Cardiovascular Risk Stratification and Renal Effects of Weight Loss and GLP-1 Receptor Agonism in Type 2 Diabetes
CARDIOVASCULAR RISK STRATIFICATION AND RENAL EFFECTS OF WEIGHT LOSS AND GLP-1 RECEPTOR AGONISM IN TYPE 2 DIABETES

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
CAND. MED. BERNT JOHAN VON SCHOLTEN
Steno Diabetes Center Copenhagen
The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in 2018.

Copenhagen, 24 January 2018
Ulla Wewer, Head of Faculty

The defence will take place:
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Steno Diabetes Center Copenhagen
Hagedorn Auditorium
Niels Steensens Vej 6, 2820 Gentofte
It all started in March 2011. I was a young doctor fresh out of medical school and was working at the department of internal medicine at Helsingør Hospital. A good friend of my parents in law (Ingelise Holstein) asked if I could be interested in doing research at Steno Diabetes Center. Endocrinology was one of my favourite specialties and so diabetes research sounded like a great idea. I applied for the job and got the position right after having completed the clinical rotations of my residency in early 2012. My research experience was at that time limited and I was curious to learn what it would be like and whether I would like it or not. It did not take me long before I realised that doing research was truly something for me and that Steno Diabetes Center was a unique place to work. What I also realised was that doing clinical research and in particular intervention studies was full of ups and downs, joys and frustrations. The road from getting the idea, writing the protocol, obtaining funding and getting approval from the required regulatory authorities was long and challenging. However, identifying the patients, convincing them to participate and following them all the way from signed informed consent to the final study visit – those were indeed the most difficult parts of clinical study conduction. Luckily, the research also led to several positive and rewarding experiences and moments, and the thrill of receiving an acceptance letter from a prominent journal was an example of how all the hard work had paid off.

This doctoral dissertation is the result of the work I have performed at Steno Diabetes Center for the past six years. I was fortunate to be part of the research group “520” led by Peter Rossing, which for the past decades has focused on prevention and treatment of complications connected to diabetes. The papers in this dissertation are a result of several different studies where I had the privilege of collaborating with research departments at Rigshospitalet, Glostrup Hospital, Herlev Hospital, Hvidovre Hospital and Novo Nordisk. These cross-sectional collaborations were rewarding and improved the quality of the studies. Needless to say, I learned a lot during my years of research at Steno Diabetes Center, which is something I benefit from every day in my current position. I am thrilled that some of the projects included in this dissertation and some I initiated during my time at Steno are still running and collecting impactful data – now with other dedicated researchers as the primary drivers.

First and foremost I would like to thank the patients and healthy controls who participated in the studies.

Ingelise, thank you for asking me to apply for the position. You are the reason why I ended up at Steno Diabetes Center and got to spend some of the best years of my career. I would also like to thank you for always taking good care of all your “520” children.

Peter - thank you for hiring me. You are indeed a scientific role model. I admire your capability to always being on top of data and I have truly appreciated your experience and intelligence when difficult questions have arisen over the years.

Tine, not sure I have realised yet that there will be no more “revision time” together with you and the rest of “Team Tine”. Thank you so much for all of your help and dedication over the years. I am grateful that I got to work with someone who is (almost) as impatient as I am,
since that for sure had an impact on the speed of completion and submission of my manuscripts.

Frederik, I guess it all started with a successful job interview at your office. You have been a great mentor for me and I have appreciated our many scientific discussions and ambitious project ideas. You truly are a science entrepreneur. We’ve had some great moments over the years and all over the world, but let’s never go bowling together again.

Signe, we started at Steno on the same day and quickly became great friends. I really appreciated having you as a colleague; always in a great mood and always with a great sense of humour. We had our fair share of work-related frustrations but more importantly we had a lot of fun over the years.

Morten, we shared office and hotel rooms for three straight years and I must start out by thanking you for always ironing my shirts. You will always be Mr. PRIORITY and the way you almost onehandedly managed to get that large study on the road was impressive.

Jens, you were at first the lucky medical student who had been given the Steno-2 study follow-up data. However, you quickly became a valuable member of the team and a good friend. I admire you for always being well-prepared, organised and helpful. I look forward to collaborating with you in the future.

Emilie, thank you for taking over some of the projects I left behind. These projects could not have landed at a better place than with you. You are a worthy member of “Team Tine” and a great friend. With your dedication in everything you do; I am convinced that you will get far. We only got to work together as full-time colleagues for one year but it was pretty remarkable what we achieved in that short time period.

Christian, although neither of us managed to do 100 push-ups in a row, we accomplished other great things together. Undoubtedly, we were the perfect supervisor team for Thilde and Regitse, and we had a lot of fun over the years. Your positive spirit and dedication are admirable and I would like to congratulate you on being the Danish champion of diabetic neuropathy.

Thomas, we started out as young and unexperienced researchers doing Investigator Sponsored Studies; then we advanced to the Key Scientific Leader Programme before you (and your world-class presenting skills) took off and landed oral presentations for each and every EASD and ADA. Attending conferences with you has always been rewarding on many levels. Your energy is contagious; I always enjoy spending time with you and appreciate our friendship.

Maria, you were an important part of “520” when I started and I want to thank you for helping out with getting my first projects up and running and for introducing me to the universe of science and statistics.

Simone, the very first paper I did at Steno (not included in this dissertation) was together with you. The findings were negative and so the paper ended up being published in a very small journal; however I am proud to say that the paper has been cited once! - in a Russian review article. You really taught me a lot during my first time so thank you for that.

Christel, our time as colleagues was short but great. I love your attitude and while you may scare some people, you don’t scare me.

Gudbjorg, I enjoyed being your colleague and it was amazing experiencing how your PhD ended up being as great as it did. Thank you for always being such a sweet person.
Henrik Reinhard and Peter Karl Jacobsen, thank you for allowing me to follow-up the patients in your elegantly designed BNPcure study.

Hans-Henrik, your impact on diabetes research has been enormous. I thank you for the always thorough, constructive and honest feedback you provided to my papers. I always felt proud once a paper had been approved by you.

Thank you to all the hard working “520” research nurses and laboratory technicians: Lone, Bente, Ulla, Anne, Tina, Berit, Jessie and Lene - had it not been for the huge amount of work performed by you, this dissertation would not have been possible.

To everyone else in “520” and to all the co-authors and collaborators not mentioned above: Thank you.

To my friends, brothers, parents in law and parents; thank you for listening to me when I have felt the urge to share some of my research specific news. I am sure it must have been very boring.

Elinora and Rose, both of you entered the world during my time at Steno and first of all thank you for that (well mostly a thank you to your mother I guess). Words cannot describe how much you mean to me. Over the years you kept reminding me that there are more important things to life than publishing papers in high-ranking journals.

Last but not least, I would like to thank you Martha for always being there for me and for being who you are. Thank you for providing me with the hard learned lesson, that there is such a thing as too much detail, when it comes to presenting scientific information to a layperson. You truly are an amazing person, the love of my life and I would honestly not know what to do without you.

Bernt Johan Illum von Scholten, February 2018
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LIST OF PUBLICATIONS

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

1) Additive prognostic value of plasma N-terminal pro-brain natriuretic peptide and coronary artery calcification for cardiovascular events and mortality in asymptomatic patients with type 2 diabetes.
   *Cardiovascular Diabetology*. 2015 May 21;14:59.

2) Markers of inflammation and endothelial dysfunction are associated with incident cardiovascular disease, all-cause mortality, and progression of coronary calcification in type 2 diabetic patients with microalbuminuria.

3) Urinary biomarkers are associated with incident cardiovascular disease, all-cause mortality and deterioration of kidney function in type 2 diabetic patients with microalbuminuria.

