the χ^2 test, or Fisher exact test as appropriate. Hypertension was defined as treatment with antihypertensive medication. HDL indicates high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; and SGLT2, sodium glucose transporter 2.

The intraobserver reliability of [18F]FDG TBR measurements were excellent for both all arteries combined (intraclass correlation coefficient [ICC], 0.97 [95% CI, 0.86–0.99]) and the individual territories: carotid arteries (ICC, 0.96 [95% CI, 0.83–0.99]), thoracic aorta (ICC, 0.97 [95% CI, 0.87–0.99]), and abdominal aorta (ICC, 0.98 [95% CI, 0.91–0.99]).

An overview of missing data for primary and secondary end points and key clinical characteristics is presented in Table II in the Data Supplement.

[18F]FDG-PET After GLP-1 Treatment

The primary end point was unchanged in both groups as mean change in active segments TBR from baseline to

Table 2. Changes in Clinical Characteristics and Secondary End Points

Group	Mean (SD) or median (IQR)		*P* value	Change mean (95% CI) or median [IQR]	*P* value
	Baseline	End-of-treatment			
Clinical characteristics					
HbA_{1c}, mmol/mol, [HbA_{1c} %]					
Liraglutide, n=49	58.8 (9.6) [7.5 (0.88)]	53.8 (14.7) [7.1 (1.34)]	0.002	−5.1 (−8.1 to −2.0) [−0.47 (−0.74 to −0.18)]	0.006
Placebo, n=48	57.3 (8.8) [7.4 (0.81)]	57.2 (8.5) [7.4 (0.78)]	0.9	−0.1 (−1.9 to 1.7) [−0.01 (−0.17 to 0.16)]	
Body weight, kg					
Liraglutide, n=49	94.5 (20.3)	90.8 (19.2)	<0.0001	−3.7 (−4.8 to −2.6)	<0.0001
Placebo, n=48	87.8 (13.8)	87.6 (14.2)	0.54	−0.18 (−0.76 to 0.40)	
Systolic blood pressure, mm Hg					
Liraglutide, n=49	134 (15)	133 (13)	1.0	0 (−5 to 5)	1.0
Placebo, n=48	138 (19)	137 (17)	1.0	0 (−4 to 4)	
LDL-cholesterol, mmol/L					
Liraglutide, n=45	2.1 (0.7)	2.2 (1.0)	0.6	0.1 (−0.2 to 0.3)	0.4
Placebo, n=46	2.2 (0.6)	2.1 (0.8)	0.5	−0.1 (−0.3 to 0.1)	
Secondary end points					
Vascular anatomy					
Coronary artery calcium score					
Liraglutide, n=44	150 [3 to 359]	145 [10 to 439]	0.41*	8 [0 to 41]	0.62*
Placebo, n=46	185 [52 to 399]	211 [60 to 405]	0.53*	7 [−1 to 31]	
Carotid intima-media thickness, mm					
Liraglutide, n=50	0.77 (0.17)	0.76 (0.17)	0.33	0.009 (−0.010 to 0.027)	0.51
Placebo, n=48	0.75 (0.14)	0.75 (0.14)	0.89	−0.001 (−0.025 to 0.023)	
Endothelial function					
Reactive hyperemia index					
Liraglutide, n=46	1.8 (0.40)	1.8 (0.38)	0.18	−0.08 (−0.19 to 0.04)	0.80
Placebo, n=44	1.8 (0.40)	1.8 (0.39)	0.43	−0.06 (−0.20 to 0.08)	
Glycocalyx integrity, µm					
Liraglutide, n=21	2.0 (0.25)	2.0 (0.23)	0.38	−0.05 (−0.18 to 0.07)	0.27
Placebo, n=23	1.9 (0.29)	2.0 (0.25)	0.48	0.05 (−0.10 to 0.20)	
Biomarkers					
High-sensitivity C-reactive protein, mg/L					
Liraglutide, n=47	1.6 [0.99 to 3.9]	1.7 [0.9 to 2.7]	0.13*	−0.11 [−0.91 to 0.27]	0.22*
Placebo, n=48	1.5 [0.75 to 2.9]	1.3 [0.7 to 4.0]	0.99*	0.03 [−0.22 to 0.42]	
Pro-B-type natriuretic peptide, pmol/L					
Liraglutide, n=34	11.2 [6.7 to 20.6]	8.4 [6.3 to 22.4]	0.41*	−2.3 [−6.4 to 4.1]	0.98*
Placebo, n=35	10.1 [6.9 to 15.7]	10.5 [3.0 to 16.4]	0.36*	−2.1 [−4.0 to 5.3]	
Interleukin-6, ng/L					
Liraglutide, n=43	1.3 [0.87 to 2.5]	1.2 [0.78 to 1.8]	0.049*	−0.19 [−0.62 to 0.28]	0.86*
Placebo, n=39	1.1 [0.87 to 1.7]	0.78 [0.66 to 1.3]	0.06*	−0.21 [−0.38 to 0.16]	
Monocyte chemoattractant protein-1, ng/L					
Liraglutide, n=49	20.6 [16.8 to 23.1]	11.6 [8.5 to 16.2]	<0.0001*	−8.2 [−11.5 to −4.1]	0.052*
Placebo, n=48	19.4 [14.1 to 23.0]	12.1 [10.0 to 15.5]	<0.0001*	−6.1 [−11.1 to −3.2]	

Data are mean (SD), median (IQR), or mean (95% CI) change. Paired *t* test for comparisons between baseline and end-of-treatment within groups and unpaired *t* test for comparison of the change from baseline to end-of-treatment between the 2 groups. IQR indicates interquartile range; and LDL, low-density lipoprotein.

*Change in log2 values.

end-of-treatment was −0.04 (95% CI, −0.17 to 0.08) for the liraglutide group and −0.09 (−0.19 to 0.01) for the placebo group, with no difference between treatment groups (mean difference, 0.05 [95% CI, −0.11 to 0.21], *P*=0.53), Table 3, Figure 3.

For the secondary objective of assessing TBR in most diseased segment and in the whole vessels, results were consistent: liraglutide versus placebo group, mean change in TBR in most diseased segment was −0.22 (95% CI, −0.40 to −0.03) versus −0.24 (−0.44 to −0.04;

Table 3. Arterial Vascular Inflammation Evaluated With [18F]FDG-PET in 102 Type 2 Diabetes Subjects Assessed in 3 Ways: Active Segments, Most Diseased Segments, and Whole Vessels

Group	Mean TBR (SD)		*P* value	Δ TBR (95% CI)	*P* value
	Baseline	End-of-treatment			
Active segments					
Liraglutide, n=50	2.1 (0.30)	2.0 (0.39)	0.50	−0.04 (−0.17 to 0.08)	0.53*
Placebo, n=49	2.0 (0.29)	1.9 (0.29)	0.07	−0.09 (−0.19 to 0.01)	
Most diseased segments					
Liraglutide, n=50	2.7 (0.72)	2.5 (0.69)	0.02	−0.22 (−0.40 to −0.03)	0.87
Placebo, n=49	2.4 (0.63)	2.2 (0.55)	0.02	−0.24 (−0.44 to −0.04)	
Whole vessels					
Liraglutide, n=50	1.9 (0.33)	2.0 (0.37)	0.27	0.07 (−0.05 to 0.18)	0.46
Placebo, n=49	1.8 (0.35)	1.8 (0.29)	0.88	0.01 (−0.10 to 0.11)	

Arterial vascular inflammation evaluated as TBR. Data are mean (SD) or change (95% CI). Paired *t* test for comparisons between baseline and end-of-treatment within groups and unpaired *t* test for comparison of the change from baseline to end-of-treatment between the 2 groups. FDG indicates fluorodeoxyglucose; PET, positron emission tomography; and TBR, target-to-background ratio.
*Primary end point.

P=0.87 between treatment groups), and mean change in TBR in whole vessels was 0.07 (95% CI, −0.05 to 0.18) versus 0.01 (−0.10 to 0.11; *P*=0.46 between treatment groups; Table 3). Results were also consistent when analyzing the carotids, thoracic aorta, and abdominal aorta separately (Table III in the Data Supplement).

Secondary End Points of Atherosclerosis

Change in supportive secondary end points for liraglutide versus placebo were as follows: median change in CACS: 8 (IQR, 0; 41) versus 7 (−1; 33), *P*=0.62 between treatment groups; mean change in CIMT: 0.009 mm (95% CI, −0.010 to 0.027) versus −0.001 mm (−0.025 to 0.023), *P*=0.51 between treatment groups. Changes in endothelial function evaluated by reactive hyperemia index or glycocalyx integrity (Table 2) or biomarkers reflecting inflammation or atherosclerosis were not different between the 2 treatment groups (Table 2 and Table IV in the Data Supplement).

When analyzing the optimal efficacy population (n=63), we observed a nearly statistical significant increase in [18F]FDG uptake in the liraglutide group compared with placebo (*P*=0.052, Table V in the Data Supplement). This trend was driven by changes in the abdominal aorta and not reproduced when analyzing the carotid arteries only (Table VI in the Data Supplement).

Adverse Events

Adverse events are reported in Table 4, Tables VI and VII in the Data Supplement. Of the 102 participants, 83 (81%) reported at least one adverse event. Most common was gastrointestinal symptoms (Table VII in the Data Supplement). In the liraglutide group 8 (16%) participants discontinued study medication due to adverse

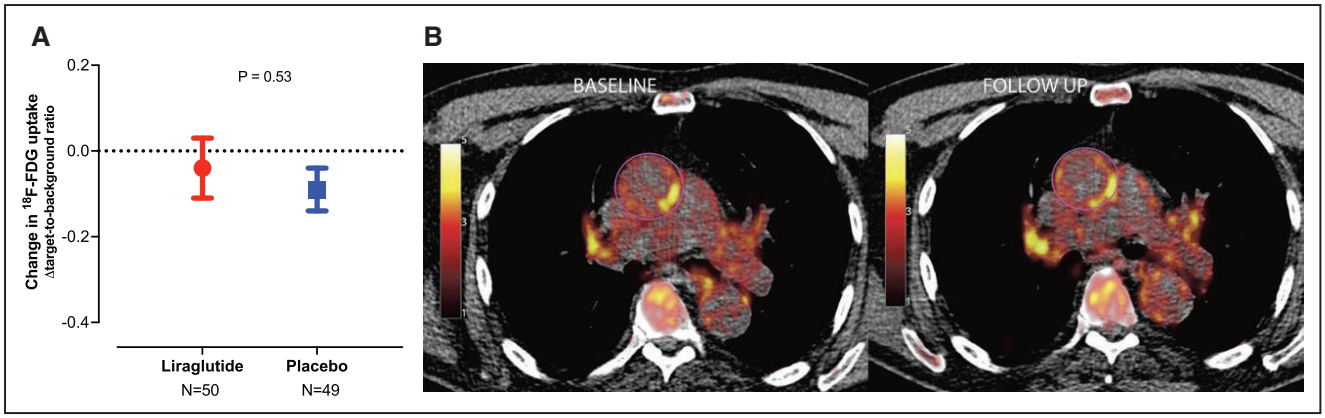

Figure 3. Arterial inflammation evaluated with [18F]fluorodeoxyglucose (FDG)-positron emission tomography (PET) in the liraglutide and the placebo-treated group.
A, Mean change from baseline to end-of-treatment in active segments target-to-background ratio for the liraglutide group and the placebo group (primary end point). Mean plots with SE. Unpaired *t* test for comparison. **B**, Representative [18F]FDG-PET/computed tomography images from a participant treated with liraglutide. The ascending thoracic aorta is outlined at baseline and follow-up examination.

Table 4. Adverse Events and Serious Adverse Events Leading to Discontinuation of the Study Drug

	Liraglutide, n=51	Placebo, n=51
Any adverse events (total number)	108	69
Any adverse events (patients with at least one)	48	35
Adverse events leading to discontinuation of study drug	8	1
Any serious adverse events (total number)	4	5
Any serious adverse events (patients with at least one)	4	4
Serious adverse events considered related to study drug	0	0
Death	0	0

Data are number of participants with a specified event. No participants died during the trial.

events (7 due to gastrointestinal symptoms, one due to sneezing attack) against 1 (2%) in the placebo group (back pain related to study examination). A total of 9 serious adverse events were registered (Table VIII in the Data Supplement); none were fatal or considered related to the study drug.

Impact of Blood Glucose

Capillary blood glucose was measured just before [18F]FDG injection and was similar in the 2 treatment groups at both baseline and follow-up (Table IX in the Data Supplement). The capillary blood glucose was not correlated with arterial [18F]FDG uptake at baseline (r=–0.03, P=0.8) or at follow-up (r=–0.1, P=0.2). Treatment group did not affect the primary end point by regression analysis either when controlling for capillary blood glucose (P=0.07) or HbA_{1c} (P=0.1). Finally, we performed adjustment for blood glucose on the quantification of arterial [18F]FDG uptake. This did not reveal any indications that liraglutide had a significant effect on arterial [18F]FDG uptake (Table X in the Data Supplement).

Impact of Previous CVD

Patients with CVD had a 10.4% higher carotid [18F]FDG uptake at baseline than the patients without CVD (P=0.16). In an exploratory analysis, we compared change in carotid [18F]FDG uptake in participants with (n=23) and without (n=79) a history of CVD and we observed a borderline significant interaction (P=0.052) between treatment group and history of CVD for predicting change in carotid [18F]FDG uptake (Figure 4). In explorative analysis including only participants with CVD, we observed a significant reduction in [18F]FDG uptake for the participants treated with liraglutide (–0.46 [95% CI, –0.89 to –0.02], P=0.04, n=13) but not for the participants treated with placebo (–0.16 [–1.03 to 0.71], P=0.68, n=9). However, the difference between

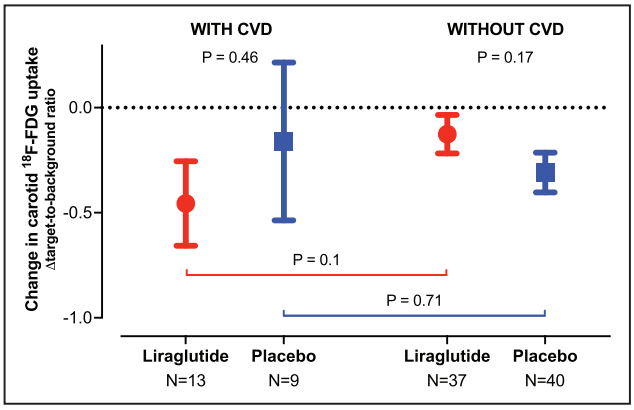

Figure 4. Arterial inflammation evaluated with [18F] fluorodeoxyglucose (FDG)-positron emission tomography (PET) in the subgroup of patients with and without cardiovascular disease.
Mean change from baseline to end-of-treatment in most diseased segment target-to-background ratio for the liraglutide group and the placebo group in subgroups of patients with and without cardiovascular disease (CVD). Mean plots with SE. Unpaired t test for comparison of the change from baseline to end-of-treatment between the groups.

participants with CVD treated with liraglutide and placebo was not statistical significant (P=0.46, Figure 4). Moreover, changes in the biomarkers reflecting inflammation or atherosclerosis were not different between the 2 treatment groups in analyses restricted to participants with a history of CVD (Table XI in the Data Supplement).

DISCUSSION

Treatment with liraglutide reduces cardiovascular events in high-risk patients,[1] but the mode of action is not fully understood. In this randomized, double-blind, placebo-controlled clinical trial, we demonstrated a significant effect of liraglutide on both HbA_{1c} and body weight, but we could not demonstrate an effect on arterial [18F]FDG uptake in this unselected type 2 diabetes population with the majority without known CVD. Similarly, we did not see any effect of liraglutide on CACS, CIMT, or endothelial function. In exploratory analyses, treatment with liraglutide in patients with manifest CVD did seem to reduce [18F]FDG uptake in the carotid arteries.

GLP-1 Treatment and Atherosclerosis

With positive results from four large cardiovascular outcome trials,[1–4] evidence has become strong for human GLP-1 receptor agonists to reduce CVD risk in type 2 diabetes patients with atherosclerotic CVD. The late separation of the Kaplan-Meier curves in the LEADER trial[1] could suggest a more indirect pleiotropic cardiovascular effect of liraglutide rather than a direct antithrombotic effect.