4) Cardiac (82)Rb PET/CT for fast and non-invasive assessment of microvascular function and structure in asymptomatic patients with type 2 diabetes.
   *Diabetologia*. 2016 Feb;59(2):371-8

5) Impaired coronary microcirculation in type 2 diabetic patients is associated with elevated circulating regulatory T cells and reduced number of IL-21R(+) T cells.

6) Cardiac autonomic function is associated with the coronary microcirculatory function in type 2 diabetic patients.

7) Time course and mechanisms of the anti-hypertensive and renal effects of liraglutide treatment.


8) Glucagon-like peptide 1 receptor agonist (GLP-1 RA): long-term effect on kidney function in patients with type 2 diabetes.


*Diabetes, Obesity and Metabolism.* 2017 Jun;19(6):901-905.

11) The influence of pharmaceutically induced weight changes on estimates of renal function: A patient-level pooled analysis of seven randomised controlled trials of glucose lowering medication.

von Scholten BJ, Ørsted DD, Svendsen AL, Persson F, Rossing P

12) Effect of large weight reductions on measured and estimated kidney function

*BMC Nephrology.* 2017 Feb 6;18(1):52

13) Effect of weight reductions on estimated kidney function: post-hoc analysis of two randomized trials

ABBREVIATIONS

ADA American Diabetes Association  
CAC Coronary artery calcium  
CAN Cardiovascular autonomic neuropathy  
CARTs Cardiovascular autonomic reflex tests  
CFR Coronary flow reserve  
CIRT Cardiovascular Inflammation Reduction Trial  
CKD-EPI Chronic Kidney Disease Epidemiology Collaboration  
CT Computed tomography  
DXA Dual-energy X-ray absorptiometry  
FACS Fluorescence-activated cell sorting (8-color flow-cytometry)  
GFR Glomerular filtration rate  
GLP-1 RA Glucagon like peptide-1 receptor agonist  
HRV Heart rate variability  
IL-21R Interleukin-21 receptor  
LEADER Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results  
MDRD Modification of Diet in Renal Disease  
MIBG Metaiodobenzylguanidine  
MR-proADM Mid-regional pro-adrenomedullin  
MR-proANP Mid-regional pro-atrial natriuretic peptide  
NT-proBNP N-terminal pro-brain natriuretic peptide  
PCSK9 Proprotein convertase subtilisin/kexin type 9  
RAAS Renin–angiotensin–aldosterone system  
$^{82}$Rb-PET/CT Rubidium-82 positron-emission-tomography/computed-tomography  
SCALE Satiety and Clinical Adiposity – Liraglutide Evidence  
SGLT2 Sodium glucose cotransporter 2  
SUSTAIN Semaglutzide Unabated Sustainability in Treatment of Type 2 Diabetes  
TNF-alpha Tumour necrosis factor-alpha  
UAER Urinary albumin excretion rate
INTRODUCTION AND AIMS

Type 2 diabetes is an emerging pandemic that is driven by the combined effects of obesity and inactivity, ageing population and a longer lifespan for patients with diabetes. Globally, an estimated 422 million adults were living with diabetes in 2014, compared to 108 million in 1980. The global age-standardized prevalence of diabetes has nearly doubled since 1980, rising from 4.7 to 8.5% in the adult population (1). Furthermore, a recent study revealed that the incidences of both type 1 and type 2 diabetes among youths increased significantly in the 2002–2012 period (2). Diabetes is a global cause of premature mortality that is probably underestimated, since only a minority of patients with diabetes dies from a cause uniquely related to the disease. The cardiovascular and renal complications of type 2 diabetes account for the majority of the social burden among patients. In broader terms, cardio-renal complications are a significant economic burden for the society. Approximately 50% of patients with type 2 diabetes die of a cardiovascular cause, whereas approximately 10% die prematurely of renal complications (3). Over the past two decades, the rates of diabetes-related complications have declined significantly, probably as a result of advances in clinical care, health care system performance and health promotion aimed at patients with diabetes (4, 5). However, despite the encouraging improvement, type 2 diabetes patients are still at considerably higher risk of cardiovascular and renal disease when compared with persons without diabetes (5).

Several studies have demonstrated that presence of microalbuminuria identifies type 2 diabetes patients with an even higher risk of cardio-renal morbidity and mortality (6-8). The Steno-2 intervention study in type 2 diabetes patients with persistent microalbuminuria demonstrated that multifactorial intervention of traditional risk factors reduced the risk of cardiovascular disease by >50% over 8 years (9), follow-up after 13 years revealed that death of cardiovascular causes and cardiovascular events were reduced by >50% (10), and the recent 21 years follow-up demonstrated a median of 7.9 years life gained (11). Furthermore, in type 2 diabetes patients with nephropathy, an improved survival and renal prognosis have recently been demonstrated and attributed to improved control of established risk factors (12). However, despite multifactorial treatment patients, it is well documented that patients with type 2 diabetes and microalbuminuria are at a significantly higher risk of cardio-renal complications compared with non-diabetic subjects (10, 13).

The underlying reason for this might partly be explained by the lack of: I) valid early markers to identify and monitor high risk individuals; II) understanding of the aetiology and pathophysiology of cardio-renal complications; and III) interventions with the ability to further reduce the risk of cardio-renal complications on top of standard of care.

For this thesis, the aim was to obtain novel information on each of the three points stated above (Part I-III); ultimately leading to tools and knowledge that can help improve prognosis for patients with type 2 diabetes. Lastly, given the increase in the prevalence of obesity, and
the focus on lifestyle factors as well as medications with impact on body weight, and the interplay between body composition and estimates of renal function, the final aim was to determine the effects of small, moderate and large weight reductions on measured and estimated renal function (Part IV).

Part I: N-terminal pro-brain natriuretic peptide (NT-proBNP) releases from the heart as a response to myocardial wall stress, and increased level is an established risk factor in patients with symptomatic heart failure (14). Further, in type 2 diabetes patients, higher levels of NT-proBNP have been associated with increased cardiovascular and all-cause mortality (15-19). The degree of coronary calcification, reported as the coronary artery calcium (CAC) score, can be measured by computed tomography (CT) scans. Numerous studies have demonstrated that CAC is a valid risk stratification tool for cardiovascular disease in the overall population, since CAC was demonstrated to add prognostic information on top of Framingham risk factors (20-22), and similar findings have been shown in type 2 diabetes patients (23).

For this thesis, the primary aim of the follow-up study (Study A, Table 1), including 200 type 2 diabetes patients with microalbuminuria, was to test whether novel and established biomarkers could identify patients at higher risk of incident cardiovascular disease and all-cause mortality.

We hypothesized that a pre-specified risk stratification - based on NT-proBNP and CAC levels - was successful in identifying patients at the highest risk during a median follow-up of 6 years (24). Endothelial dysfunction is an early marker of atherosclerosis, and it is has been shown that inflammatory processes are involved in the development of atherosclerotic cardiovascular disease (25). In Study A, we also evaluated a pre-selected comprehensive panel of biomarkers of endothelial dysfunction and low-grade inflammation and their association with incident cardiovascular disease, all-cause mortality and progression in CAC (26). Lastly, in the continuing search for biomarkers to demonstrate prognostic power beyond traditional risk factors and to provide new targets for intervention, we also evaluated urinary hepatocyte growth factor and adiponectin as predictors of cardiovascular disease and all-cause mortality. The two urinary biomarkers were pre-selected and represent different aspects of renal pathophysiology (27).

Part II: Despite extensive research on the topic of diabetic cardiovascular complications during the past decades, a better understanding of the mechanisms underlying diabetic vascular disease is required, since it may provide novel approaches to early prevention or to decelerate progression in cardiovascular complications. The hypothesis for Part II of this thesis was that new cardiovascular imaging modalities may provide novel and useful information. The coronary flow reserve (CFR) is a measure of the cardiac circulation. CFR is reflecting the microcirculation in addition to the function of the large arteries in the epicardium; and among patients with diabetes, CFR has been demonstrated to predict cardiac mortality (28). For this thesis, the cross-sectional study (Study B, Table 1) aimed to assess CFR and CAC in type 2
diabetes patients with and without albuminuria and without known cardiovascular disease. A Rubidium-82 positron-emission-tomography/computed-tomography ($^{82}$Rb-PET/CT) scan was used for this purpose (29). Further, we used 8-color flow-cytometry (FACS) analysis of the peripheral blood with the aim to uncover early inflammatory signatures associated with impaired coronary microcirculatory function (30). Lastly, cardiovascular autonomic neuropathy (CAN) is an important diabetes complication that is often overlooked. CAN is associated with risk of cardiovascular disease, including arrhythmias and sudden death. We used cardiac I-123 metaiodobenzylguanidine (MIBG) scintigraphy, heart rate variability (HRV) indices and cardiovascular autonomic reflex tests (CARTs) to assess the degree of cardiac autonomic neuropathy, and we related these findings to the measures of coronary microcirculatory function, obtained from the $^{82}$Rb-PET/CT scan (31).

**Part III:** Hyperglycaemia, hypertension, dyslipidaemia and obesity are all established cardiovascular risk factors associated with type 2 diabetes. National policies recommend multifactorial treatment including glucose-lowering, antihypertensive, lipid-lowering and antiplatelet treatment as standard of care. As previously stated, this multifactorial approach, including the widespread use of renin–angiotensin–aldosterone system (RAAS) blocking treatment, has played an important role in reducing risk of complications in type 2 diabetes. Until recently, interventions with the capability to further reduce the residual cardio-renal risk have been lacking. The results of the EMPA-REG OUTCOME study of the sodium glucose co-transporter 2 (SGLT2) inhibitor, empagliflozin (32, 33), together with the findings of the LEADER trial of the glucagon like peptide-1 receptor agonist (GLP-1 RA) liraglutide (34) and the SUSTAIN-6 trial of the GLP-1 RA semaglutide (35), all demonstrating beneficial cardio-renal effects, were well-received and provided treating physicians with additional optimism. The hypothesis of Part III of this thesis was that treatment with liraglutide has anti-hypertensive effects as well as blood pressure-independent anti-albuminuric proprieties. The first intervention study of this thesis was an open-label time course study (Study C, Table 1) with the aim to investigate liraglutide’s effect on ambulatory blood pressure and renal function including potential mechanistic explanations (36); based on early findings showing blood pressure lowering potential of liraglutide (37). The second intervention study (Study D, Table 1) was designed following findings from Study C with the aim to analyse the effects of liraglutide on albuminuria in a randomized, double-blind, placebo-controlled, cross-over trial including type 2 diabetes patients with persistent micro- or macroalbuminuria (38). Lastly, in a pre-specified sub-study of Study D, the effect of liraglutide on five pre-defined cardiovascular risk biomarkers was evaluated (39).

**Part IV:** The rising prevalence of overweight and obesity is a major global health challenge and concern (40). During recent years focus on weight-lowering treatments has increased. While several weight-lowering drugs have been approved by the Food and Drug Administration and are available for prescription, the weight-lowering potential of SGLT2 inhibitors and
GLP-1 RA is of particular interest in the light of the recent outcome trials (32, 34). Besides weight-lowering drugs, bariatric surgery is implemented more often and is associated with faster and larger weight reductions compared to pharmaceutically induced weight reductions.

Precise assessment of glomerular filtration rate (GFR) is pivotal, both to assess the effect of weight loss or gain on kidney function, as well as to assess progression in nephropathy and to monitor safety. Still, methods for monitoring renal function in the presence of weight loss over time have not been validated. The main determinant of creatinine generation/production is skeletal muscle mass, where creatinine is the final catabolite of the energetic metabolism of the muscle (41). Therefore, if body weight - and particularly muscle mass - fluctuates over time, this would influence estimates of renal function, if serum creatinine is also affected, without actual changes in measured GFR. The hypothesis for Part IV of this thesis was that the use of serum creatinine for estimation of GFR may be a limitation in the setting of weight changes. The effect of different degrees of weight loss on estimated and measured kidney function was therefore assessed. For this purpose, we first performed a post hoc analysis of seven randomised clinical trials with liraglutide 0.6-1.8 mg/d (Study E, Table 1) to determine the effect of a small (mean ~ 2 kg), yet clinically relevant, weight reduction on serum creatinine and estimated GFR (42). Next, we designed a study using bariatric surgery as the weight-lowering intervention (Study F, Table 1), in order to determine the impact of a rapid and large weight reduction on estimated and measured GFR. Dual-energy X-ray absorptiometry (DXA) scans were performed to assess changes in body composition (i.e. muscle mass) and relate these changes to changes in serum creatinine (43). Lastly, a post hoc analysis of the two large randomized clinical trials from the Satiety and Clinical Adiposity – Liraglutide Evidence (SCALE) programme with liraglutide 3.0 mg/d was performed to evaluate the impact of a moderate reduction in body weight (mean ~ 8 kg) - including ‘super-responders’ (reduction > 15 kg) – on serum creatinine and estimated GFR (44).

**Patients, Designs and Methods**

**Patients**

All patients gave written informed consent prior to participation. The study protocols were in accordance with the Declaration of Helsinki and ethical approval was obtained for all studies. The patients included in Study A-G (Table 1) had type 2 diabetes according to the WHO definition, whereas Study F and G included obese patients with or without type 2 diabetes (Table 1).

In Study A (24, 26, 27) the 200 patients were between 20 and 70 years old and had persistent microalbuminuria (defined as a urinary albumin excretion rate (UAER) of >30 mg/24 h in at least two of three consecutive measurements). In Study B (29-31) patients were between 35 and 80 years old; 30 patients had normoalbuminuria, 30 had persistent albuminuria (UAER ≥30 mg/24 h) and, 30 controls without diabetes were matched for sex and age to the 30
normoalbuminuric patients. For Study C (36, 45), 35 hypertensive patients older than 18 years with an estimated GFR of > 60 ml/min/1.73 m² were enrolled. Study D (38, 39) included 32 patients older than 18 years, with persistent microalbuminuria and estimated GFR of > 30 ml/min/1.73m², and Study E (42) included 5100 patients aged between 18 and 80 years. For Study F (43) 23 obese patients older than 18 years and with a scheduled gastric bypass surgery were included, and for Study G (44) 4354 patients with obesity, prediabetes or type 2 diabetes older than 18 years were enrolled.

**Designs**

Five different designs were used for this thesis. Study A (24, 26, 27) was prospective with 6 years of follow-up, and Study B (29-31) was cross-sectional. Study C and F (36, 43, 45) were open-label intervention studies, where Study C was a time course study evaluating response to initiation and withdrawal of treatment. Study D (38, 39) was a randomized, placebo-controlled, double-blind, cross-over trial with treatment periods of 12 weeks. Study E and G (42, 44) were post hoc analyses of multicentre, parallel-group, placebo or active-controlled trials.

**Methods**

Standard laboratory assays, biomarkers assays and clinical measurements are described in the publications (24, 26, 27, 29-31, 36, 38, 39, 42-45).

**Coronary artery calcium score:**

In brief, the Agatston scoring method was applied and is a semi-automated tool that calculates a score based on the extent of coronary artery calcification detected by a multidetector-row CT scanner. CAC determination was performed in each patient during a single breath-hold (46).

**Hybrid cardiac PET/CT imaging:**

A detailed description has previously been published (29). In brief, a gated cardiac PET was performed using a hybrid PET/CT-scanner in 3D-mode following the administration of 1,100 MBq ⁸²Rubidium. Maximal hyperaemia was induced with infusion of adenosine at 140 μg kg⁻¹ min⁻¹ for 6 minutes. The myocardial blood flow was calculated using the Siemens Syngo MBF 2.3.

**Flow cytometry analysis:**

A detailed description has previously been published (30). In brief, flow cytometry analysis was accomplished and samples acquired on a FACS LSRFortessa, equipped with blue, red
and violet laser, according to standardised procedures. FACSdiva software (BD Biosciences) was applied for data analysis.