A number of animal studies support this hypothesis showing that GLP-1 inhibits the formation of

atherosclerotic plaques significantly,[12] inhibits the formation of macrophage-derived foam cells in the plaque,[13] and decreases the adhesion of mononuclear cells to the vessel wall.[14] This leads to an inhibition of atherogenesis by liraglutide in mice by decreased lipid deposition and reduction in intima-media thickness while the number of smooth muscle cells increase.[15] The attenuated development of plaque lesions in mice seems partly independent of weight and cholesterol lowering[7] and requires the endothelial GLP-1 receptor.[16] Similarly, a human study demonstrated that carotid plaques ex vivo were less inflamed in patients treated with GLP-1 receptor agonists, suggesting a more stable phenotype.[17] Also, a decrease in serum concentrations of the inflammatory marker sCD163 and fewer inflammatory macrophages were demonstrated in patients with type 2 diabetes treated for 6 months with liraglutide.[18] A similar effect is not seen with intense lowering of blood glucose without GLP-1 treatment.[19]

[18F]FDG-PET After GLP-1 Treatment

To our knowledge, this study is the first to investigate in vivo arterial [18F]FDG uptake, a marker of arterial inflammation, following treatment with GLP-1 receptor agonist in patients with type 2 diabetes. Inflammation plays a pivotal role in the development of atherosclerosis and recently 2 large randomized trials provided direct evidence that this risk is modifiable by anti-inflammatory therapy,[20,21] also in patients with type 2 diabetes.[22] The hypothesis of our study was that the known cardiovascular benefit of liraglutide[1] is at least in part caused by a reduction in arterial inflammation. [18F]FDG-PET/CT was chosen as primary end point since several studies, over the last decade, have shown that this method can be used to directly quantify arterial inflammation reliably and noninvasively.[11] [18F]FDG is actively taken up by cells with a high glycolytic activity, such as inflammatory cells, and the [18F]FDG signal correlates with metabolically active macrophages.[23] [18F]FDG-PET has been used to assess the direct impact of pharmaceutical interventions on arterial inflammation in a number of trials such as statin treatment.[24] The [18F]FDG-PET/CT method has similarly been used in a population with impaired glucose tolerance or type 2 diabetes to compare the effect of pioglitazone to glimepiride on plaque inflammation.[25] This study found an attenuation of arterial [18F]FDG uptake by pioglitazone but not by glimepiride despite equal effect on blood glucose in the 2 treatment arms[25]; these results are congruent with the known cardiovascular risk benefit of pioglitazone[26] as compared with no risk benefit of glimepiride.[27]

Based on the previous observations regarding the beneficial effect of liraglutide on CVD, our primary result is somewhat surprising. However, compared with the LEADER trial, we did not enrich the population with high-risk subjects and our study thus included only 23% with known CVD compared with 81% in the LEADER trial.[1] The majority of our population was, therefore, in a relatively lower risk of CVD, and it is our hypothesis that the low arterial inflammation resulted in a reduced potential for attenuation of the inflammatory signal. This hypothesis is supported by the fact that compared with previous studies assessing carotid uptake of [18F]FDG, our population had a low uptake at baseline.[10,24] In an exploratory analysis of the patients with known CVD, we observed that these subjects had a 10% higher carotid [18F]FDG uptake at baseline than the subjects without CVD. In an analysis of the total population, we observed a borderline significant interaction between presence of CVD and treatment supporting our hypothesis that the neutral results could have been caused by inclusion of relatively lower-risk patients. When only including subjects with known CVD in the analysis (n=23), we observed a numeric difference in the reduction of [18F]FDG uptake in subjects treated with liraglutide compared with placebo, but the difference did not reach statistical significance perhaps due to lack of power. However, as this was an explorative analysis the results are only hypothesis generating and need to be tested in a future study.

An alternative hypothesis for explaining the unchanged [18F]FDG uptake in our population is that [18F]FDG may not be a specific surrogate of inflammation in nonatherosclerotic arteries. The biological basis of cellular [18F]FDG uptake is complex and influenced by a number of metabolic factors. Small animal and clinical studies have investigated the biological link between arterial inflammation and [18F]FDG signal as reviewed by Sadeghi.[28] Several of these studies have demonstrated a correlation between [18F]FDG uptake and in vivo macrophage markers,[23] but without indications that inflammation should be the main determinant of arterial [18F]FDG uptake. This notion is supported by a study showing that uptake in medial smooth muscle cells was a significant contributor to the arterial [18F]FDG signal in minipigs.[29] However, at the same time positive results in clinical studies of anti-inflammatory drugs, like statins, in subjects with known CVD or at high risk for atherosclerosis[24] indicate that the arterial [18F]FDG signal does contain relevant clinical information despite its limited specificity.

Impact of Blood Glucose

A change in glucose metabolism could theoretically affect vascular [18F]FDG uptake and consequently influence the conclusion of our trial since [18F]FDG is a glucose analogue that is transported into the cells by the plasma membrane glucose transporters. However, a study of patients with diabetes or impaired glucose tolerance demonstrated no change in vascular [18F]

FDG uptake despite a significant reduction in both fasting plasma glucose and HbA_{1c} following treatment with glimepiride.[25] As expected, we found that liraglutide decreased HbA_{1c} compared with placebo, but when comparing the capillary blood glucose measured immediately before the [18F]FDG injection we found no difference between the 2 groups. Further support for the robustness of the [18F]FDG method in the presence of type 2 diabetes is that no correlation between arterial [18F]FDG uptake and blood glucose (either measured as HbA_{1c} or capillary blood glucose) was demonstrated in our study. This result is in line with observations in mice, where presence of type 2 diabetes does not confound evaluation of plaque inflammation with [18F]FDG.[30] In addition, patients with type 2 diabetes are routinely included in other studies of vascular inflammation using [18F]FDG-PET.[10,24] Glucose adjustment of [18F]FDG imaging is possible but generally not recommended for arterial [18F]FDG uptake.[9] Using blood glucose corrected [18F]FDG uptake in our study did not change the conclusions.

Secondary Supportive End Points

In support of our primary end point, we found that 26 weeks of treatment with liraglutide did not significantly change CACS, CIMT or measures of endothelial function compared with placebo. Circulating biomarkers of atherosclerosis and inflammation was not affected by liraglutide, and revealed that the included population had a low level of inflammation. Our study was not powered to detect changes in these secondary end points, and the treatment period of 26 weeks is probably too short for significant changes in the vascular anatomy, including CACS and CIMT, to occur.

As expected from previous studies with GLP-1 receptor agonists, gastrointestinal symptoms were the most common side effect and the most common reason to discontinue study treatment. More participants in the liraglutide group than in the placebo group discontinued the trial regimen due to adverse events. A total of 8 participants experienced a serious adverse event, none of which were fatal or considered related to liraglutide.

Study Limitations

Our trial has some limitations, and issues related to the design may have impacted our result. First, we included an unselected population of patients with type 2 diabetes rather than solely patients at very high CVD risk and we did not include a prescreening [18F]FDG-PET/CT to identify subjects with a [18F]FDG uptake indicating presence of vascular inflammation. The rationale for this was that type 2 diabetes is associated with increased cardiovascular risk,[31] and we thus expected a higher baseline [18F]FDG uptake than we observed. Second, we quantified the [18F]FDG uptake using active segments analysis of both aorta and the carotid arteries rather than using most diseased segment analysis of the carotid arteries only as in most of the recent studies. Recent studies indicate that the carotid artery [18F]FDG uptake has less day-to-day variation[11] and a study in pigs demonstrated [18F]FDG signal in the aorta irrespective of the presence of macrophage-containing lesions.[29] Based on these considerations, we performed the exploratory analysis in the subpopulation with known CVD using most diseased segment analysis of the carotid artery.

Conclusions

In conclusion, our study found no effect of liraglutide on arterial [18F]FDG uptake in patients with type 2 diabetes and low prevalence of vascular inflammation indicating that liraglutide did not attenuate arterial inflammation in these patients. An explorative analysis indicated a possible effect in the subgroup of patients with a history of CVD, in line with current guidelines where liraglutide is recommended to patients with history of CVD.

ARTICLE INFORMATION

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Disclosures

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Supplemental Materials

Data Supplement Methods
Data Supplement Tables I–XI
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Paper II

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Article

Effect of Liraglutide on Vascular Inflammation Evaluated by [^{64}Cu]DOTATATE

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Abstract: Quantification of vascular inflammation before and after treatment with glucagon-like peptide-1 receptor agonists (GLP-1 RAs) may help reveal mechanistic pathways underlying the cardiovascular benefits of these drugs. We assessed change in vascular inflammation in the carotid arteries over 26 weeks by copper-64-labeled [1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid]-D-Phe1, Tyr3-octreotate ([^{64}Cu]DOTATATE) PET in 30 participants included in a substudy of a double-blind trial where persons with type 2 diabetes (T2D) were randomized to liraglutide (n = 15) or placebo (n = 15) for 26 weeks. Mean age (SD) was 66.4 (7.2) years, HbA_{1c} 56.4 (9.2) mmol/mol and BMI 28.9 (4.6) kg/m^2. Weight and HbA_{1c} were significantly reduced by liraglutide vs. placebo ($p \leq 0.01$). The [^{64}Cu]DOTATATE uptake (mean standardized uptake values) was significantly reduced in the liraglutide-treated group (−0.11 [95% confidence interval −0.19 to −0.03], p = 0.01) and not changed significantly in the placebo group (−0.07 [−0.14 to 0.01], p = 0.08). The mean difference between groups did not reach significance (−0.04 [−0.15 to 0.07], p = 0.44). In conclusion, [^{64}Cu]DOTATATE uptake was reduced in persons with T2D treated with liraglutide. However, the reduction compared to placebo did not reach statistical significance, perhaps due to limited power. A reduction in vascular inflammation with liraglutide could help explain the cardiovascular protection observed with GLP-1 RAs in outcome studies but warrants further and larger studies.

Keywords: GLP-1 RA; vascular inflammation; PET; type 2 diabetes

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1. Introduction

Inflammation is a key process in progressive atherosclerosis and quantification of vascular inflammation in atherosclerosis may reveal mechanistic pathways for drugs with the ability to reduce the risk of cardiovascular disease. The majority of clinical research aimed at in vivo evaluation of vascular inflammation has used fluorine-18-labeled fluorodeoxyglucose positron emission tomography ([^{18}F]FDG PET). A large body of evidence links the [^{18}F]FDG uptake to the abundance of macrophages in atherosclerotic plaques, including studies of excised human plaques [1]. Carotid [^{18}F]FDG uptake has also been demonstrated to identify a reduction in vascular inflammation in response to statin treatment [2]. Lack of cell specificity is one limitation of [^{18}F]FDG imaging, which has fueled interest in more cell-specific probes, including copper-64-labeled [1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid]-D-Phe1, Tyr3-octreotate ([^{64}Cu]DOTATATE). [^{64}Cu]DOTATATE targets the G-protein-coupled receptor somatostatin receptor subtype-2 (SST2) that is selectively

expressed on the surface of activated macrophages. Preclinical [3] and retrospective studies [4,5] suggest that DOTATATE binding is a specific marker of macrophage activity, and a study in 10 persons undergoing endarterectomy demonstrated that [^{64}Cu]DOTATATE accumulates in atherosclerotic plaques of the carotid artery [6]. No clinical trials have yet evaluated change in vascular inflammation using [^{64}Cu]DOTATATE PET [6] and head-to-head comparisons of the FDG and DOTATATE PET tracer are limited to a single study [7].

Several large cardiovascular outcome trials have demonstrated that glucagon-like peptide-1 receptor agonists (GLP-1 RAs) reduce the risk of cardiovascular disease in type 2 diabetes [8–11]. Animal studies have demonstrated that GLP-1 RAs reduce atherosclerotic plaque formation [12,13] and affect key steps in the development of atherosclerotic plaques, including macrophage-derived foam cell formation [14] and the adhesion of mononuclear cells to the endothelium [15]. We therefore conducted a randomized placebo-controlled clinical trial to evaluate the GLP-1 RA liraglutide's effect on vascular inflammation.

We found no effect of liraglutide on the primary endpoint, which was change in vascular inflammation assessed using [^{18}F]FDG PET in 102 participants, however, an exploratory analysis indicated a possible effect of liraglutide on [^{18}F]FDG uptake in the subgroup of participants with a history of cardiovascular disease [16].

Here, we present a substudy of this trial aimed to investigate liraglutide's effect on vascular inflammation, evaluated as [^{64}Cu]DOTATATE uptake in the carotid arteries, in a subgroup of 30 participants.

2. Materials and Methods

2.1. Study Design and Participants

The main trial included 102 participants with type 2 diabetes in a double-blind, randomized controlled clinical setting, as previously described in detail [16]. In short, participants were randomized in a 1:1 ratio to treatment with liraglutide or placebo for 26 weeks. Starting dose was 0.6 mg once daily for 1 week, followed by 1.2 mg once daily for 1 week, followed by 1.8 mg once daily for the remainder of the trial. The dose escalation was flexible, and participants were kept on the highest tolerated dose.

Major inclusion criteria were age > 50 years; $HbA_{1c} \geq 48$ mmol/mol (6.5%); and eGFR ≥ 30 mL/min/1.73 m^2 (estimated by the CKD-EPI formula). Glucose- and cholesterol-lowering treatment had to be stable for min. 4 weeks prior to enrollment in the study. Major exclusion criteria were type 1 diabetes; other treatment (90 days prior to enrollment in the study) or disorders (except for conditions associated with type 2 diabetes history), which could interfere with the results of the trial. The full list of inclusion and exclusion criteria has previously been published [16].

Here, we report results from a prespecified substudy in 30 participants where vascular inflammation was evaluated by both [^{18}F]FDG uptake and [^{64}Cu]DOTATATE uptake. The number of included participants in this substudy was limited by [^{64}Cu]DOTATATE tracer availability, and inclusion was random. [^{18}F]FDG PET/CT imaging was performed at baseline and after 26 weeks of liraglutide/placebo treatment. [^{64}Cu]DOTATATE PET/CT imaging was performed on a separate day, preferably within the same week as [^{18}F]FDG PET/CT imaging, but timing was pragmatic due to limited [^{64}Cu]DOTATATE tracer availability. The median [IQR] interval between the 2 imaging studies was 8 [6–14] days for the baseline examination and 6 [2–15] days for the follow-up examination

The study was approved by the local ethics committee (H-16044546) and the Danish Medicines Agency (2016110109) and was in compliance with the principles of the Declaration of Helsinki. All participants provided informed consent.

2.2. Blood and Urine Analysis

HbA_{1c} was measured using high-performance liquid chromatography. Plasma creatinine was measured by an enzymatic method (Hitachi 912, Roche Diagnostics, Mannheim, Germany) and to calculate eGFR we used the CKD-EPI equation [17]. Urinary albumin creatinine rate (UACR) was measured by an enzyme immunoassay in two consecutive

morning urine samples. High-sensitivity C-reactive protein was measured in lithium–heparin–plasma using Cobas 8000 e801 (Roche Diagnostics, Rotkreuz, Switzerland) assays.

2.3. [^{64}Cu]DOTATATE PET/CT

We used a combined PET/CT scanner (Siemens Biograph mCT64, Siemens, Berlin, Germany). Patients were scanned one hour (±10 min) after [^{64}Cu]DOTATATE injection. The PET was acquired in three-dimensional list mode for 10 min centered at the carotid bifurcation. A low-dose CT scan (120 keV, mAs 50) was applied for attenuation correction and anatomical location of the carotid arteries. The PET images were reconstructed using CT-based attenuation correction, with both resolution–recovery (point spread function, TrueX) and time-of-flight (2 iterations, 21 subsets, zoom 1.0) giving 400 × 400 image slices (voxel size 2.00 × 2.04 × 2.04). A 2 mm full-width-at-half-maximum Gaussian filter was then applied.

The PET quantification was carried out using OsiriX MD 11.0 (Pixmeo, Bernex, Switzerland). The baseline and follow-up examinations were analyzed in parallel to ensure correct alignment between timepoints, however, the reader was blinded to the order of the examinations. The carotid arteries were identified and traced with freehand or ellipse regions of interest (ROIs) on the axial CT slices without use of the PET images. Afterwards, the ROIs were copied onto the spatially aligned PET examination. The carotids were traced from 2 cm proximal to the bifurcation and as distal as the internal carotid artery was identifiable on the non-contrast enhanced CT.

For each ROI, we quantified the DOTATATE uptake as the standardized uptake value (SUV) by measuring a maximum pixel activity value (SUVmax) and mean pixel activity (SUVmean). For sensitivity, we also calculated target-to-background ratio (TBR) as a ratio of SUVmax and the average blood SUV estimated from venous blood in the superior cava vein or the jugular vein.

2.4. [^{18}F]FDG PET/CT

Details of the procedure are described in [16]. In brief, [^{18}F]FDG-PET/CT imaging of the carotid arteries was undertaken two hours (±15 min) after injection of 4 MBq/kg 18F-FDG. Prior to injection of [^{18}F]FDG, patients fasted for six hours. Insulin-treated patients could eat a small meal up to two hours before the examination, however, fast-acting insulin was not to be taken in the two hours before the examination. Before injection of the [^{18}F]FDG, blood glucose was measured. Patients were instructed to rest lying in a bed between injection and scanning. There was no limit on exercise prior to the visit. Mean standardized uptake value (SUV) of [^{18}F]FDG was measured in the carotids using an approach similar to the [^{64}Cu]DOTATATE PET/CT. PET/CT images were analyzed by a masked, experienced reader.

2.5. Statistical Analysis

No formal power calculation was carried out for this substudy. The sample size for the entire study was based on the FDG PET/CT imaging. We aimed at including as many participants from the main study as possible and the number of participants in the substudy was determined by tracer availability at the time of inclusion in the main study. By chance, we ended up including 15 participants from each treatment group.

We present normal distributed data as the mean (standard deviation (SD)), continuous-scale non-normal distributed data (diabetes duration, urinary albumin creatinine ratio, high-sensitivity C-reactive protein (hsCRP)) as the median (interquartile range (IQR)) and categorical variables as numbers and percentages. An unpaired *t*-test and χ^2 test or Fisher's exact test, as appropriate, were used to find differences in clinical characteristics between treatment groups at baseline. The continuous-scale non-normal distributed variables were log2 transformed in all analyses. A paired *t*-test was used to compare baseline and end-of-treatment values within groups and an unpaired *t*-test to compare the change from baseline to end-of-treatment between treatment groups. We used linear regression to analyze the

correlation between [^{64}Cu]DOTATATE and [^{18}F]FDG PET uptake and provide R^2 to present the correlation between the two, and the F-test was applied to determine whether this relationship was statistically significant.

$p < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA).

3. Results

3.1. Patients

We included 30 participants in this substudy, 15 treated with liraglutide and 15 with placebo. Baseline characteristics (Table 1) were as follows: mean age (SD) 66.4 (7.2) years; median [IQR] diabetes duration 12.3 [5.7–19.8] years; mean HbA_{1c} 56.4 (9.2) mmol/mol; mean eGFR 85.8 (13.3) mL/min/1.73m^2; and 8 (26.7%) had a history of cardiovascular disease. Baseline characteristics were well balanced between the liraglutide and the placebo treated group, except for triglycerides, which were higher in the liraglutide group ($p = 0.01$). The baseline characteristics for the participants included in the substudy were comparable to the 72 participants only included in the main study (Table 2).

Table 1. Characteristics of the participants at baseline.

	Total (n = 30)	Liraglutide (n = 15)	Placebo (n = 15)	p Value
Sex (Women)	5 (16.7%)	2 (13.3%)	3 (20.0%)	1.0
Age (years)	66.4 (7.2)	65.9 (8.3)	66.9 (6.3)	0.69
Body mass index (kg/m^2)	28.9 (4.3)	29.5 (4.0)	28.2 (4.7)	0.41
Type 2 diabetes				
Known duration of DM (years)	12.3 [5.7–19.8]	13.9 [5.9–20.9]	8.8 [5.5–17.2]	0.43
HbA_{1c} (mmol/mol)	56.4 (9.2)	59.1 (10.4)	53.7 (7.0)	0.11
Kidney function				
Estimated glomerular filtration rate (mL/min/1.73 m^2)	85.8 (13.3)	86.2 (14.4)	85.4 (12.6)	0.88
Urinary albumin creatinine ratio (mg/g)	5.5 [4.5–12.5]	5.5 [2.5–12.5]	5.5 [5.0–14.5]	0.74
Cardiovascular risk factors				
Systolic blood pressure (mmHg)	134 (19)	133 (14)	136 (24)	0.68
LDL cholesterol (mmol/L)	2.2 (0.51)	2.2 (0.54)	2.1 (0.49)	0.70
Triglycerides (mmol/L)	1.7 (0.88)	2.1 (0.97)	1.3 (0.55)	0.01
Current smoker	1 (3.3%)	1 (6.7%)	0 (0%)	1.0
Hypertension	22 (73.3%)	12 (80.0%)	10 (66.7%)	0.68
High-sensitivity C-reactive protein (mg/L)	1.4 [0.9–3.1]	1.4 [1.0–3.3]	2.0 [0.8–2.8]	0.88
History of cardiovascular disease *	8 (26.7%)	6 (40.0%)	2 (13.3%)	0.21
Glucose-lowering medication				
Insulin use	12 (40.0%)	8 (53.3%)	4 (26.7%)	0.14
SGLT2 inhibitors	6 (20.0%)	3 (20.0%)	3 (20.0%)	1.0
Cardiovascular medication				
Aspirin treatment	10 (33.3%)	4 (26.7%)	6 (40.0%)	0.44
Lipid-lowering treatment	27 (90.0%)	14 (93.3%)	13 (86.7%)	1.0

Data are n (%), mean (SD) or median [IQR]. Hypertension was defined as treatment with anti-hypertensive medication. * A history of cardiovascular atherosclerotic disease was defined as a history of acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, stroke, peripheral arterial thrombosis, claudication and/or nitroglycerin-requiring angina pectoris. DM: diabetes mellitus, SGLT2: sodium glucose transporter 2.

Mean change in HbA_{1c} was −6.1 mmol/mol (95% confidence interval (CI) −10.1 to −2.1) in the liraglutide-treated group compared to −0.1 mmol/mol (−2.6 to 2.4) in the placebo group, resulting in a mean difference of −6.1 mmol/mol (−10.6 to −1.5) between groups ($p = 0.01$). Mean change in body weight was −3.0 kg (−4.7 to −1.2) in the liraglutide-treated group compared to −0.2 kg (−1.0 to 0.65) in the placebo group, resulting in a mean difference of −2.8 kg (−4.7 to −0.90) between groups ($p = 0.006$). Mean change in LDL cholesterol was −0.26 mmol/L (−0.48 to −0.05) in the liraglutide-treated group compared to −0.29 mmol/L (−0.50 to −0.07) in the placebo-treated group, with a mean difference

of 0.02 mmol/L (−0.27 to 0.31) between groups (p = 0.88). Mean change in systolic blood pressure was 6.7 mmHg (1.8 to 11.6) in the liraglutide-treated group compared to 3.1 mmHg (−4.2 to 10.4) in the placebo group, with a mean difference of 3.5 mmHg (−4.9 to 12.0) between groups (p = 0.40). The level of hsCRP was unchanged in both groups, with no difference between groups (p = 0.51).

Table 2. Participants in the substudy compared to participants only included in the main study.

	Included in Substudy (n = 30)	Not Included in Substudy (n = 72)	p-Values
Sex (Women)	5 (16.7%)	11 (15.3%)	1.0
Age (years)	66.4 (7.2)	66.4 (8.6)	1.0
Body mass index (kg/m^2)	28.9 (4.3)	30.3 (4.7)	0.15
Type 2 diabetes			
Known duration of DM (years)	12.3 [5.7–19.8]	10.6 [5.7–18.2]	0.30
HbA1c (mmol/mol)	56.4 (9.2)	57.0 [52.0-64.0]	0.20
Kidney function			
Estimated glomerular filtration rate (mL/min/1.73 m^2)	85.8 (13.3)	82.1 (17.3)	0.30
Urinary albumin creatinine ratio (mg/g)	5.5 [4.5–12.5]	6.3 [3.5–16.0]	0.30
Cardiovascular risk factors			
Systolic blood pressure (mmHg)	134 (19)	136 (17)	0.61
LDL cholesterol (mmol/L)	2.2 (0.51)	2.1 (0.73)	0.48
Triglycerides (mmol/L)	1.7 (0.88)	1.9 (1.1)	0.33
Current smoker	1 (3.3%)	13 (18.1%)	0.06
Hypertension	22 (73.3%)	57 (79.2%)	0.52
HsCRP (mg/L)	1.4 [0.9–3.1]	1.6 [0.86–4.2]	0.10
History of cardiovascular disease **	8 (26.7%)	15 (20.8%)	0.52
Glucose-lowering medication			
Insulin use	12 (40.0%)	27 (37.5%)	0.81
SGLT2 inhibitors	6 (20.0%)	14 (19.4%)	0.95
Cardiovascular medication			
Aspirin treatment	10 (33.3%)	27 (37.5%)	0.69
Lipid-lowering treatment ***	27 (90.0%)	61 (84.7%)	0.75

Data are n (%), mean (SD) or median [IQR]. Hypertension was defined as treatment with anti-hypertensive medication. ** A history of cardiovascular atherosclerotic disease was defined as a history of acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, stroke, peripheral arterial thrombosis, claudication and/or nitroglycerin-requiring angina pectoris. *** Lipid-lowering treatment defined as statins or ezetimibe. DM: diabetes mellitus, SGLT2: sodium glucose transporter 2.

3.2. [^{64}Cu]DOTATATE Uptake in Carotid Arteries

Vascular inflammation in the carotid arteries was evaluated as change in [^{64}Cu]DOTATATE uptake assessed as change in SUV_{mean} from baseline to end-of-treatment (Figures 1–3, Table 3). We observed a significant decrease in SUV_{mean} in the liraglutide-treated group (−0.11 [95% CI −0.19 to −0.03] p = 0.01) and no significant change in SUV_{mean} in the placebo group (−0.07 [−0.14 to 0.01], p = 0.08). The mean difference between change in SUV_{mean} in the two groups did not reach statistical significance (−0.04 [−0.15 to 0.07], p = 0.44). We confirmed these findings when we assessed [^{64}Cu]DOTATATE uptake as change in SUV_{max} (liraglutide-treated group: mean change in SUV_{max} −0.15 [−0.27 to −0.03] p = 0.02; placebo-treated group: mean change in SUV_{max} −0.09 [−0.20 to 0.02], p = 0.11; mean difference between groups: −0.06 [−0.22 to 0.10], p = 0.45). There was no difference between groups when assessing [^{64}Cu]DOTATATE uptake as change in TBR (liraglutide-treated group: mean change 0.20 [−0.05 to 0.46] p = 0.11; placebo-treated group: mean change 0.14 [−0.15 to 0.43], p = 0.32; mean difference between groups: 0.06 [−0.31 to 0.43], p = 0.73).

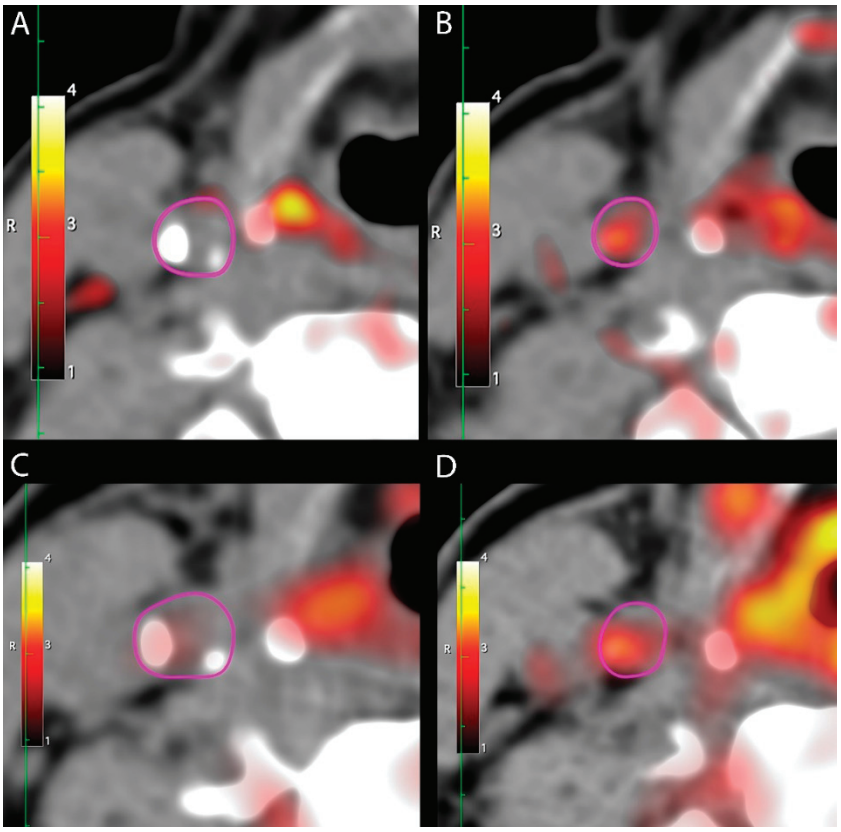

Figure 1. PET/CT images at baseline with high and low uptake of [^{64}Cu]DOTATATE. Representative baseline axial images showing the carotid artery outlined in pink. (**A**). An axial slice with calcifications and low uptake of [^{64}Cu]DOTATATE (SUV_{mean} = 0.9). (**B**) An axial slice with minimal calcifications and high uptake of [^{64}Cu]DOTATATE (SUV_{mean} = 1.4). (**C**,**D**) [^{18}F]FDG uptake in the same locations for comparison.

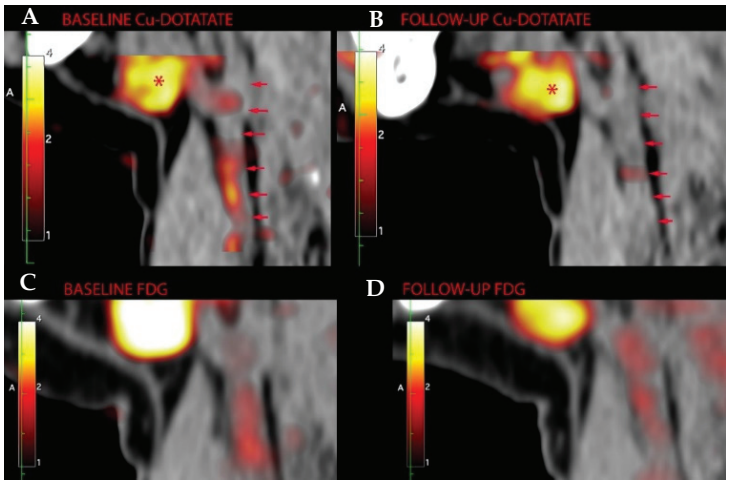

Figure 2. Sagittal [^{64}Cu]DOTATATE-PET/CT images from a participant treated with liraglutide. The left carotid artery is outlined at (**A**) baseline (SUV_{mean} = 1.2) and (**B**) follow-up examination (SUV_{mean} = 0.92). * Physiological uptake in submandibular gland. The lower panels show [^{18}F]FDG uptake from the same participant for comparison at (**C**) baseline and (**D**) follow-up.

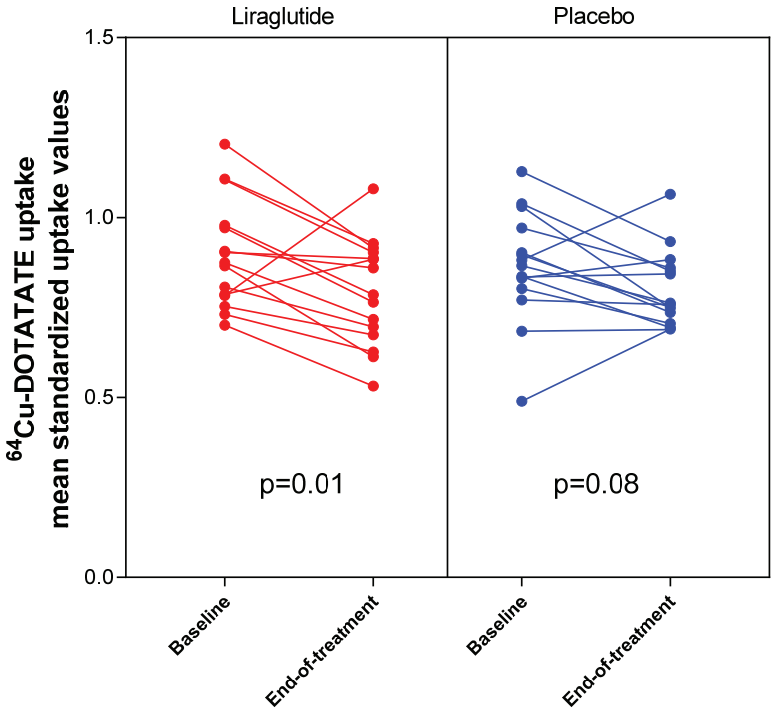

Figure 3. Effect of liraglutide and placebo on vascular inflammation evaluated with [^{64}Cu]DOTATATE PET in the carotid arteries. [^{64}Cu]DOTATATE uptake evaluated as mean standardized uptake values at baseline and end-of-treatment for the individual participants. Paired *t*-test was used to compare baseline and end-of-treatment values within groups.