**Measurements of cardiac autonomic function by heart rate analyses:**

A detailed description has previously been published (31). In brief, all electrocardiographic signals for HRV analyses and CARTs were measured with the Vagus device (Medicus Engineering, Aarhus, Denmark). Following five minutes supine resting, five minutes resting heart rate measures for HRV analyses were acquired. Time-domain HRV indices as well as frequency-domain HRV indices were determined.

The CARTs included response to: 1) standing (30-to-15 ratio); 2) deep breathing (E-to-I ratio); and 3) Valsalva test. The E-to-I ratio and 30-to-15 ratio are mainly measures of parasympathetic function, while the Valsalva test measures both sympathetic and parasympathetic function (47).

The CARTs were evaluated in relation to age-related reference intervals (48), and the American Diabetes Association (ADA) criteria (49) was used to define CAN as; no CAN: no pathological CARTs; borderline CAN: one abnormal CART; definite CAN: two or three abnormal CARTs. A standard protocol was used in accordance with recommendations (50).

**Cardiac $^{123}$I-MIBG scintigraphy:**

A detailed description has previously been published (31). In brief, planar cardiac scintigraphy was attained 15 min (early) and 240 min (late) after a tracer injection of 200 MBq $^{123}$I-MIBG. In accordance with published guidelines, a region of interest was drawn above the heart and the mediastinum on early as well as late images (51). The myocardial washout rate from early to late images was calculated in accordance with guidelines of the European Association of Nuclear Medicine Cardiovascular Committee and the European Council of Nuclear Cardiology (51).

The use of the late heart-to-mediastinum ratio for assessment of symptomatic CAN is supported by evidence (52, 53). An abnormal late heart-to-mediastinum ratio was defined as <1.6 (54).

**Glomerular filtration rate:**

Glomerular filtration rate was measured as plasma clearance of $^{51}$Cr-EDTA by standard methods (55). Five equations were applied for the estimation of GFR:

1) Creatinine equation (Cockcroft & Gault): CG eGFR (56); 2) Creatinine equation (MDRD): MDRD eGFR (57); 3) Creatinine equation (CKD-EPI): CKD-EPI-pCr eGFR (58); 4) Cystatin C equation (CKD-EPI): CKD-EPI-cysC eGFR (59); and 5) Creatinine-cystatin C equation (CKD-EPI): CKD-EPI-pCr-cysC eGFR (59).
Components of the renin-angiotensin-aldosterone-system (RAAS):
A detailed description has previously been published (38). In brief, components of the RAAS were measured in experienced academic labs with special attention to sample handling and analysis. Samples for plasma renin concentration, renin activity, angiotensin II and aldosterone levels were determined after 30 minutes of supine rest.
**Results:**

In Paper 1 (Study A, Table 1), a pre-specified risk stratification - based on levels of NT-proBNP and CAC – identified patients at elevated risk of incident cardiovascular disease and all-cause mortality. The 200 patients with type 2 diabetes and microalbuminuria were stratified in the high-risk group if NT-proBNP was $> 45.2$ ng/L and/or CAC was $\geq 400$ at baseline ($n=133$). After a median follow-up of 6 years, risk of both cardiovascular disease and all-cause mortality was increased for these high-risk individuals compared with the patients in the low-risk group ($n=67$). A total of 40 cardiovascular events were recorded; 38 occurred in the high-risk group; and a total of 26 deaths occurred; 24 in the high-risk group (Figure 1).

![Figure 1](image)

<table>
<thead>
<tr>
<th>No. at risk</th>
<th>Cumulative proportional incidence of combined cardiovascular events</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-risk</td>
<td>133 117 105 72</td>
</tr>
<tr>
<td>Low-risk</td>
<td>67   67  66 48</td>
</tr>
</tbody>
</table>

a Risk of cardiovascular events by classification into high- and low-risk at baseline. HR 11.4 (95% CI: 2.7-47.3); $p < 0.0001$.

<table>
<thead>
<tr>
<th>No. at risk</th>
<th>Cumulative proportional incidence of all-cause mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-risk</td>
<td>133 126 120 92</td>
</tr>
<tr>
<td>Low-risk</td>
<td>67 67 66 49</td>
</tr>
</tbody>
</table>

b Risk of all-cause mortality by classification into high- and low-risk at baseline. HR 6.4 (95% CI: 1.5-27.1); $p = 0.004$. 

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Both NT-proBNP and CAC were independent predictors of cardiovascular disease and all-cause mortality, also when analysed as continuous variables (24).

In Paper 2 (Study A, Table 1), higher circulating levels of biomarkers of low-grade inflammation and endothelial dysfunction were associated with incident cardiovascular disease and all-cause mortality. Several markers were of prognostic interest, among these the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-alpha) and the integral membrane protein thrombomodulin were the strongest determinants. Even in a comprehensively adjusted model including traditional cardiovascular risk factors plus NT-proBNP and CAC, higher TNF-alpha remained associated with our defined endpoints (26).

In Paper 3 (Study A, Table 1), the aim was to determine whether two urinary biomarkers, reflecting different renal pathophysiology, provided prognostic information. Higher urinary hepatocyte growth factor was an independent determinant of cardiovascular disease and all-cause mortality and improved risk prediction. Patients were in an exploratory analysis divided into two groups according to the median level of hepatocyte growth factor, and patients with levels above the median had a significantly higher risk of the cardiovascular endpoint and all-cause mortality (Figure 2). Urinary adiponectin was associated with risk of cardiovascular disease and deterioration in renal function (evaluated as a reduction in eGFR of >30% at any time point during the follow-up period) (27).

Figure 2

a Risk of cardiovascular events for patients with urinary levels of HGF above the median value (dashed line); and for patients with urinary levels of HGF below the median value (solid line)
b Risk of all-cause mortality for patients with urinary levels of HGF above the median value (dashed line); and for patients with urinary levels of HGF below the median value (solid line)
**Discussion:**

Despite the beneficial effects achieved by implementation of multifactorial treatment in patients with type 2 diabetes and microalbuminuria (9, 11), the residual risk of cardio-renal complications is still pronounced. Risk prediction models are important components of prevention strategies, and they are implemented in many medical fields. The UKPDS risk engine and the Framingham Risk Score are good examples of risk scores for cardiovascular disease, and these algorithms have led to improvements in prevention and treatment, which have reduced the burden of disease (60, 61). However, there is room for improvement as the low and intermediate risk groups often contribute a high numerical amount of events. Hence, the search for new biomarkers to improve risk prediction and to potentially provide new therapeutic targets is ongoing.

The inevitable and central question that arises is how to evaluate the incremental contribution of a new biomarker to a conventional risk prediction model best. Standard methods have been either too liberal: in genetic studies or in larger samples, a significant p-value is easily achieved, or too conservative: c-statistic or area under the curve are difficult to move when the model already contains a few good, traditional risk factors (62). A distinction between risk prediction and risk classification was introduced in a paper from 2007, suggesting that other measures were necessary that could go beyond statistical significance and c-statistic (63). In recent years, net reclassification improvement and integrated discrimination improvement have been proposed as measures with the ability to determine the degree of correct reclassification (62, 64). In Study A, both improved risk prediction and risk classification for cardiovascular disease and all-cause mortality by addition of CAC or NT-proBNP to traditional risk factors were demonstrated.