Table 3. Carotid inflammation evaluated with PET in 30 persons with type 2 diabetes.

	Baseline Mean (SD)	End-of-Treatment Mean (SD)	*p* Value	Δ (95% CI)	*p* Value
[^{64}Cu]DOTATATE uptake					
Carotid SUV_{mean}					
Liraglutide, *n* = 15	0.90 (0.15)	0.79 (0.15)	0.01	−0.11 (−0.19; −0.03)	0.44
Placebo, *n* = 15	0.86 (0.15)	0.80 (0.11)	0.08	−0.07 (−0.14; 0.01)	
[^{18}F]FDG uptake					
Carotid SUV_{mean}					
Liraglutide, *n* = 15	1.4 (0.24)	1.4 (0.23)	0.28	−0.04 (−0.12; 0.04)	0.83
Placebo, *n* = 15	1.5 (0.20)	1.5 (0.21)	0.25	−0.06 (−0.16; 0.04)	

Vascular inflammation evaluated as mean standardized uptake values (SUV_{mean}). Data are mean (SD) or change (95% CI). Paired *t*-test for comparisons between baseline and end-of-treatment within groups and unpaired *t*-test for comparison of the change from baseline to end-of-treatment between the two groups.

3.3. [^{64}Cu]DOTATATE Uptake vs. [^{18}F]FDG Uptake in Carotid Arteries

The vascular inflammation in the carotid arteries evaluated as change in [^{18}F]FDG uptake from baseline to end-of-treatment was unchanged (SUV_{mean} in the liraglutide-treated group: −0.04 [95% CI −0.12 to 0.04], $p = 0.28$ and in the placebo group: −0.06 [−0.16 to 0.04], $p = 0.25$; without difference between groups 0.01 [−0.11 to 0.14], $p = 0.83$).

At baseline, the correlation between [^{64}Cu]DOTATATE and the [^{18}F]FDG uptake in the carotid arteries evaluated as SUV_{mean} was small and without statistical significance ($R^2 = 0.11$, $p = 0.07$, Figure 4). The change in uptake from baseline to end-of-treatment assessed with these two tracers was also not correlated ($R^2 = 0.06$, $p = 0.20$).

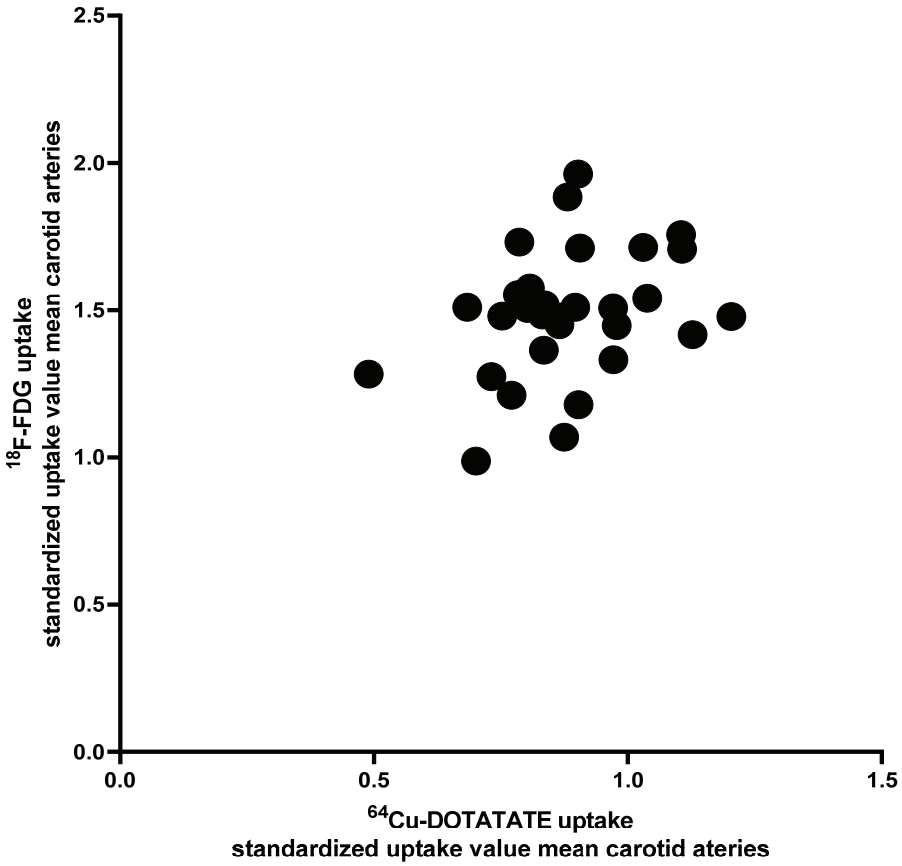

Figure 4. Correlation between [^{64}Cu]DOTATATE and [^{18}F]FDG PET uptake evaluated as mean standardized uptake value in the carotid arteries. Linear regression analysis revealed $R^2 = 0.11$ and no correlation between the two measures ($p = 0.07$, evaluated by F-test).

4. Discussion

In a substudy of a randomized placebo-controlled clinical trial, we demonstrated that vascular inflammation assessed using [^{64}Cu]DOTATATE was reduced over 26 weeks in the 15 participants treated with liraglutide. In the 15 participants receiving placebo, the [^{64}Cu]DOTATATE uptake was unchanged. However, the difference in vascular inflammation between the two treatment groups did not reach statistical significance, possibly due to lack of power. To the best of our knowledge, this is the first study to assess vascular inflammation using [^{64}Cu]DOTATATE in an intervention trial. Our findings hint at a mechanistic pathway, which could contribute to the cardiovascular benefit observed with GLP-1 RAs in outcome studies.

The interest in the effect of GLP-1 RAs on vascular inflammation is fueled by an increasing amount of preclinical and, to a lesser extent, clinical data that point toward an anti-inflammatory effect of these agents [18]. Preclinical data, primarily in mice, have demonstrated that GLP-1 RAs inhibit the formation of atherosclerotic plaques [12,19], suppress macrophage-derived foam cell formation in the plaques [14] and decrease the adhesion of mononuclear cells to the vessel wall [15]. This effect on atherosclerotic plaque formation requires the endothelial GLP-1 receptor [20] and seems partly independent of a reduction in weight and cholesterol [13]. Clinical data have demonstrated that liraglutide treatment reduces the levels of the inflammatory marker sCD163 and inflammatory macrophages [21] and one study demonstrated reduced inflammation in carotid plaques in patients treated with GLP-1-based therapy who had undergone endarterectomy [22].

As recently published, we observed no effect of liraglutide on arterial [^{18}F]FDG uptake in the main LiraFlame study including 102 participants [16]. [^{18}F]FDG PET has been successfully used to assess the impact of pharmaceutical interventions on arterial

inflammation in a number of clinical trials [2,23,24], and several studies over the last decade have shown that [^{18}F]FDG PET can be used to quantify arterial inflammation reliably and noninvasively [25–27]. Sadeghi et al. has reviewed a number of small animal and clinical studies that have investigated the biological link between arterial inflammation and the [^{18}F]FDG signal, and several of these studies have demonstrated a correlation between [^{18}F]FDG uptake and in vivo macrophage markers, but without indications that inflammation is the main determinant of arterial [^{18}F]FDG uptake [28]. This is supported by a recent study in minipigs demonstrating that [^{18}F]FDG uptake in medial smooth muscle cells was a significant contributor to the arterial [^{18}F]FDG signal [29]. Overall, the participants in the LiraFlame study had a low level of vascular inflammation at baseline, and therefore the specificity of the tracer could be detrimental in order to detect subtle changes in vascular inflammation induced by liraglutide.

The [^{64}Cu]DOTATATE tracer is a novel marker of vascular inflammation, and binds specifically to somatostatin receptor-2 expressed by activated inflammatory macrophages. This specificity potentially provides significant advantage for detecting subtle changes in vascular biology and therefore represents an interesting supplement to the [^{18}F]FDG tracer when evaluating effects on vascular inflammation [7]. Only a few studies have used somatostatin receptor imaging in atherosclerosis, and most have used the [^{68}Ga]DOTATATE tracer. The difference between labeling with ^{68}Ga and ^{64}Cu mainly relates to substantially better spatial resolution using ^{64}Cu due to the physical properties of the isotopes. When studying inflammation in small structures, such as the carotid artery wall, this difference in spatial resolution may be of utmost importance.

The two studies that have used the [^{64}Cu]DOTATATE tracer have demonstrated an association between [^{64}Cu]DOTATATE uptake and the Framingham risk score [5] and [^{64}Cu]DOTATATE uptake in carotid atherosclerotic plaques in patients undergoing endarterectomy and markers of macrophages measured ex vivo [6].

We observed that liraglutide reduced vascular inflammation assessed as [^{64}Cu] DOTATATE uptake, both when evaluated as SUVmean and SUVmax, but not as TBR. Even though the difference between the liraglutide- and placebo-treated group did not reach statistical significance, we consider this finding to be relevant in terms of explaining the cardiovascular protection observed with GLP-1 RAs. This is because (1) we revealed a signal in only 15 participants and (2) this signal was comparable to the signal observed in the main LiraFlame study, where the [^{18}F]FDG uptake was reduced in participants with a history of cardiovascular disease (n = 23). This subgroup also had a higher level of vascular inflammation at baseline, providing the possibility to detect a change in [^{18}F]FDG PET, whereas [^{64}Cu]DOTATATE PET with higher specificity could also sense a signal in persons with lower levels of vascular inflammation.

Given that the existing evaluation of [^{64}Cu]DOTATATE in human vascular inflammation is sparse, we also evaluated the correlation between the [^{64}Cu]DOTATATE and the [^{18}F]FDG uptake in the carotid arteries and observed only a small correlation without statistical significance, underscoring that uptake of these two tracers probably reflects different components of vascular inflammation.

The main strengths of this study are the randomized placebo-controlled design and the use of a novel high-resolution PET tracer to specifically quantify macrophages in vascular inflammation and their response to treatment. The primary limitation is the small number of included patients in the substudy. Another limitation is the patient population. Liraglutide has been shown to reduce cardiovascular events in patients with established cardiovascular disease, but in this study, we included an unselected type 2 diabetes population and only eight of the 30 participants had established cardiovascular disease.

5. Conclusions

In conclusion, our study demonstrated a significant decrease in [^{64}Cu]DOTATATE uptake in the carotid arteries in persons with type 2 diabetes treated with liraglutide.

However, the difference between the liraglutide- and placebo-treated group did not reach statistical significance. A potential reduction in vascular inflammation with liraglutide could help explain the cardiovascular protection observed with GLP-1 RAs in outcome studies but warrants further and larger studies.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the regional Ethics Committee (H-16044546 30 January 2017) and the Danish Medicines Agency (2016110109).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author. The data are not publicly available due to risk of re-identification.

Conflicts of Interest: E.H.Z. and V.R.C. declare no competing interest. A.K. has received consultancy fees from Novo Nordisk and is an inventor on a patent of ^{64}Cu-DOTATATE. R.R., B.J.v.S. and T.W.H. have shares in Novo Nordisk A/S and B.J.v.S. is now an employee of Novo Nordisk A/S. P.R. has received the following: consultancy and/or speaking fees (to Steno Diabetes Center Copenhagen) from Astellas, AstraZeneca, Bayer, Boehringer Ingelheim, Eli Lilly, Gilead, MSD, Merck, Novo Nordisk, Vifor and Sanofi Aventis; research grants to the institution from AstraZeneca and Novo Nordisk. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Paper 12

BMJ Open Diabetes Research & Care

Ceramides and phospholipids are downregulated with liraglutide treatment: results from the LiraFlame randomized controlled trial

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ABSTRACT

Introduction Treatment with glucagon-like peptide-1 receptor agonists (GLP-1 RAs) can reduce risk of cardiovascular disease (CVD) in persons living with type 2 diabetes, however the mechanisms explaining this cardiovascular benefit are still debated. We investigated changes in the plasma lipidome following treatment with the GLP-1 RA liraglutide.

Research design and methods In a double-blind placebo-controlled trial, we randomized 102 persons with type 2 diabetes to liraglutide or placebo for 26 weeks. Fasting blood plasma was collected at baseline and at end-of-treatment. The lipidome was measured using liquid-chromatography-coupled mass-spectrometry as a secondary end point in the study. Treatment response of each lipid was tested with lipid-specific linear mixed-effect models comparing liraglutide with placebo. Bonferroni p<7.1e-03 was employed. The independence of the findings from clinical covariates was evaluated with adjustment for body mass index, HbA_{1c}, fasting status, lipid-lowering treatment and change in lipid-lowering treatment during the trial.

Results In total, 260 lipids were identified covering 11 lipid families. We observed significant decreases following liraglutide treatment compared with placebo in 21 lipids (p<7.1e-03) from the following lipid families: ceramides, hexocyl-ceramides, phosphatidylcholines, phosphatidylethanolamines and triglycerides. We confirmed these findings in adjusted models (p≤0.01). In the liraglutide-treated group, the individual lipids were reduced in the range of 14%–61% from baseline level, compared with 19% decrease to 27% increase from baseline level in the placebo group.

Conclusions Compared with placebo, liraglutide treatment led to a significant downregulation in ceramides, phospholipids and triglycerides, which all are linked to higher risk of CVD. These findings were independent of relevant clinical covariates. Our findings are hypothesis generating and shed light on the biological mechanisms underlying the cardiovascular benefits observed with GLP-1 RAs in outcome studies, and further strengthen the evidence base for recommending GLP-1 RAs to prevent CVD in type 2 diabetes.

Trial registration number NCT03449654.

Significance of this study

What is already known about this subject?

- Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) can reduce the high risk of cardiovascular disease in persons living with type 2 diabetes.
- The mechanism behind this effect is largely unknown.

What are the new findings?

- This is the first randomized placebo-controlled clinical trial to assess GLP-1 RAs' effect on the plasma lipidome using untargeted liquid-chromatography-coupled mass-spectrometry.
- The present results suggested that treatment with the GLP-1 RA liraglutide led to a downregulation in ceramides, phospholipids and triglycerides.
- Ceramides, phospholipids and triglycerides are lipids linked to risk of cardiovascular disease.

How might these results change the focus of research or clinical practice?

- These results are hypothesis generating and may inspire research that aims to understand the cardiovascular mode-of-action of GLP-1 RAs to further investigate GLP-1 RAs' lipid-regulating effect.

INTRODUCTION

Recent outcome studies have demonstrated that treatment with glucagon-like peptide-1 (GLP-1) receptor agonists can reduce the high risk of cardiovascular disease in persons living with type 2 diabetes.[1–4] To date, there is no convincing evidence that points to a single mechanism as dominant for this risk reduction.[5]

Favorable within-trial changes have been demonstrated in traditional cardiovascular risk factors including glycemia, body weight, systolic blood pressure, urinary albumin-to-creatinine ratio and low-density lipoprotein (LDL)-cholesterol. A recent analysis of The

Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results trial evaluated these cardiovascular risk factors as potential mediators of the effect of the GLP-1 receptor agonist liraglutide on major cardiovascular events.[6] The analysis indicated potential mediation by HbA_{1c} and to a lesser extent by urinary albumin-to-creatinine ratio. However, the authors concluded that HbA_{1c} was unlikely to be a true mediator based on the totality of evidence including other cardiovascular outcome trials, where the choice of therapies used to reduce HbA_{1c}, and not just HbA_{1c} reduction by itself, have important impact on cardiovascular outcomes.[6]

A mediation analysis can only evaluate candidate mediators that were measured during the trial. LDL-cholesterol was not found to be a mediator, but the lipid metabolism associated with type 2 diabetes and cardiovascular disease is complex, and other lipids play direct roles in development of atherosclerosis. With new lipidomic technologies using mass spectrometry, it is now achievable to study the whole range of lipids (lipidome) in a biological system.[7] Lipidomics has been applied to discover new disease biomarkers and to unravel mechanisms and pathways underlying various diseases and therapies.

In a randomized, double-blind, placebo-controlled trial, we analyzed the lipidome before and after treatment with the human GLP-1 receptor agonists liraglutide as a secondary end point. Our aim was to explore downstream effects of liraglutide on lipid metabolism that could link to the cardiovascular benefit.

RESEARCH DESIGN AND METHODS

Study design and participants

The trial was conducted between October 26, 2017 and August 16, 2019 at the Steno Diabetes Center Copenhagen. We included men and women with type 2 diabetes in a randomized, double-blind, placebo-controlled, parallel-group trial. Participants were recruited from the outpatient clinic at the Steno Diabetes Center Copenhagen and through newspaper advertisements.

Inclusion criteria were as follows: age >50 years; HbA_{1c} ≥ 48 mmol/mol (6.5%); estimated glomerular filtration rate ≥30 mL/min/1.73 m^2 (estimated by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula); stable glucose-lowering and cholesterol-lowering treatment (minimum 4 weeks). Key exclusion criteria were: type 1 diabetes; chronic or previous acute pancreatitis; other treatment (90 days prior to screening) which in the investigator's opinion could interfere with the effect of liraglutide (including oral glucocorticoids, calcineurin inhibitors, dipeptidyl peptidase 4 inhibitors, GLP-1 receptor agonists); cancer or disorders (except for conditions associated with type 2 diabetes history), which could interfere with the results of the trial; clinical signs of diabetic gastroparesis; previous bowel resection; impaired liver function; inflammatory bowel disease; weight >150 kg (full list see online supplemental materials).

The primary end point of the trial was change in vascular inflammation assessed using ^{18}F-fluorodeoxyglucose PET/CT,[8] and here we report results from the secondary end point of change from baseline to week 26 in the lipidome for the liraglutide-treated group compared with placebo. The protocol for this analysis was developed before the last participant was enrolled in the study.

Randomization and masking

All participants were randomized in a 1:1 ratio to daily subcutaneous injections of liraglutide or matching placebo for 26 weeks. All investigators and participants were blinded to treatment allocation. Treatment pens and computer-generated allocation sequence were provided by Novo Nordisk (Bagsvaerd, Denmark) and two clinicians not otherwise involved in the trial handled the treatment allocation. EHZ enrolled participants and assigned participants to interventions.

Procedures

Starting dose was 0.6 mg/day and was escalated to 1.2 mg/day after 1 week and to 1.8 mg/day after 2 weeks. Maintenance-dose was 1.8 mg/day. We allowed the dose escalation to be flexible in order to reach the maximum tolerated dose for each participant. Participants were instructed to be fasting for 4 hours prior to the study visits. Blood samples were collected at randomization and after 26 weeks treatment and were stored at −80°C until analysis, which was 4 months after last patient last visit.

Lipid quantification

Plasma samples were prepared and analyzed at the Steno Diabetes Center Copenhagen. Lipids were extracted using a modified Folch extraction method[9] and were analyzed by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Samples were analyzed in a randomized order, with QC pooled plasma samples. Details can be found in the online supplemental materials.

The acquired data were preprocessed using MZmine 2 (V.2.28)[10] and postprocessed in R V.3.5.1 (R Core Team, Vienna, Austria) by normalization to internal standards, batch correction, truncation of outliers, imputation of missing values and $\log_{10}$ transformation.[11]

Statistical analysis

The lipidome analysis was prespecified and hypothesis generating. The sample size was a consequence of the sample size of the main study and no specific sample size estimation was done for this substudy.

Clinical data were assessed with SAS software (V.9.4; SAS Institute, Cary, North Carolina, USA). All data analysis and visualization related to the lipidome was done with R (V.3.5.1; R Core Team).

For the clinical data, continuous variables are given as means with SD, non-normally distributed variables are

Table 1 Characteristics of the participants at baseline

	Total (n=102)	Liraglutide (n=51)	Placebo (n=51)	P value
Sex (woman)	16 (15.7%)	6 (11.8%)	10 (19.6%)	0.28
Age (years)	66.4 (8.2)	65.9 (8.6)	66.9 (7.8)	0.56
Body mass index (kg/m^2)	29.9 (4.6)	30.5 (5.3)	29.3 (3.8)	0.16
Type 2 diabetes				
Known duration in years	10.9 (5.7; 18.2)	12.2 (5.4; 18.2)	10.2 (5.7; 19.2)	0.65
HbA_{1c} (mmol/mol)	58.4 (10.1)	58.7 (9.6)	58.0 (10.6)	0.73
Kidney function				
Estimated glomerular filtration rate (mL/min/1.73 m^2)	83.2 (16.3)	82.7 (17.6)	83.7 (15.0)	0.75
Urinary albumin-to-creatinine ratio (mg/g)	6.0 (3.5–14.5)	6.0 (3.5–15)	6.0 (3.5–14.5)	0.65
Cardiovascular risk factors				
Systolic blood pressure (mm Hg)	135 (17)	133 (15)	137 (20)	0.25
Diastolic blood pressure (mm Hg)	79 (8)	80 (7)	79 (8)	0.57
Total cholesterol (mmol/L)	4.1 (0.81)	4.1 (0.80)	4.1 (0.82)	0.85
LDL-cholesterol (mmol/L)	2.1 (0.67)	2.1 (0.72)	2.1 (0.62)	0.48
HDL-cholesterol (mmol/L)	1.2 (0.36)	1.2 (0.38)	1.3 (0.34)	0.14
Triglycerides (mmol/L)	1.8 (1.0)	2.1 (1.2)	1.6 (0.78)	0.01
Current smoker	14 (13.7%)	10 (19.6%)	4 (7.8%)	0.08
History of cardiovascular event				
Myocardial infarction	13 (12.8%)	8 (15.7%)	5 (9.8%)	0.37
Stroke	6 (5.9%)	3 (5.9%)	3 (5.9%)	1.00
Peripheral arterial thrombosis	2 (2.0%)	2 (3.9%)	0 (0.0%)	0.50
History of cardiovascular symptoms				
Claudication	4 (3.9%)	3 (5.9%)	1 (2.0%)	0.62
Angina pectoris	5 (4.9%)	4 (7.8%)	1 (2.0%)	0.36
Any cardiovascular disease	23 (22.6%)	14 (27.5%)	9 (17.7%)	0.24
Glucose-lowering medication				
Insulin use	39 (38.2%)	20 (39.2%)	19 (37.3%)	0.84
SGLT2 inhibitors	20 (19.6%)	8 (15.7%)	12 (23.5%)	0.32
Cardiovascular medication				
Aspirin treatment	37 (36.3%)	16 (31.4%)	21 (41.2%)	0.30
Statins and/or ezetimibe	88 (86.3%)	46 (90.2%)	42 (82.4%)	0.25
Fibrates	1 (1.0%)	1 (2.0%)	0 (0.0%)	1.00

Adapted from Ripa *et al*.[8] Data are n (%), mean (SD) or median [IQR]. Differences in baseline characteristics between the liraglutide and the placebo group were tested using unpaired t-test and the $\chi 2$ test.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; SGLT2, sodium glucose transporter 2.

summarized as medians with IQR and categorical variables are reported as percentages. Unpaired t-test and χ^2 test was used to test differences in clinical characteristics between the two groups at baseline, and unpaired t-test was also used to compare change from baseline to end-of-treatment in HbA_{1c}, weight and clinical lipid measurements between the two groups.

To identify which lipid levels changed in response to treatment, linear mixed-effect models were built for each individual lipid species to explain observed standardized levels with time, treatment and their interaction as fixed effects and participant ID as random effect. A full list of lipids, estimated coefficients and p values was reported. Lipid class-level changes were assessed with the same approach of modeling on the average level in each lipid class. The independence of the findings from clinical covariates was evaluated with adjustment for body mass index (BMI), HbA_{1c}, fasting status, lipid-lowering treatment and change in lipid-lowering treatment during the trial by adding each of these covariates as additional independent variables to the linear mixed-effect model. Using similar approach, we evaluated the independence of the findings from the baseline measurement of total triglycerides.

Models were fitted using the limma[12] and lme4 packages.[13] Nominal associations at p<0.05 were reported

for exploratory associations at the lipid class level also reported as family level. Bonferroni p<7.1e-03 was employed for the single lipid linear mixed-effect models. Results were visualized as a lipidome-wide heatmap using the LipidomeR package.[14]

Selected lipid species (n=21) with a response to the treatment in the linear mixed-effect models were investigated further: for each lipid species, relative change from baseline to end-of-treatment in each of the two arms was calculated. The result was visualized as a diverging bar graph with the ggplot2 package.[15] Baseline lipid levels were compared between participants with and without a history of cardiovascular disease. Standardized mean difference of the selected lipids was calculated using the 'effsize' package.[16] Linear regression was used to investigate associations between the selected individual lipids and sex, age, BMI, HbA_{1c} and lipid-lowering treatment at baseline. Mediation analysis was performed with linear regression models, the significance and the tested mediation effect was calculated with the 'mediation' package in R,[17] using bootstrapping to simulate 500 samples.

Data availability statement

The datasets analyzed during the current study are not publicly available due to the risk of patient re-identification. De-identified participant data or anonymized clinical study reports can be obtained from the first author on reasonable request. Necessary data protection agency and ethical committee approvals must be provided in compliance with relevant legislation.

RESULTS

Participants

The study included 102 participants, and all were eligible for lipidomic analysis. Five participants did not have blood samples available for lipidomic analysis at end-of-treatment: one participant randomized to liraglutide dropped out of the study after experiencing gastrointestinal side effects, and one participant randomized to liraglutide discontinued the study treatment due to gastrointestinal side effects, remained in the study, but did not wish to have blood samples taken at the end-of-treatment visit. Three participants randomized to placebo did not have blood samples taken at end-of-treatment: one dropped out of the study due to concurrent cancer disease, one discontinued study treatment after experiencing shoulder pain in relation to the first study visit, remained in the study, but did not wish to have blood samples taken at the end-of-treatment visit, and one did not show up for the visit where the end-of-treatment blood samples were taken due to work-related stress. These five participants were only included in the baseline analysis.

The population included 16 (15.7%) women and the mean (SD) age was 66 (8.2) years, median (IQR) known diabetes duration was 10.9 (5.7; 18.2) years, HbA_{1c} 58.4 (10.1) mmol/mol and BMI 29.9 (4.6) kg/m^2. The baseline clinical characteristics of the 51 participants receiving liraglutide and the 51 receiving placebo were balanced (table 1), except for triglycerides (mean (SD): liraglutide 2.1 (1.2) vs placebo 1.6 (0.78) mmol/L, p=0.01). A history of cardiovascular disease (defined as history of acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, stroke, peripheral arterial thrombosis, claudication and/or nitroglycerin requiring angina pectoris) was reported in 23 (22.6%) participants at baseline (balanced between groups, p=0.24).

Mean changes (95% CI) over the 26 weeks for the liraglutide-treated group compared with placebo in body weight were −3.7 (−4.8 to −2.6) vs −0.18 (−0.76 to 0.40) kg (p<0.0001); and HbA_{1c} −5.1 (−8.0 to −2.0) vs −0.08 (−1.9 to 1.7) mmol/mol (p=0.006).[8] Mean changes (95% CI) for the liraglutide-treated group compared with placebo for clinical lipid measurements were as follows: LDL-cholesterol 0.06 (−0.17 to 0.30) vs −0.08 (−0.29 to 0.13) mmol/L (p=0.37);[8] HDL-cholesterol 0.06 (0.004 to 0.12) vs 0.05 (−0.02 to 0.11) mmol/L (p=0.72); total cholesterol 0.08 (−0.18 to 0.35) vs −0.03 (−0.26 to 0.21) mmol/L (p=0.54); triglycerides −0.11 (−0.44 to 0.21) vs 0.01 (−0.19 to 0.21) mmol/L (p=0.52).

Lipids reduced by liraglutide treatment compared with placebo

In total, 260 lipids were identified (figure 1, online supplemental table S1) covering 11 lipid families including ceramides, diglycerides, hexocyl-ceramides, lactosyl ceramides, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositoles, sphingomyelins and triglycerides. Of the 260 lipids, 21 were significantly decreased after liraglutide treatment compared with placebo (figure 1, online supplemental table S1). These 21 lipids were from the following lipid families: ceramides, hexocyl-ceramides, phosphatidylcholines, phosphatidylethanolamines and triglycerides. We observed a pattern with noticeably reduction in short ceramides and large, poly-unsaturated triglycerides (figure 1). The size of the reductions was ranging from 14% to 61% from baseline level, in the liraglutide-treated group, compared with 19% decrease to 27% increase from baseline level in the placebo group, as visualised in figure 2. We confirmed the reduction in these 21 individual lipids in models adjusted for BMI, HbA_{1c}, fasting state (4 were not fasting for baseline measurements, and 3 were not fasting for end-of-treatment measurements), lipid-lowering treatment at baseline (yes or no) and change in lipid-lowering treatment during the study period (n=9) (p≤0.01).

To provide a context for the 21 lipids that changed significantly in the liraglutide-treated group, we examined how they were related to selected baseline characteristics. At baseline, there were few correlations between these 21 individual lipids and sex, age, BMI, HbA_{1c} and lipid-lowering treatment (online supplemental table S2). The baseline levels of these 21 individual lipids were

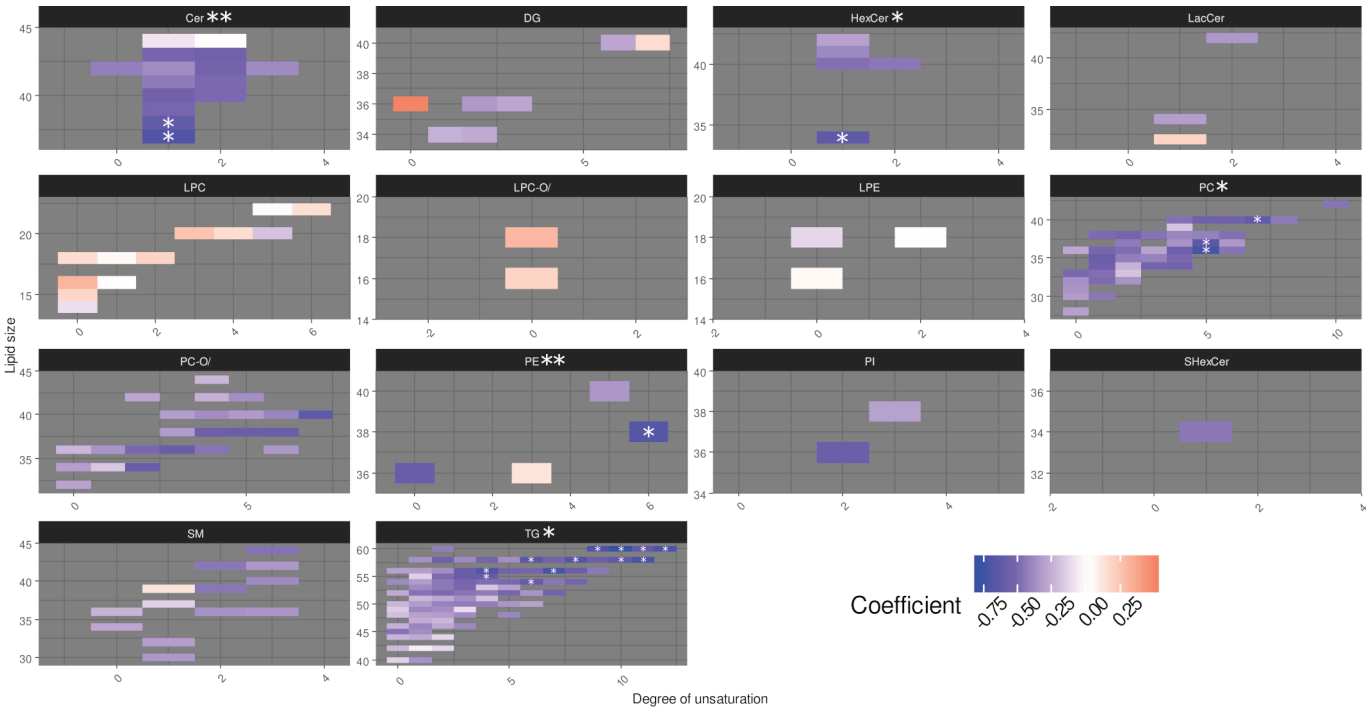

Figure 1 Lipidome-wide responses to liraglutide treatment compared with placebo. Lipid species grouped in panels according to lipid families (title of each panel). X-axis is the number of fatty-acid double bonds for the specific species (level of unsaturation) and Y-axis is the number of carbon atoms (total fatty acid chain length), and therefore each cell in the heat maps represents one lipid species (one individual lipid). Color indicates the coefficients from lipid-specific linear mixed-effect models. Blue colors represent lipids that are reduced in level by liraglutide treatment compared with placebo. Red colors represent lipids that are increased in level by liraglutide treatment compared with placebo. *p<0.05; **p<0.01. Cer, ceramides; DG, diglycerides; HexCer, hexocyl-ceramides; LacCer, lactosyl-ceramides; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositoles; SM, sphingomyelins; TG, triglycerides.

generally higher in participants with versus without a history of cardiovascular disease (figure 3).