Higher NT-proBNP has also previously been shown to predict cardiovascular outcomes and to improve the accuracy in risk prediction for cardiovascular events or death in type 2 diabetes patients (15-19). Similarly, higher CAC has been shown to predict future cardiovascular disease in diabetes patients and was superior to both the UKPDS risk engine and the Framingham Risk Score (23, 65-67). Despite these convincing data on the added prognostic power of both NT-proBNP and CAC in type 2 diabetes, our study was the first to stratify patients in a high- and low-risk group according to NT-proBNP and CAC levels at baseline. Our simple screening algorithm was very efficacious in identifying the patients with highest risk of adverse outcome; however a major concern is how to minimize or even eliminate this risk burden. The most recent ADA standards of medical care in diabetes (68) do not recommend screening of asymptomatic patients with high risk of atherosclerotic cardiovascular disease, in part because intensive medical treatment should already be prescribed for these patients. Furthermore, whether or not a more intensified treatment approach in patients with increased levels of NT-proBNP and/or CAC, remains to be confirmed. In a randomized controlled study in 300 type 2 diabetes patients who had elevated levels of NT-proBNP (>125 pg/ml), it was
demonstrated that treatment with RAAS- and beta-blockers was an efficacious and harmless intervention in preventing cardiovascular events (69). In relation to CAC, a randomized controlled trial of atorvastatin, vitamin C and vitamin E, versus matching placebos included a total of 1005 asymptomatic individuals, but no significant reduction in the primary endpoint of atherosclerotic cardiovascular disease was demonstrated. However, in a subgroup of patients with elevated CAC scores (>400, n=469) treatment with atorvastatin, vitamin C and E reduced number of cardiovascular events significantly (70). In a randomized controlled trial, 102 patients with calcific aortic stenosis were assigned to atorvastatin 80 mg or matched placebo. Forty-eight non-diabetes patients with a median CAC level of 195 (IQR: 57–448) were randomized to atorvastatin, which did not prevent progression of CAC, compared to placebo, during a follow-up of median 24 months (71). Also, it is appealing to hypothesize that treatment with coronary angiography and revascularization at an early stage could be beneficial in this patient population at higher risk, when the best medical treatment strategy has not been elucidated. The effect of coronary revascularization in asymptomatic diabetes patients is however heavily debated, and at present time, invasive procedures are not recommended, since the evidence proving beneficial effects is lacking (72).

Endothelial dysfunction and low-grade chronic inflammation have been associated with higher risk of cardiovascular mortality during 12 years of follow-up in a study of 194 patients with type 2 diabetes, who did not receive multifactorial intervention (73). When we demonstrated several biomarkers of endothelial dysfunction and low-grade inflammation as determinants of cardiovascular disease and all-cause mortality in our study of asymptomatic patients receiving multifactorial treatment, it expands on the existing literature and highlights the harmful role of endothelial dysfunction and low-grade inflammation. It was then speculated and concluded that multifactorial intervention apparently does not stop or neutralize these adverse effects.

Thrombomodulin has previously been shown to be elevated in various diseases, where levels of systemic or local levels of inflammatory cytokines, including TNF-alpha, have also been increased (74). Also, levels of thrombomodulin were found to be higher in atheromatous arterial disease and with the presence of type 1 and type 2 diabetes, and elevated levels of thrombomodulin have been associated with vascular complications (75, 76). In Paper 2, higher thrombomodulin was a determinant of cardiovascular disease and all-cause mortality in asymptomatic type 2 diabetes patients, indicating a link between endothelial-cell damage and adverse outcome.

Disorders in the metabolism of TNF-alpha are implicated in metabolic disorders, such as obesity and insulin resistance (77, 78), and elevated plasma levels of TNF-alpha have been identified in non-diabetic patients with coronary artery disease (79), and have been associated with degree of early atherosclerosis (80). In type 2 diabetes patients, higher TNF-alpha was a strong determinant of cardiovascular disease, all-cause mortality and progression in CAC. It is
tempting to consider targeting markers of inflammation (e.g. TNF-alpha) based on our findings. Currently it is unclear if inhibition of inflammation will lead to reductions in cardiovascular events, however dedicated ongoing intervention studies are aiming to reduce cardiovascular risk by anti-inflammatory drugs in type 2 diabetes (81, 82). Also, the possible cardiovascular benefit of anti-inflammatory drugs should be balanced against adverse effects with this medical approach, and cost of treatment could be an obstacle for its implementation in clinical practice (26).

A novel finding from Paper 3 was that higher urinary hepatocyte growth factor predicted our cardiovascular endpoint and all-cause mortality. Previous studies – in non-diabetic patients - have shown circulating hepatocyte growth factor to be a determinant of mortality in patients with cancer or cardiovascular disease (83, 84) and with incident cardiovascular events (85, 86). However, no previous studies report on the predictive value of urinary levels of hepatocyte growth factor, and in Paper 3, this biomarker is proposed as a new, simple and promising screening tool with the potential to improve risk prediction in type 2 diabetes, but also with the ability to serve as a target of treatment. However, the way from bench to bedside is extensive, and our finding needs first to be validated in a larger cohort. Furthermore, intervention studies targeting hepatocyte growth factor in type 2 diabetic patients may provide important information.

Levels of urinary adiponectin have been shown to be positively correlated with intima-media thickness in a cross-sectional study in 156 patients with type 2 diabetes (87). These findings were extended in Paper 3, where it was demonstrated that higher levels of urinary adiponectin were associated with our cardiovascular endpoint and deterioration in renal function. Also, higher levels of serum adiponectin have been associated with cardiovascular mortality in male type 2 diabetes patients (88). In patients with type 1 diabetes, higher levels of serum adiponectin have been linked to cardiovascular and all-cause mortality and with progression to end-stage renal disease (89, 90). We did not measure serum adiponectin, which was a limitation for this study (27).

Taken together, our findings from Study A suggest that combining NT-proBNP and CAC is a useful tool for the identification of patients at higher risk of cardiovascular disease and all-cause mortality; however the cost and radiation exposure (for CAC) should be weighed against the potential clinical benefit for the patients and the potential profit for the society. A strong association between endothelial dysfunction/low-grade inflammation and development of cardiovascular disease was confirmed. Also, a novel urinary biomarker, hepatocyte growth factor, was proposed to predict cardiovascular disease and all-cause mortality; however this finding needs to be validated in a larger cohort of type 2 diabetic patients.
CARDIAC IMAGING FOR EVALUATION OF CORONARY MICROcirculatory FUNCTION AND CARDIAC AUTONOMIC FUNCTION [PART II]

Results:
In Paper 4 (Study B, Table 1), it was demonstrated that asymptomatic type 2 diabetes patients had impaired CFR and elevated CAC when compared with healthy controls. The findings were even more distinct in patients with elevated levels of albuminuria. It was found that 83% of the type 2 diabetes patients with albuminuria had reduced CFR (< 2.5), compared to 40% of normoalbuminuric patients and 17% of the control subjects. Furthermore, a CAC level higher than 300 was found in 53% of the patients with albuminuria, compared to 27% of normoalbuminuric and 7% of the healthy controls. Also, CFR was demonstrated to be negatively correlated with CAC and UAER and positively correlated with eGFR (Figure 3) (29).

Figure 3

Correlations between coronary flow reserve and (a) coronary artery calcium score, (b) urinary albumin excretion rate and (c) estimated glomerular filtration rate. All correlations were significant ($p \leq 0.001$).
In Paper 5 (Study B, Table 1) impaired CFR was shown to be functionally associated with increased expression of the toll-like receptor-4 on CD8 T cells, increased regulatory T cell number, regulatory T cell maturation and lower interleukin-21 receptor (IL-21R) expression on CD8 T cells. Furthermore, presence of type 2 diabetes was associated with a reduction in overall T cell, T helper 17 cell, IL-21R expression, regulatory T cells and toll-like receptor-4 T cells, while toll-like receptor-4 expression was enhanced (30).