We also analysed changes on a lipid family level for the liraglutide-treated group compared with placebo, and observed significant decreases in 5 of the 11 lipid families namely ceramides (26 weeks average reduction liraglutide vs placebo: −27% vs 8%, p=0.01), hexocyl-ceramides (−28% vs −10%, p=0.02), phosphatidylcholines (−5% vs 2%, p=0.04), phosphatidylethanolamines (−14% vs 7%, p=0.004) and triglycerides (−27% vs 14%, p=0.03).

The primary end point of the LiraFlame trial, change in vascular inflammation assessed using ^{18}F-fluorodeoxyglucose PET/CT, was unchanged.[8]

Sensitivity analysis

In a sensitivity analysis, we adjusted for the clinical measurement of total triglycerides at baseline, since this level was not balanced between the two groups, despite randomization. The change in the 21 lipid levels in the liraglutide-treated group remained significant compared with placebo after this adjustment, the highest adjusted p value was 0.009 (PE(O-34:2)/PE(P-34:1)).

Moreover, we performed a sensitivity analysis, where zero change from baseline to end-of-treatment was assumed for the five participants with lipidomics data missing at end-of-treatment. Overall results were similar, but six single lipids (TG(58:10); TG(60:11); Cer(d38:1); TG(54:6); TG(55:4) and PE(O-34:2)/PE(P-34:1)) were no longer significantly reduced by liraglutide treatment compared with placebo after multiple adjustment (online supplemental table S3).

Mediation analysis

In a mediation analysis including the top 10 most associated lipids, we tested if the observed effect of liraglutide on these lipids were mediated through change in BMI or HbA_{1c}. None of the p values for mediation effect were significant (p≥0.06; online supplemental table S4).

DISCUSSION

In this randomized clinical trial, we demonstrate that individual ceramide and phospholipid lipid species were reduced in the liraglutide-treated group compared with placebo. In addition, we confirmed a reduction in individual triglyceride lipid species in the liraglutide-treated group and we were able to demonstrate that this was primarily a downregulation of large, poly-unsaturated triglycerides. In support of this, when analyzing the lipid species as lipid families, we observed significant reductions in the ceramide, hexocyl-ceramide, phosphatidylcholine, phosphatidylethanolamine and triglyceride

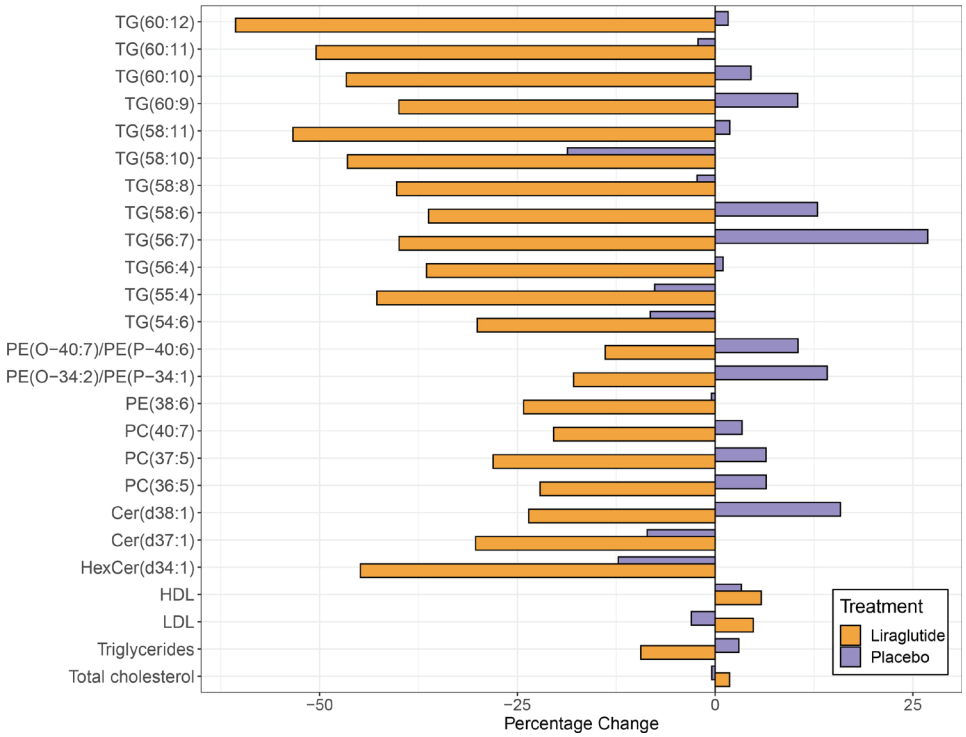

Figure 2 Percentage change after 26 weeks in average amount of individual lipids and in the traditional clinical lipid measurements following liraglutide and placebo treatment. Included are the 21 individual lipids significantly reduced by liraglutide compared with placebo and the traditional clinical lipid measurements, including HDL-cholesterol, LDL-cholesterol, triglycerides and total cholesterol. The traditional clinical lipid measurements were not significantly reduced by liraglutide compared with placebo treatment. Cer, ceramides; HDL, high-density lipoprotein; HexCer, hexocyl-ceramides; LDL, low-density lipoprotein; PC, phosphatidylcholines; PE, phosphatidylethanolamines; TG, triglycerides.

lipid family in the liraglutide-treated group compared with placebo. Moreover, mediation analyses indicated that the lipid-regulating effect of liraglutide was likely not mediated by change in BMI or HbA_{1c}.

Ceramides, phospholipids and triglycerides are lipids identified as biomarkers—and potential causes—of cardiovascular disease. Given the robustness and magnitude of the reduction in these pro-atherogenic biomarkers observed, we consider that our findings are interesting, and that liraglutides effect on lipids should be explored further and could improve our understanding of the mechanisms explaining the cardiovascular protection observed with human GLP-1 receptor agonists.

We investigated 260 lipids of which 21 was significantly reduced in the group treated with liraglutide compared with placebo. In the discussion, we focus only on the lipids that changed.

Liraglutide, ceramides and atherosclerosis: a pathway to reduced risk of cardiovascular disease?

As a novel finding, we demonstrated that ceramide levels were reduced in the group treated with liraglutide. Ceramides are a class of bioactive lipids implicated in cardiovascular disease and with interesting similarities to LDL-cholesterol. Ceramides are present in the serum in much lower concentrations than cholesterol (approximately 1/1000) and can be measured with sensitive techniques such as mass spectrometry. Like cholesterol, ceramides (the backbone of all sphingolipids) accumulate in atherosclerotic lesions.[18] Experimental data have revealed how ceramides are formed at the surface of atherogenic lipoproteins via sphingomyelinase activity.[19 20] An increased ceramide content promotes lipoprotein aggregation, which in turn promotes the subendothelial retention or trapping of lipoproteins within the vessel wall, a key event in early atherogenesis.[19 20] Ceramides are involved in the transcytosis of lipoproteins across the endothelium[21] and are further perceived as an important second messenger in several aspects of the inflammatory process.[18] Several studies have demonstrated that higher circulating levels of ceramides are risk factors for future cardiovascular events in apparently healthy individuals, in individuals with known coronary artery disease as well as in persons with diabetes.[22–28] Ceramides were recently reported as part of the Cardiovascular Event Risk Test (CERT) and the derivative CERT2 risk-score developed and validated in large cohorts by Hilvo *et al* as a simple cardiovascular risk estimation score based on ceramides and phospholipids.[29 30]

In $ApoE^{-/-}$ mice and rabbits fed a high fat and cholesterol diet, inhibiting the biosynthesis of ceramides ameliorates atherosclerosis and arterial stiffness.[31 32] Evidence that therapeutic interventions can reduce ceramides in humans is sparse. The PREDIMED trial recently revealed a strong association between ceramides, and risk

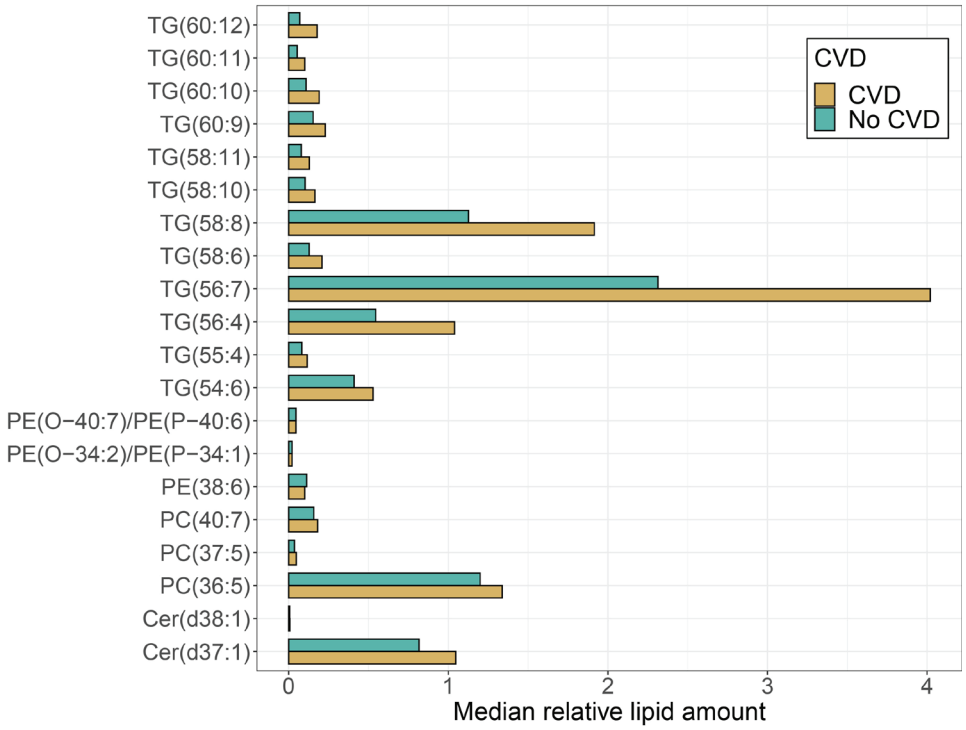

Figure 3 Baseline level of individual lipids for participants with versus without a history of cardiovascular disease. Shown are selected lipids that were significantly reduced by liraglutide compared with placebo treatment for participants with (n=23) vs without a history of cardiovascular disease (CVD). Levels are $\log_{10}$ transformed. Levels were generally higher for participants with a history of cardiovascular disease, although none of the differences were significant. A history of cardiovascular disease was defined as a history of acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, stroke, peripheral arterial thrombosis, claudication and/or nitroglycerin requiring angina pectoris. Cer, ceramides; PC, phosphatidylcholines; PE, phosphatidylethanolamines; TG, triglycerides.

of cardiovascular disease that was mitigated by a Mediterranean dietary intervention.[33]

Liraglutide and phospholipids

We report significant reductions for the liraglutide-treated group compared with placebo in single-lipid phospholipids from the two lipid families phosphatidylethanolamines and phosphatidylcholines. In comparisons with ceramides, the body of literature that links phospholipids to cardiovascular disease is limited, but emerging. A study in a case-cohort (n=3779) subset from the Action in Diabetes and Vascular Disease: Preterax and Diamicron-MR trial demonstrated that higher levels of phospholipids among other lipids were associated with risk of cardiovascular morbidity and mortality[28] and as already mentioned, phospholipids were part of the recently published CERT2 cardiovascular risk estimation score.[29]

Liraglutide and large triglycerides

Others have demonstrated, before us, that liraglutide lowers the triglyceride level measured as a traditional clinical lipid.[5 34 35] We identified 88 distinct triglyceride lipid species, which enabled us to add new information that especially large, poly-unsaturated triglycerides were reduced in the liraglutide-treated group. The precise mechanism behind liraglutide's effect on triglycerides has yet to be determined. One hypothesis that has been put forward is that GLP-1 receptor signaling mediates a decreased secretion of apoB48-containing chylomicron particles in the intestinal mucosa, which subsequently reduces the intestinal absorption of triglycerides.[34 36]

The overall size of triglyceride reduction with liraglutide has been reported as modest when triglycerides were measured as a traditional clinical lipid.[5 35 37] Our data indicate that this modest reduction could cover more pronounced reductions in large, poly-unsaturated triglycerides together with less pronounced effects on other triglyceride lipid species. Our findings were independent of changes in BMI and thus weight changes cannot explain this finding.

It remains controversial that triglyceride reduction could translate into a substantial reduced cardiovascular risk. A long-standing association exists between triglycerides and cardiovascular disease in observational studies, but, whether the triglyceride level is a risk marker for development of cardiovascular disease or more directly promote cardiovascular disease has been discussed for decades.[38] Mendelian randomization studies support a casual role of triglycerides for risk of cardiovascular disease.[39] The many randomized clinical trials using medications that lower triglycerides, such as fibrates, niacin and omega-3 fatty acids, have failed to show conclusive evidence of further cardiovascular risk reduction after LDL-cholesterol levels were 'optimally

controlled'.[40] However, a positive outcome has recently been reported in the Reduction of Cardiovascular Events with Icosapent Ethyl–Intervention Trial (REDUCE-IT).[41] In REDUCE-IT, the omega-3 fatty acid product icosapent ethyl reduced the cardiovascular risk regardless of the triglyceride level attained, contributing to the ongoing debate whether triglyceride lowering per se reduces the cardiovascular risk and pointing out to the importance of determining single lipid biochemistry.

Strengths and limitations

Our study is hypothesis generating. Only similar analysis of a cardiovascular outcome trial could provide evidence that liraglutide prevents cardiovascular events by decreasing ceramides and phospholipids. By using lipidomics we were able to investigate liraglutide's possible effect on a broad panel of lipid families and individual lipids. The strengths of our study include the double-blinded, randomized, placebo-controlled design and the robust technology applied to measure a comprehensive panel of lipids. Participants were instructed to fast for 4 hours. We can speculate that 12–14 hours overnight complete dietary restriction would give lower overall levels of the lipids measured, including triglycerides, but as we are measuring changes from baseline within individuals, we do not think this impacted our findings. Despite the randomized design, we observed a higher baseline triglyceride level in the liraglutide-treated group compared with placebo. As we are measuring changes from baseline within individuals, we do not think this impacts our findings for the single-lipid triglycerides, but we cannot rule out that it is easier to clear more triglycerides with higher levels. We confirmed our findings in a sensitivity analysis, adjusted for the clinical measurement of total triglycerides at baseline. Lipidomics is a tool to study fingerprints with hundreds of lipids but currently it is platform-dependent and does not detect all the lipids in the lipidome. Other relevant variables (ie, glycemic control, body weight) were improved in the liraglutide-treated group, and therefore it is not possible to assume a direct relationship between the observed changes in the single lipids and liraglutide therapy. In order to evaluate if the effect on the lipidome is a direct effect of liraglutide, or secondary to weight loss or improved glycemic control following liraglutide treatment, an active comparator should have been included (ie, insulin as a comparator for liraglutides effect on glycemic control or diet-induced weight loss as a comparator for liraglutides effect on body weight).

Conclusion

Ceramides, phospholipids and triglycerides were downregulated in the group treated with liraglutide compared with placebo. Lipids which all are linked to risk of cardiovascular disease. This lipid-regulating effect of liraglutide should be examined further and may contribute to the cardiovascular benefits observed in outcome studies.

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Contributors EHZ, AW, RSR, BJvS, TS, TWH, AK, CL-Q and PR contributed to study design and data interpretation. EHZ recruited participants to the study. EHZ, VRC and TWH contributed to running of the study and data collection. AW performed the lipidomics sample analysis. AW and TS processed and analyzed the lipidomics data. AW, TS and CL-Q interpreted the lipidomics data. EHZ performed statistical analysis of the clinical data. EHZ drafted the manuscript and the final version was critically reviewed and approved by all authors. Guarantor statement: EHZ is the guarantor of the work, and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Competing interests AW, VRC, TS and CL-Q declares no competing interests. AK has received consultancy fees from Novo Nordisk. RSR, BJvS, TWH and PR have shares in Novo Nordisk and BJvS and EHZ is now an employee of Novo Nordisk, but work related to this article was conducted while EHZ was employed by Steno Diabetes Center Copenhagen. PR has received the following: consultancy and/or speaking fees (to Steno Diabetes Center Copenhagen) from AbbVie, Astellas, AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, MSD, Novo Nordisk and Sanofi Aventis; research grants to institution from AbbVie, AstraZeneca and Novo Nordisk.