In Paper 6 (Study B, Table 1), cardiac autonomic function, assessed by both HRV tests, CARTs and cardiac $^{123}$I-MIBG scintigraphy, was positively correlated with CFR (Figure 4). When adjusting for age and heart rate, the late heart-to-mediastinum ratio, assessed by $^{123}$I-MIBG scintigraphy, and low frequency power, a HRV measure, were associated with CFR. Even after adjustment for appropriate risk factors, the late heart-to-mediastinum ratio was still significantly associated with CFR. Prevalence of cardiac autonomic neuropathy was 7%, defined by a late heart-to-mediastinum ratio $<1.6$ (54) and 11% based on CARTs and according to the ADA criteria (49). Further, the cardiac autonomic function was reduced in patients with type 2 diabetes compared with control subjects; both when assessed by cardiac $^{123}$I-MIBG scintigraphy (late heart-to-mediastinum ratio) and by HRV indices (31).

**Figure 4**

![Coronary flow reserve and correlations with various measures](image)

Coronary flow reserve and correlations with: late heart-to-mediastinum ratio (A), total power (B), and the Valsalva test (C). $R^2 = 0.32$ (A), $R^2 = 0.27$ (B), and $R^2 = 0.11$ (C). All correlations were significant ($p \leq 0.005$).
**Discussion:**

The findings from Study B provide novel and useful information on the potential underlying mechanisms and prevalence of impaired coronary microcirculatory function, by the use of cardiovascular imaging modalities and flow cytometric analyses of peripheral blood.

In Paper 4, it was demonstrated that even in asymptomatic type 2 diabetes patients, the coronary microcirculatory function is poorer and the overall atherosclerotic burden is higher when compared with non-diabetic controls. A CFR below 2.5 is considered reduced (91), and has been demonstrated to be a predictor of cardiac mortality in patients with diabetes (28). A CAC score above 300 is considered elevated (92) and has been demonstrated to predict cardiovascular disease and mortality in type 2 diabetes patients (23, 93). The high frequency of reduced CFR and increased CAC in the albuminuric group highlights the jeopardy of the combination of type 2 diabetes and albuminuria.

It was suggested that microvascular impairment occurs in multiple beds, since impaired CFR was more common in the diabetic patients with elevated levels of albuminuria, and previous studies have linked impaired CFR with the severity of retinopathy (94) and renal impairment (95). CFR was shown to correlate negatively with CAC, also shown in other cross-sectional studies. In a cohort of 222 patients without known cardiovascular disease, CFR was also shown to correlate negatively with presence and magnitude of CAC (96), and among patients with suspected coronary artery disease, higher CAC was significantly associated with impaired CFR (97). Taken together, coronary microvascular dysfunction may be an essential part in the progression of atherosclerosis, however the link between functional and structural modifications in measures of atherosclerosis is not necessarily forthright and they may reflect dissimilar pathophysiological processes as well as differences in the time course of the disease. This was to some degree mirrored in our study, where three participants with a CAC score of 0 had reduced CFR (< 2.5), indicating that a CAC score of 0 could not be used as a gatekeeper. Cardiac PET imaging is advantageous with regards to radiation safety, comfort and image acquisition duration (<1 hour, as compared to ≈ 2 days for classic cardiac single-photon emission CT), and PET is a promising method to guide personalised care. Furthermore, given the prognostic value of CAC and CFR, cardiac PET/CT scanning could be a useful screening tool with the ability to identify asymptomatic type 2 diabetes patients who have the highest risk of cardiovascular disease. However, the procedure is costly and must find its way into clinical practice by demonstrating its cost-effectiveness. The benefit of newer non-invasive cardiovascular disease screening methods, such as PET or angio-CT, to identify subgroups of patients for different treatment strategies remains unproven. Taken together, the balance of cost, benefit and risks of implementing novel screening modalities in asymptomatic diabetes patients remains controversial, especially in the modern setting of intensive control of known risk factors.
The data from Paper 5 imply that impaired CFR is related to a diminished inflammatory signature combined with increased regulatory activity and therefore provides central clues concerning the link between inflammation and cardiovascular complications in type 2 diabetes. The lower number of systemic IL-21R+ CD8 T cells in patients with impaired CFR suggests that these cells may protect the vasculature in type 2 diabetes. Elevated levels of IL-21 have been suggested to be associated with progression in atherosclerosis in other studies, whereas a recent study demonstrated IL-21R to be directly involved in modulating infarct volume in cerebral ischemia via neuroprotection (98, 99). However, previous studies on this topic have not been conducted in asymptomatic type 2 diabetes patients, and our findings add knowledge to the understanding of the cardiovascular complications associated with type 2 diabetes. Also in Paper 5, it was observed that enhanced numbers of regulatory T cells and an increased expression level of FoxP3 on the regulatory T cells were linked with reduced CFR. A previous study observed that circulating regulatory T cell numbers were elevated in patients with ST-segment elevation myocardial infarction, while reduced in patients with non-ST-segment elevation acute coronary syndrome (100). However, varying results have been reported in other studies. A study found the number of FoxP3 regulatory T cells to be low in all stages of human atherosclerotic lesions (measured in surgical or biopsy samples) (101), and a reduced number of regulatory T cells was associated with carotid atherosclerotic plaque vulnerability (102). Further, a study demonstrated lower levels of regulatory T cells in patients with vulnerable coronary arterial plaques compared with healthy controls (103). Several studies have shown that patients with acute coronary syndrome have fewer regulatory T cells compared with healthy individuals (104-106). Different study populations and cytometry techniques used may explain the conflicting findings (107). Nevertheless, the data from Paper 5 may be valuable, when designing novel drugs, and may provide novel biomarkers useful to identify patients at cardiovascular risk. However, and of importance, this was a hypothesis generating and thought provoking sub-study, thus an extensive series of biomarkers were investigated, which increases the risk of chance findings and validation studies are warranted (30).

In Paper 6, the link between a wide-ranging panel of cardiac autonomic function measures and CFR was investigated, and all measures (except the washout rate estimated from the 123I-MIBG scintigraphy) were shown to be positively correlated with CFR. Similar associations have been evaluated in patients with diabetes in few previous studies. One study included 28 patients with both type 1 and type 2 diabetes, and sympathetic function was assessed by the norepinephrine analog 11C-hydroxyephedrine. In the subjects with sympathetic dysfunction, sympathetically mediated dilation of coronary resistance vessels was reduced (108). Another small study, including type 1 diabetes patients only, concluded that individuals with preclinical microangiopathy had irregularities of cardiac sympathetic innervation and blood flow regulation (109). Our data revealed that the cardiac autonomic function tests that mainly reflected
sympathetic autonomous control and activity were strongly associated with CFR. It was suggested that increased cardiac sympathetic tone and impaired adrenergic receptors may play a significant pathogenetic role in the development of myocardial injury in type 2 diabetes. It has been speculated that increased cardiac sympathetic tone may decrease myocardial vascularity, increase production of reactive oxygen species, untimely myocardial apoptosis and stimulate remodelling in the myocardium (109), ultimately leading to reduced vascular performance of the heart and reduced coronary blood flow (110). A rather low prevalence of CAN (11%) was demonstrated compared to older studies (from 1985 and 1986) reporting prevalences varying between 17% and 20%, which may partly be explained by improvement in clinical care (111, 112). However, cardiac autonomic function was worse in type 2 diabetes patients compared with healthy individuals in Paper 6, and detection of reduced cardiac autonomic function in early stages, to eventually intensify treatment, may prove to be of prognostic benefit.

Despite the thorough evaluation of the association between a large panel of cardiac autonomic function measures and CFR, the sample size is a limitation of our study which increases the likelihood of a type II error, and confirmation of the results in larger studies is warranted (31).
**Results:**

In Paper 7 (Study C, Table 1), open-label liraglutide treatment was associated with acute (after 3-7 days) increases in 24–h systolic blood pressure and 24-h heart rate. These changes were followed by reductions in 24-h systolic blood pressure once treatment was up-escalated to maximum dose (1.8 mg/day) after 21 days. After maximum dose for four weeks, the antihypertensive effect had diminished. Blood pressure changes were more noticeable during the daytime compared to night-time and for office compared to 24–h blood pressure (Figure 5).