Patient consent for publication Not required.

Ethics approval The study was approved by the local ethics committee (H-16044546) and the Danish Medicines Agency (2016110109) and was performed in compliance with the principles of the Declaration of Helsinki.

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Paper 13

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ORIGINAL ARTICLE

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The importance of addressing multiple risk markers in type 2 diabetes: Results from the LEADER and SUSTAIN 6 trials

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Abstract

Aims: To investigate to what extent multiple risk marker improvements confer lower risk of cardiovascular and kidney complications in a contemporary type 2 diabetes population.

Materials and methods: Post-hoc analysis of the LEADER (n = 8638; median follow-up 3.8 years) and SUSTAIN 6 (n = 3040; median follow-up 2.1 years) cardiovascular outcome trials. Participants were those with baseline and year-1 assessment of at least one of the parameters of interest; we pooled the liraglutide-/semaglutide- and placebo-treated groups and categorized them by number of risk markers with clinically relevant improvements after 1 year of study participation. We investigated risk of major adverse cardiovascular events (MACE), expanded MACE, cardiovascular death and nephropathy. Predefined clinically relevant changes: body weight loss ≥5%; reductions in: glycated haemoglobin ≥1%, systolic blood pressure ≥5 mmHg and low-density lipoprotein cholesterol ≥0.5 mmol/L; estimated glomerular filtration rate change ≥0 ml/min/1.73 m^2 and urinary albumin-to-creatinine ratio change ≥30% of baseline value. Cox regression analysed risk of outcomes adjusted for baseline risk marker levels and treatment group and stratified by trial.

Results: Participants with two, three, or four or more improved risk markers versus participants with no risk marker improvement had reduced risk of expanded MACE [hazard ratio (95% confidence interval) 0.80 (0.67-0.96); 0.80 (0.66-0.97); 0.82 (0.66-1.02)], cardiovascular death [0.66 (0.45-0.96), 0.67 (0.45-0.99), 0.60 (0.38-0.94)] and nephropathy [0.71 (0.52-0.97), 0.48 (0.34-0.68), 0.43 (0.29-0.65)].

Conclusions: In persons with type 2 diabetes, improvements in ≥2 risk markers conferred cardiovascular risk reduction versus none or one improved risk marker. The nephropathy risk decreased with improvement in more risk markers. These findings stress the importance of multifactorial interventions targeting all risk markers.

KEYWORDS
cardiovascular disease, diabetic nephropathy, type 2 diabetes

1 | INTRODUCTION

Type 2 diabetes is a complex disease with a high rate of both micro- and macrovascular complications and an increased risk of death compared with the general population.[1] When it comes to reducing this high risk of complications and death, evidence is centred around pharmacotherapies that target isolated risk markers, including glycated haemoglobin (HbA1c), low-density lipoprotein-cholesterol (LDL-C) and blood pressure.[2–5]

In the randomized Steno 2 study, multifactorial intervention for 8 years targeting several modifiable risk markers resulted in a median increase in long-term survival of 7.9 years after 21 years of follow-up and 50% reduced risk of cardiovascular disease as compared with standard of care.[6] Adding to the interest in multiple risk markers, a recent observational cohort study including all patients in the Swedish National Diabetes Register who had type 2 diabetes indicated that those with HbA1c, LDL-C, albuminuria and blood pressure levels within the target range and non-smokers had little or no excess risk of death, myocardial infarction or stroke, compared with the general population.[7]

The extent to which improvements in multiple risk markers affect outcomes in type 2 diabetes has been sparsely investigated. Therefore, in two large cardiovascular outcome trials, we evaluated post hoc the importance of multiple risk marker improvement. We pooled the active treatment and placebo-treated groups in all analyses to investigate, in a contemporary type 2 diabetes population and independent of specific treatments, to what degree clinically relevant improvement in multiple risk markers confers lower risk of micro- and macrovascular disease.

2 | MATERIALS AND METHODS

The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER, NCT01179048) and the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN 6, NCT01720446) trial designs and methods have been previously published in detail.[8,9] Briefly, both were multicentre, double-blind, placebo-controlled trials, including persons with type 2 diabetes and high cardiovascular risk (N = 9340 in LEADER and N = 3297 in SUSTAIN 6). Participants were randomly assigned to a glucagon-like peptide-1 receptor agonist (GLP-1RA) or placebo, both added to the standard of care. In LEADER, liraglutide up to 1.8 mg subcutaneous once daily or matching placebo was given in a 1:1 ratio. In SUSTAIN 6, semaglutide 0.5 or 1.0 mg, or matching placebo, was given subcutaneous once weekly in a 1:1:1:1 ratio, pooled as semaglutide versus placebo for this analysis. The mean duration of follow-up was 3.8 years in LEADER and 2.1 years in SUSTAIN 6.

Investigators in both trials were directed to treat all participants to the standard of care according to guidelines. Both studies were conducted according to the Declaration of Helsinki and approved by the relevant authorities.

2.1 | Participants

This current post-hoc analysis included participants from LEADER and SUSTAIN 6 who had baseline and year-1 assessment of at least one of the six risk markers of interest: body weight, HbA1c, systolic blood pressure (SBP), LDL-C, estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR).

LEADER and SUSTAIN 6 included persons with type 2 diabetes and an HbA1c level of 7.0% (53 mmol/mol) or above. Participants were eligible if they were drug-naïve or treated with oral antihyperglycaemic agents or insulin. Key inclusion criteria were (a) age ≥50 years with cardiovascular morbidity (previous cardiovascular, cerebrovascular or peripheral vascular disease), or (b) age ≥60 years with one or more cardiovascular risk factors [persistent microalbuminuria (30-299 mg/g) or proteinuria, hypertension and left ventricular hypertrophy by electrocardiogram or imaging, left ventricular systolic or diastolic dysfunction by imaging, or ankle/brachial index <0.9]. The published original trial reports contain the complete list of inclusion and exclusion criteria.[8,9]

2.2 | Risk markers

Six well-established cardio-renal risk markers that are easily accessible for many clinicians in the daily evaluation of patients were selected for evaluation, including body weight, HbA1c, SBP, LDL-C, eGFR and UACR. We used SBP because it is the predominant risk factor among older adults and because the mean age of participants in this study was 64.3 years.[10] SBP and body weight were measured by standard methods at study sites. HbA1c, LDL-C, serum creatinine and UACR were measured using routine methods in central laboratories (Dublin, Ireland; New York, USA; Tianjin, China; Singapore; Bangalore, India), and eGFR was calculated [Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation] at baseline and at regular intervals during the trials, including at week 52 (12 months) in LEADER and week 56 in SUSTAIN 6 (referred to as year 1). Urine albumin or creatinine measurements less than the lower limit of quantification (LLoQ, 3.0 mg/L) were imputed using a value of 0.5× LLoQ; measurements greater than the higher limit of quantification (HLoQ) were imputed using the HLoQ value in the calculation of UACR.

We defined clinically relevant risk marker improvement at year 1 from baseline as body weight loss ≥5%, HbA1c reduction ≥1%, SBP reduction ≥5 mmHg, LDL-C reduction ≥0.5 mmol/L, eGFR change ≥0 ml/min/1.73 m^2 and UACR change ≥30% of baseline value. Participants were categorized into five groups based on the number of risk marker improvements at year 1: Group G0 (reference): zero risk marker improvement; Group G1: one risk marker improvement; Group G2: two risk marker improvements; Group G3: three risk marker improvements; Group G4: four or more risk marker improvements.

2.3 | Outcomes

We investigated the number of risk marker improvements at year 1 and the incidence of development of first outcome after year 1.

To evaluate the association between risk marker improvement and outcome, outcomes developed in the first year of participation in both studies were excluded. For participants with an event in the first year, events developed after 1 year of participation were included. The four outcomes of interest were: (a) major adverse cardiovascular events (MACE), including cardiovascular death, non-fatal myocardial infarction and non-fatal stroke; (b) expanded MACE, including cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, revascularization (LEADER: coronary; SUSTAIN 6: coronary or peripheral) and hospitalization for unstable angina pectoris or heart failure; (c) cardiovascular death; and (d) a composite nephropathy endpoint defined as the new onset of macroalbuminuria or a doubling of serum creatinine level and an eGFR ≤45 ml/min/1.73 m^2 , or the need for continuous renal-replacement therapy or death from renal disease.

2.4 | Statistical analyses

Normally distributed data are presented as mean ± standard deviation, continuous-scale skewed data (UACR) are presented as geometric mean (coefficient of variation) and median (interquartile range) and were log-transformed for all analyses; categorical variables are presented as numbers and percentages.

A Cox regression model stratified by trial was used to analyse the time to first MACE, expanded MACE, cardiovascular death and the composite nephropathy endpoint from 1 year onwards according to risk marker categories, adjusted for randomized treatment (liraglutide/semaglutide vs. placebo) and continuous baseline levels of the risk markers.

Test for trend was evaluated in a Cox regression model stratified by trial, with number of improved risk markers as a continuous variable (hence the test for slope equals 0) and adjusted for randomized treatment and continuous baseline levels of the risk markers.

We also analysed the relative importance of the six risk marker improvements. Relative importance provides an estimate of how important each risk marker improvement is in terms of predicting each of the outcomes after year 1.[7] We calculated the relative importance as measured by the R^2 values (explained relative risk in the Cox regression model) using an approach that has previously been described[11]; we evaluated the R^2 by using the full model as the Cox regression model for each endpoint with all six risk marker improvements (yes vs. no) adjusted for the continuous baseline levels of the six risk markers and treatment stratified by trial. A sensitivity analysis imputing missing values (single imputation) at year 1 for those participants with at least one measurement at year 1 was performed using the participant-wise predicted values from a random slope model for each risk marker independently, with baseline value and treatment by a linear time interaction as fixed effects. After the imputation, the clinically relevant improvements were derived for each risk marker.

The statistical package used for these analyses was SAS, version 9.4 (SAS Institute Inc.).

3 | RESULTS

3.1 | Study population

In total, 11 678 persons with type 2 diabetes were included in this study (N = 8638 in LEADER and N = 3040 in SUSTAIN 6).

The baseline characteristics of all participants and of the five groups (G0-G4) based on number of improved risk markers are presented in Table 1. There was a tendency towards a higher number of women and higher baseline LDL-C, SBP and UACR with an increasing number of improved risk markers. Separate baseline data for LEADER and SUSTAIN 6 are presented in Tables S1 and S2.

3.2 | Risk marker improvement

The number of participants with one, two, three and four or more clinically relevant risk marker improvements at year 1 was 3162 (27.1%), 3540 (30.3%), 2515 (21.5%) and 1406 (12.0%), respectively (Table 1). There were 1055 (9.0%) participants without any clinically relevant risk marker improvements at year 1. In the LEADER trial, 138 (1.6%) participants died or discontinued the trial before the 1-year visit and 564 (6.5%) participants were still in-trial but missed the 1-year assessment (or at baseline). In SUSTAIN 6, the corresponding numbers were 72 (2.4%) and 185 (6.1%) participants, respectively. For those participants with at least one assessment at year 1, almost 90% had all six assessments related to the risk markers at year 1.

3.3 | Risk of outcomes

In the two studies combined, a total of 1111 (9.5%) participants experienced MACE, 1771 (15.2%) expanded MACE, 406 cardiovascular death (3.5%) and 621 (5.3%) developed the composite nephropathy endpoint after year 1 of study participation (Figure 1).

The number of events, incidence rates and hazard ratios for all outcomes in participants with one, two, three, or four or more (G1-G4) risk marker improvements at year 1, compared with participants without any risk marker improvements (G0), are presented in Figure 1, and separately for LEADER and SUSTAIN 6 participants in Figures S1 and S2. In a pooled analysis of LEADER and SUSTAIN 6, the hazard ratios for expanded MACE, cardiovascular death and the composite nephropathy endpoint were lower in the groups of participants with two, three or four or more risk marker improvements (G2-G4), compared with the zero risk marker improvement group (G0: reference group). The hazard ratios for all four endpoints were comparable in the group of participants with one risk marker improvement (G1) and the group of participants with no risk marker improvements at year 1 (G0: reference group). There was a trend of decreased hazard ratios across the groups for MACE ($p = .08$), expanded MACE ($p = .004$), cardiovascular death ($p = .005$) and the composite nephropathy endpoint ($p < .0001$). Collapsing G0 with G1 versus collapsing G2, G3 and G4 and testing if these two groups were equal, we obtained the following p-values for the endpoints: MACE ($p = .048$),

TABLE 1 Baseline characteristics of the participants by risk marker improvement subgroups

Baseline	G0	G1	G2	G3	G4	Total
No. of participants, n (%)[a]	1055 (9.0)	3162 (27.1)	3540 (30.3)	2515 (21.5)	1406 (12.0)	11 678 (100)
Treated with liraglutide or semaglutide, n (%)	322 (30.5)	1202 (38.0)	1727 (48.8)	1550 (61.6)	1059 (75.3)	5860 (50.2)
Placebo, n (%)	733 (69.5)	1960 (62.0)	1813 (51.2)	965 (38.4)	347 (24.7)	5818 (49.8)
Age, years	64.2 ± 7.4	64.3 ± 7.1	64.2 ± 7.2	64.4 ± 7.2	64.5 ± 7.2	64.3 ± 7.2
Female, n (%)	320 (30.3)	1061 (33.6)	1278 (36.1)	991 (39.4)	613 (43.6)	4263 (36.5)
HbA1c, %[d]	8.2 ± 1.2	8.5 ± 1.4	8.8 ± 1.6	8.8 ± 1.5	8.8 ± 1.4	8.7 ± 1.5
HbA1c, mmol/mol	66.0 ± 12.9	69.6 ± 15.5	72.5 ± 17.4	73.0 ± 16.7	72.8 ± 15.6	71.3 ± 16.3
Body weight, kg[d]	91.1 ± 20.7	91.6 ± 20.6	91.4 ± 20.6	92.4 ± 20.9	92.2 ± 21.5	91.7 ± 20.8
Diabetes duration, years, median (IQR)	11.5 (6.8-16.9)	11.8 (7.0-17.7)	11.6 (7.0-17.1)	11.7 (7.1-17.2)	12.4 (7.4-18.3)	11.8 (7.1-17.5)
Current smoker, n (%)	125 (11.8)	413 (13.1)	376 (10.6)	301 (12.0)	170 (12.1)	1385 (11.9)
SBP, mmHg[d]	131 ± 16	133 ± 17	136 ± 18	139 ± 18	141 ± 18	136 ± 17
LDL-C, mg/dl	84.6 ± 32.5	84.3 ± 33.3	89.5 ± 35.5	92.6 ± 37.4	101.4 ± 41.6	89.8 ± 36.3
LDL-C, mmol/L[d]	2.2 ± 0.8	2.2 ± 0.9	2.3 ± 0.9	2.4 ± 1.0	2.6 ± 1.1	2.3 ± 0.9
eGFR (CKD-EPI), ml/min/ 1.73 m^2 [d]	81.4 ± 21.3	80.6 ± 21.6	80.4 ± 21.5	79.7 ± 22.0	79.2 ± 22.0	80.3 ± 21.7
UACR, median (IQR)[d]	11.9 (3.4-64.8)	12.5 (3.9-57.5)	14.6 (4.6-63.1)	17.2 (5.6-69.9)	22.3 (7.5-96.0)	15.3 (4.6-67.5)
Established CVD, n (%)	873 (82.7)	2559 (80.9)	2863 (80.9)	2059 (81.9)	1156 (82.2)	9510 (81.4)
Presence of CVD risk factor, n (%)[a,b]	182 (17.3)	603 (19.1)	677 (19.1)	456 (18.1)	250 (17.8)	2168 (18.6)
Lipid-lowering treatment, n (%)	820 (77.7)	2431 (76.9)	2728 (77.1)	1886 (75.0)	1023 (72.8)	8888 (76.1)
RAAS inhibition treatment, n (%)	857 (81.2)	2548 (80.6)	2883 (81.4)	2032 (80.8)	1123 (79.9)	9443 (80.9)
Metformin treatment, n (%)	796 (75.5)	2486 (78.6)	2727 (77.0)	1887 (75.0)	1033 (73.5)	8929 (76.5)
Insulin treatment, n (%)	487 (46.2)	1498 (47.4)	1550 (43.8)	1111 (44.2)	631 (44.9)	5277 (45.2)
SGLT-2 inhibitor treatment, n (%)[c]	1 (<0.1)	0 (0.0)	2 (<0.1)	1 (<0.1)	1 (<0.1)	5 (<0.1)
Aspirin treatment, n (%)	682 (64.6)	2022 (63.9)	2264 (64.0)	1576 (62.7)	868 (61.7)	7412 (63.5)

Note: Adapted from Zobel EH et al. The importance of addressing multiple risk markers in type 2 diabetes: results from the LEADER and SUSTAIN 6 trials.[12]. Abstract/FC 058 ©ERA-EDTA GROUP. Reproduced by permission of Oxford University Press on behalf of the ERA-EDTA. Table is not published under this article's licence and permission must be sought for any form of reuse.