**Figure 5**

Effect of liraglutide treatment on: (a) 24–h systolic blood pressure, (b) daytime systolic blood pressure, (c) nighttime systolic blood pressure and (d) office systolic blood pressure.

*Represents a significant change ($p < 0.05$) from baseline.
Significant reductions in UAER, GFR and fractional albumin excretion were found; all independent of blood pressure changes. Further, 24-h heart rate was increased, while extracellular volume and mid-regional pro-atrial natriuretic peptide (MR-proANP) were reduced during liraglutide treatment. During 3 weeks washout of liraglutide treatment, levels of UAER, GFR and fractional clearance of albumin returned to baseline values (Figure 6) (36). After extension of the study (Study C, Table 1, Paper 8), it was observed that, in patients (75%) restarting liraglutide after the washout period, 1 year of treatment resulted in reductions in measured GFR, UAER and fractional clearance of albumin (45). Also, reductions in 24-h systolic blood pressure and MR-proANP were observed, while extracellular volume was unchanged.

**Figure 6**

Effect of liraglutide treatment on:
(a) urinary albumin excretion rate,
(b) glomerular filtration rate and
(c) estimated glomerular filtration rate.
*Represents a significant change ($p < 0.05$) from baseline.
In Paper 9 (Study D, Table 1), 12 weeks of liraglutide treatment, on top of stable RAAS-inhibition, significantly reduced UAER of >30% in type 2 diabetes patients with persistent microalbuminuria (Figure 7). Changes in UAER correlated with changes in 24-h systolic blood pressure and HbA1c. Further, during liraglutide treatment a significant reduction was observed in plasma renin concentration and angiotensin II; however, these changes did not differ significantly from changes during placebo treatment. Liraglutide treatment did not significantly change GFR or 24-h systolic blood pressure (38).

**Figure 7**

![Change in urinary albumin excretion rate (UAER) from baseline to end of treatment. UAER was lowered by 26 (95% CI: 5; 43)% during liraglutide treatment and increased by 9 (95% CI: −6; 22)% during placebo treatment.](image)

In a sub-analysis of Study D (Table 1, Paper 10) the effect of liraglutide treatment on pre-selected biomarkers well-known to be linked with cardiovascular risk in type 2 diabetes were assessed, and we demonstrated treatment to be associated with reductions in TNF-alpha, mid-regional pro-adrenomedullin (MR-proADM) and MR-proANP (Figure 8). These changes occurred independently of changes in blood pressure, UAER, HbA1c and body weight. No effect of treatment on plasma or urinary levels of urokinase plasminogen activator receptor, copeptin or norepinephrine was observed (39).
Figure 8

Individual effects of 12 weeks of liraglutide treatment on:
A) levels of tumour necrosis factor-alpha (TNF-alpha) and
B) levels of mid-regional pro-atrial natriuretic peptide (MR-proANP).
Distribution of both variables was skewed and log10-transformation was applied.

Discussion:
Interventions with the capability to lower the cardio-renal risk in type 2 diabetic patients have been lacking. However, recently the ADA standards of medical care in diabetes (113) included a new recommendation to consider liraglutide treatment (in addition to empagliflozin) to lower the risk of mortality in high-risk type 2 diabetes patients with known cardiovascular disease; this endorsement was based on the results from the LEADER trial (34). Also, the LEADER trial demonstrated a marked lower incidence of new onset nephropathy in the liraglutide treated group (34). Research aiming to expand the understanding of the mechanistic explanations behind these micro- and macrovascular benefits of liraglutide treatment is needed.

The antihypertensive and positive chronotropic effects of liraglutide treatment are well described. A pooled analysis including 2,792 type 2 diabetes patients demonstrated liraglutide (1.2-1.8 mg/day) to be associated with lowering of systolic blood pressure of $\approx 3$ mmHg after 26 weeks of treatment (37). In a systematic review and network meta-analysis, liraglutide (1.2-1.8 mg/day) was associated with a heart rate increase of $\approx 2$ beats per minute versus placebo treatment (114). In Paper 7 (Study C, Table 1) the time course and effect of liraglutide
treatment on ambulatory blood pressure and heart rate was investigated. The initial increase in 24-h systolic blood pressure and 24-h heart rate was speculated to be a result of increased cardiac output and/or side effects. It may also be that GLP-1 receptor stimulation, through activation of autonomic regulatory neurons, increases blood pressure and heart rate, which was suggested based on findings in a preclinical study (115). The subsequent reduction in 24-h systolic blood pressure may be partly explained by reductions in extracellular volume and MR-proANP. The 24-h heart rate was increased throughout the liraglutide treatment period, possibly explained by the existence of GLP–1 receptors in the sinoatrial node (116). The effect of liraglutide on measured GFR and UAER was investigated, and significant and reversible reductions were demonstrated. Based on the novel and potentially clinically relevant findings of changes in renal function, the study was extended to include a 1 year follow-up visit (Study C, Table 1, Paper 8). Twenty-three patients (75%) of the original patients re-started liraglutide, and a decline in GFR was confirmed, which was similar to that observed during short-term treatment. It was therefore likely due to metabolic or hemodynamic reversible effects, and not permanent (structural) changes. In addition, the anti-albuminuric effects were confirmed, which together with glucose-lowering, weight-lowering and antihypertensive effect – suggested renoprotective properties of liraglutide. However, the open-label design without a comparator or placebo control was a limitation and restricted the robustness of our findings, although other preclinical and non-randomized studies had also suggested renoprotective effects of liraglutide (117, 118). Study D was a randomized, placebo-controlled study to investigate the effect of liraglutide treatment on albuminuria in type 2 diabetic patients with persistent micro- or macroalbuminuria. Liraglutide treatment, on top of RAAS-blocking treatment, reduced UAER of >30%. This effect may be partly explained by reductions in 24-h systolic blood pressure and HbA1c, and linked to an effect on the RAAS. While other and larger studies have shown the same tendency of reductions in albuminuria, the changes were insignificant (119, 120). This may be explained by lower baseline levels of albuminuria, and fewer urine samples at less time points. In a recent meta-analysis it was suggested that short-term reduction in albuminuria is associated with long-term renal protection across different interventions and populations (121). Also, the authors suggested that for new interventions, reduction in albuminuria of at least 30% - on top of recommended standard of care - seems required to confer a detectable renoprotective effect (121). However, whether the >30% reduction in albuminuria associated with liraglutide treatment in our study will translate into clinical renoprotection needs to be demonstrated in long-term outcome studies in relevant populations. Furthermore, despite the randomized, placebo-controlled design, Study D is limited by the rather small sample size and the short duration. No statistical change in GFR was observed during liraglutide treatment, which was conflicting compared to our time course study (Study C), however a similar finding was demonstrated in a recent study including 19 overweight type 2 diabetes patients also treated with liraglutide for 12 weeks (122). Also, a
study using integrated data from nine trials including a total of 6,005 type 2 diabetes patients found no differences in eGFR during dulaglutide treatment vs. placebo, insulin glargine or active comparators (123). The difference in baseline GFR levels may be an explanation of the diverging results, since a larger reduction may be expected with a higher baseline level, as in Study C (36).

In Paper 10, the effect of liraglutide treatment on cardiovascular risk biomarkers was investigated, and by reducing levels of TNF-alpha and MR-proADM, anti-inflammatory actions of liraglutide were suggested, which may contribute to the beneficial cardiovascular effects demonstrated in the LEADER trial (34, 39). A previous study in type 2 diabetic patients did not detect significant changes in TNF-alpha during liraglutide treatment, but demonstrated reduction in two other cardiovascular risk biomarkers: plasminogen-activator inhibitor-1 and BNP (124). A significant reduction in MR-proANP was found, also observed in the time course study (Study C), which might reflect a reduced atrial and ventricular distention, and perhaps a prognostic benefit in heart failure, although this was not identified in the LEADER trial, in the FIGHT study or in the LIVE study (34, 125, 126). Whether the observed reductions in cardiovascular risk biomarkers are linked to cardiovascular benefit cannot be determined by our short-term study, however may provide novel pathophysiological information on the cardio-protective effects of liraglutide treatment.
EFFECT OF WEIGHT REDUCTIONS ON MEASURED AND ESTIMATED RENAL FUNCTION [PART IV]

Results:
In Paper 11 (Study E, Table 1), a mean body weight reduction of ≈ 2 kg in type 2 diabetic patients treated with liraglutide (1.2-1.8 mg/day) for 26 weeks, did not yield changes in serum creatinine. Hence, no change in weight independent estimates of GFR (MDRD and CKD-EPI equations) was observed, whereas the weight dependent estimate (Cockcroft-Gault equation) was influenced.