Pooled data from the LEADER and SUSTAIN 6 trials. Data are presented as mean ± standard derivation, unless stated otherwise. Participants were categorized according to number of risk markers with an improvement at year 1 [none (group G0), one (G1), two (G2), three (G3) and four or more (G4)].

Abbreviations: CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin; IQR, interquartile range; LDL-C, low-density lipoprotein-cholesterol; RAAS, renin-angiotensin-aldosterone system; SBP, systolic blood pressure; SGLT-2, sodium-glucose cotransporter-2; UACR, urinary albumin-to-creatinine ratio.

[a]Calculated as a percentage of the overall total (all other percentages were calculated out of the risk marker improvement subgroups).

[b]Presence of CVD risk factor was defined as persistent microalbuminuria (30-299 mg/g) or proteinuria, hypertension and left ventricular hypertrophy by electrocardiogram or imaging, left ventricular systolic or diastolic dysfunction by imaging, or ankle/brachial index <0.9.

[c]SGLT-2 inhibitors were not marketed before randomization in the LEADER trial, hence relatively few participants in the pooled population received this medication at baseline.

[d]These parameters are risk markers that were evaluated in this post-hoc analysis.

expanded MACE ($p = .0006$), cardiovascular death ($p = .0031$) and the composite nephropathy endpoint ($p < .0001$).

We observed similar results in a separate analysis of LEADER and SUSTAIN 6 (Figures S1 and S2) and when the liraglutide/semaglutide and placebo groups were analysed separately (Figures S3 and S4). We also confirmed our findings in a sensitivity analysis with missing responses imputed (i.e. single imputation of missing risk markers at year 1; Figure S5), although results (trends) were slightly attenuated.

3.4 | Relative importance of the individual risk marker improvement

Figure S6 shows the six risk marker improvements ranked in order of the highest to lowest R^2 according to MACE, expanded MACE, cardiovascular death and the composite nephropathy endpoint. The R^2 in this analysis could be interpreted in the same way as the coefficient of determination in the linear model. The R^2 for all the improvements

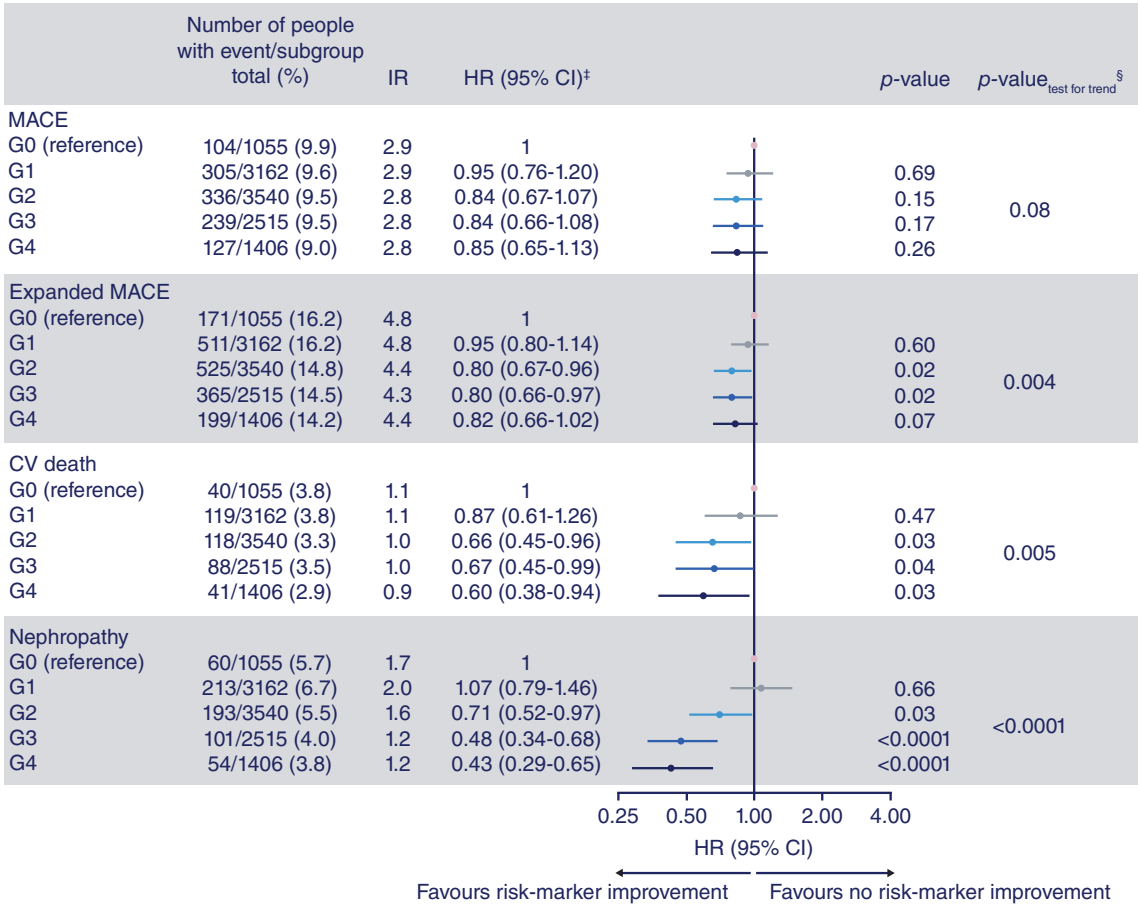

FIGURE 1 Outcomes according to number of risk marker improvements (adjusted by baseline variables) among persons with type 2 diabetes. From Zobel EH et al. The importance of addressing multiple risk markers in type 2 diabetes: results from the LEADER and SUSTAIN 6 trials.[12] Abstract/FC 058 ©ERA-EDTA GROUP. Reproduced by permission of Oxford University Press on behalf of the ERA-EDTA. Figure is not published under this article's licence and permission must be sought for any form of reuse. HRs show risk for outcomes according to number of risk markers with a clinically relevant improvement among participants with type 2 diabetes. Post-hoc analysis of data from the LEADER and SUSTAIN 6 trials included 11 678 persons with type 2 diabetes. Participants were categorized according to number of risk markers with an improvement at year 1 [none (group G0), one (G1), two (G2), three (G3) and four or more (G4)]. ‡Compared G1-G4 with G0 (the reference group); §test for trend was evaluated in a Cox regression model with number of risk marker improvements as a continuous variable adjusted for treatment and baseline levels of the risk markers. CI, confidence interval; CV, cardiovascular; HR, hazard ratio; IR, incidence rate per 100 patient years of observation; MACE, major adverse cardiovascular events

adjusted for baseline variables was modest and varied from 2% to 19% for first MACE and the composite nephropathy endpoint, respectively. For the individual improvements, HbA1c contributed most to the R^2 for first MACE (56%) and expanded MACE (39%); improvement in eGFR contributed most to the R^2 for cardiovascular death (47%) and improvement in UACR contributed most to the R^2 for the composite nephropathy endpoint (58%).

3.5 | Glucagon-like peptide-1 receptor agonist treatment

We observed an increase in the number of participants treated with GLP-1RA versus placebo in the groups of participants with none, one, two, three, or four or more risk marker improvements as follows: 30.5% in G0, 38.0% in G1, 48.8% in G2, 61.6% in G3 and 75.3% in G4 (Table 1). Hence, 24.7% of the participants with four or more risk marker improvements were treated with placebo.

In two separate analyses, one including participants treated with semaglutide/liraglutide and one including participants treated with placebo, we observed similar results as in the pooled analysis (Figures S3 and S4).

In analyses without adjustment for randomized treatment, we confirmed our main findings (data not shown).

3.6 | Sensitivity analysis on a modified nephropathy endpoint

We confirmed our findings for the composite nephropathy endpoint using a modified nephropathy endpoint defined as a doubling of serum creatinine level and an eGFR ≤45 ml/min/1.73 m^2, or the need

for continuous renal-replacement therapy or death from renal disease. Participants with two, three, or four or more improved risk markers versus participants with no risk marker improvement had a reduced risk of the modified nephropathy endpoint: hazard ratio (95% confidence interval) 0.65 (0.39-1.07), 0.43 (0.25-0.74) or 0.32 (0.17-0.61), respectively.

4 | DISCUSSION

Our analyses of the two large cardiovascular outcome trials LEADER and SUSTAIN 6, including >11 000 persons with type 2 diabetes, show the association between the number of risk markers with a clinically relevant improvement within 1 year and the risk of developing micro- and macrovascular complications.

The risk markers evaluated were HbA1c, body weight, SBP, LDL-C, UACR and eGFR. For the cardiovascular outcomes, a clinically relevant improvement at year 1 in two or more of these risk markers was associated with reduced cardiovascular risk, as compared with participants without a clinically relevant improvement in any of these risk markers. More specifically, risk reduction was more noticeable when two or more improvements were obtained as compared with only having no or one improvement. For the risk of developing the composite nephropathy endpoint, a clinically relevant improvement at year 1 in two or more risk markers resulted in lower risk. For this outcome, we observed a pronounced decrease in risk for each additional risk marker improved. Data were not strong enough to conclude that a stepwise association exists for cardiovascular risk reduction. Findings from separate analyses of LEADER and SUSTAIN 6 were similar to the findings from the pooled analysis.

Dedicated outcome trials to evaluate the importance of addressing multiple risk markers are laborious and expensive. Accordingly, while awaiting such trials to inform us on multiple risk marker improvement, we pursued insights from a post-hoc analysis of two large, recent cardiovascular outcome trials. Our findings imply that, in a clinical setting, it is important to consider multiple factors. As such, our results are in line with findings from the randomized Steno 2 study.[6,13,14] The Steno 2 study (n = 160) combined lifestyle intervention targeting exercise, obesity, diet and smoking, as well as pharmacological therapy targeting glucose, lipids and blood pressure, including angiotensin-converting enzyme inhibitors and aspirin, in persons with type 2 diabetes and microalbuminuria.[14] A 50% reduction in cardiovascular outcome of this multifactorial intervention was shown after 8 years[14] and a 20% reduction in risk of mortality after 13 years.[13] In addition, the benefit was sustained after 21 years of follow-up, although all were treated similarly after the first 8 years.[6,15–17]

The Japan Diabetes Optimal Integrated Treatment for three major risk factors of cardiovascular diseases (J-DOIT3) study randomized 2542 persons with type 2 diabetes to receive conventional or intensive therapy for glucose, blood pressure and lipid control for a median of 8.5 years.[18] The study showed that intensified multifactorial intervention significantly reduced onset and progression of diabetic kidney disease, while the benefit on cardiovascular disease and mortality was less clear.[18,19]

Epidemiological data support that the number of well-controlled risk markers matters for the outcome. A recent cohort study including 271 174 persons with type 2 diabetes and 1 355 870 healthy controls followed up for a median of 5.7 years showed that the excess risk of cardiovascular disease seen in persons with type 2 diabetes, compared with the healthy controls, decreased stepwise for each risk marker within the target range at baseline.[7] The risk markers evaluated were HbA1c, LDL-C, albuminuria, smoking and SBP, and thus overlap the risk markers we evaluated.[7]

When we analysed the relative importance of the six risk markers in terms of R^2 for each endpoint, HbA1c was the largest contributor for two of the four outcomes, followed by eGFR and UACR for one endpoint each. However, we did not observe that improvement in one risk marker constituted a major part of the explained relative risk, indicating that it was the multiple risk marker response that associated with improved outcome. There is extensive evidence that pharmacotherapies targeting only one of the six risk markers evaluated in this study reduce the risk of both micro- and macrovascular complications in type 2 diabetes.[3,4,20–22]

The greater proportion of subjects treated with GLP-1RAs in the groups with multiple improved risk markers indicates that treatment with GLP-1RAs may improve these risk markers; however, 24.7% of the participants with four or more risk marker improvements were treated with placebo, showing how the risk markers in scope improved despite being in a placebo group.

Our study has several strengths, including the systematic, standardized measurement of risk markers and collection of outcomes and a large number of participants studied for a considerable length of time. One potential limitation is that two of the risk markers (UACR and eGFR) were also part of the renal outcome. However, we analysed improvement in the risk markers at year 1 and studied outcomes developed after year 1, and thereby mitigated the risk of reverse causation in the interpretation of the results.

This is a post-hoc analysis with inherent risk of bias, hence the results should be interpreted with caution. We defined a clinically relevant improvement as a binary variable; on the one hand, this is an approach applicable to the clinic, but, on the other hand, the assessment could potentially be improved if we applied more advanced statistical models with the risk markers as continuous variables. The cut-offs for clinically relevant risk marker improvement within 1 year were defined by the authors and agreed upon before any data analysis. Where possible, we tried to select previously used cut-offs and, although chosen without data analysis, aiming at a potentially similar impact on outcome. We acknowledge that other cut-offs could also be clinically relevant. For eGFR, a yearly reduction of approximately 1 ml/min/1.73 m^2 could be expected from ageing, and it could be argued that a higher cut-off value than the one chosen would better discriminate reduction in eGFR from real progression in kidney disease. However, we chose a cut-off of 0, as eGFR was stable during 26 weeks of treatment with a GLP-1RA in the PIONEER 5 study[23] and the LIRA-RENAL study.[24] The mean

level of kidney function in this cohort was in the normal range and, at these levels of renal function, the accuracy of the CKD-EPI equation is limited and measures of eGFR based on cystatin C would potentially be more accurate.

The trial setting limits the generalizability of our findings. Because of the randomized study design, GLP-1RA treatment was restricted to half of the population and improvement in the other half was because of initiation or intensification of other treatments or natural changes. Acknowledging that we pooled two randomized studies, our analysis was adjusted for treatment group (liraglutide/semaglutide and placebo), which also addresses the fact that more participants were on active treatment in the higher G classes (G0→G4). Therefore, our findings are not specifically related to the effects of GLP-1RA treatment.

In conclusion, in a contemporary population with type 2 diabetes, improvements in two or more risk markers were associated with cardiovascular risk reduction, as compared with zero or one improved risk marker. This trend with increasing number of improvements and decreased risk was most noticeable for risk of nephropathy. Our findings stress the importance of multifactorial intervention in individuals with type 2 diabetes.

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CONFLICT OF INTEREST

EHZ is now a Novo Nordisk A/S full-time employee, but work related to this article was carried out when EHZ was employed full-time by Steno Diabetes Center Copenhagen. BW, BJvS and SR are Novo Nordisk A/S full-time employees and BW, BJvS, SR and TWH are Novo Nordisk shareholders. FP reports research grants from AstraZeneca and lecture fees from AstraZeneca, MSD, Janssen, Eli Lilly, Boehringer Ingelheim, Novo Nordisk A/S and Novartis, as well as being a consultant/advisory board member for AstraZeneca, Bayer, Amgen and MSD. PR has received honoraria from Steno Diabetes Center Copenhagen for advisory work, and for consultancy/education from Astellas, AstraZeneca, Bayer, Boehringer Ingelheim, Eli Lilly, MSD, Merck, Mundipharma, Novo Nordisk, Vifor and Sanofi.

AUTHOR CONTRIBUTIONS

Emilie Hein Zobel, Tine W. Hansen, Frederik Persson, Bernt J. von Scholten, Benjamin Wolthers and Peter Rossing conceived and designed the research, analysed and interpreted the data; Søren Rasmussen performed the statistical analysis; Emilie Hein Zobel drafted the manuscript; Tine W. Hansen, Frederik Persson, Bernt J. von Scholten, Benjamin Wolthers and Peter Rossing critically revised the manuscript for key intellectual content; all authors approved the final version of the manuscript. Emilie Hein Zobel is the paper's guarantor, had full access to the data and is responsible for the integrity of the work as a whole.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The subject level analysis data sets for the research presented in this publication are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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