In Paper 12 (Study F, Table 1), a rapid and large weight loss of mean 27 kg, obtained 6 months after Roux-en-Y gastric bypass surgery, was associated with a significant reduction in plasma creatinine which led to increases in creatinine-based eGFR (MDRD and CKD-EPI), while there was no change in cystatin C-based eGFR. The weight reduction was associated with a reduction in absolute measured GFR, while there was no change in measured GFR, when adjusted for concurrent body surface area. Lean limb mass is considered a surrogate measure of muscle mass and can be assessed from DXA scans. Lean limb mass was reduced by mean 3.5 kg and was associated with reduction in creatinine.

In Paper 13 (Study G, Table 1), body weight reductions of mean 6–8 kg in persons with obesity, prediabetes or diabetes - treated with liraglutide 3.0 mg/day for 56 weeks - were not associated with changes in serum creatinine (Figure 9). Findings were similar in analyses restricted to high weight-loss responders (reduction >15%), both after 28 and 56 weeks of treatment.

Figure 9

Association between changes in body weight and changes in serum creatinine in the SCALE Obesity and Prediabetes trial after 56 weeks. Reference lines are from ANCOVA model with change from baseline in body weight and age as covariates, sex and treatment group as factors, and interaction between change in body weight and treatment group.
Discussion:

The optimal method of monitoring renal function in obese patients is currently being debated, since the different measures and equations have never been validated in this patient population. The precise way of measuring renal function consecutively in the setting of weight changes is also undetermined. Since eGFR is considered an important prognostic marker of both renal and cardiovascular disease (127, 128), it is essential to gain knowledge on how to best monitor renal function in patients experiencing weight changes. Taken together, our findings from Study E-G provide novel and important physiological information on the effect of weight reductions (small, moderate, large) on circulating levels of creatinine, estimations of GFR and measured GFR.

In Paper 11, a small weight reduction induced by liraglutide treatment did not affect levels of serum creatinine, and it was concluded that the MDRD and CKD-EPI eGFR equations can be used in patients experiencing a pharmaceutically induced body weight reduction of ≈ 2 kg. In contrast, estimates of renal function using the Cockcroft-Gault equation, which includes both body weight and creatinine, were reduced in patients treated with liraglutide, since body weight was lowered and creatinine remained unaltered. The study strength was the large individual level data on more than 5,000 patients, but conclusions on the influence of liraglutide on kidney function were limited by the absence of information on measured GFR (42). Our general hypothesis of a link between body weight changes and changes in creatinine levels was however demonstrated in Paper 12, where a fast and large weight reduction was associated with significant reductions in creatinine. Previous studies had demonstrated reductions in creatinine following bariatric surgery, and reductions in muscle mass have been proposed as an explanation (129, 130). However, an actual association between reduction in muscle mass and reduction in creatinine was demonstrated in Paper 12. Further, the large weight reduction did not yield changes in measured GFR or cystatin-C based eGFR, when corrected for body surface area, whereas absolute measured GFR was reduced. The findings emphasize the need for non-creatinine based eGFR equations when monitoring renal function in the setting of bariatric surgery. We speculated if a moderate weight reduction could also be associated with reductions in creatinine, which was the rationale for Paper 13 (Study G, Table 1). No association was demonstrated between moderate weight reduction and creatinine. Analyses restricted to patients with the largest weight reduction (>15 % ≈ 15 kg) were confirmatory. One explanation of the discrepancy between the results in Paper 12 and 13 could be that the greater caloric deficit after bariatric surgery may lead to a greater loss of muscle mass. Furthermore, while body weight was gradually decreased in patients included in the SCALE programme over the time course of one year, bariatric surgery is associated with a more rapid and larger weight reduction. After bariatric surgery, the body enters a catabolic state, where the body prefers to use muscle as energy before consuming fat, which contributes to muscle mass reduction (131). It might therefore be that fast and large weight reductions following bariatric
surgery are associated with greater reductions in muscle mass when compared to the more gradual and moderate weight reductions over a longer time window obtained in the SCALE programme. Despite the large individual level data, Study G lacked information on changes in body composition (i.e. DXA scans) and measured GFR, limiting the ability to compare findings. However, the results are in line with findings from Paper 11 (42), indicating that both small and moderate liraglutide-induced weight reductions do not change serum creatinine, and creatinine-based eGFR equations can be applied to monitor treatment safety (44).

Taken together, the findings from Part IV of this thesis suggest that in large-scale clinical trials where patients are experiencing small to moderate weight reductions, there is apparently no (or limited) impact of body weight/muscle mass reduction on levels of circulating creatinine, which suggests that creatinine-based estimations of GFR are suitable to use for safety monitoring. On the other hand, creatinine-based estimations should be avoided in the setting of bariatric surgery, where the fast and large weight reduction results in reductions in creatinine producing increased creatinine-based eGFR which is not reflected by measured GFR or cystatin C-based eGFR. Although measured GFR is regarded as the gold standard, this method is both time consuming and expensive, and we therefore suggest cystatin C as the preferred method of estimating GFR in the setting of fast and large weight changes.
CONCLUSIONS / PERSPECTIVES

The first aim of this thesis was to find valid early markers to identify type 2 diabetes patients at highest risk of cardiovascular disease and mortality, and we identified several biomarkers with this ability. NT-proBNP, CAC and urinary hepatocyte growth factor were especially strong determinants and addition of these biomarkers to traditional risk factors improved diagnostic accuracy. Based on our findings, together with previous large-scale studies in asymptomatic type 2 diabetes patients (66, 67, 132-138), the role of CAC as a robust predictor of cardiovascular disease and all-cause mortality seems documented. Therefore, despite the radiation exposure and cost, CAC may be a helpful screening tool in a broader population of asymptomatic type 2 diabetes patients. Combining the prognostic information of CAC with NT-proBNP may even prove to improve risk stratification further. It is pivotal to identify patients at highest risk of cardiovascular disease, since a lower target setting for traditional risk factors, such as blood pressure and LDL-cholesterol, may improve the prognosis for these individuals. However, this remains speculative and dedicated studies with this purpose are warranted. As an example, we have proposed a study to enrol asymptomatic type 2 diabetic patients, where we will stratify the patients in a high-risk group based on levels of CAC > 100. These patients will be randomized to intensified lipid-lowering treatment (PCSK9-inhibitors) or placebo on top of guideline recommended treatment, including maximal tolerated statin treatment. Patients stratified in the low-risk group (CAC ≤ 100) will be included as control subjects. The study is designed as a pilot cardiovascular outcome trial with the primary endpoint being 4-point major adverse cardiovascular events. The study has not yet been initiated as sufficient funding has not been obtained. Presence of low-grade inflammation and endothelial dysfunction were also illustrated to be essential risk indicators in type 2 diabetes patients; and it may be that treatment exerting anti-inflammatory effects can reduce the burden of cardiovascular disease associated with type 2 diabetes. The results of the Cardiovascular Inflammation Reduction Trial (CIRT) are highly anticipated. The trial was designed to test if treatment with low-dose methotrexate can reduce rates of cardiovascular disease among stable coronary artery disease patients with type 2 diabetes or metabolic syndrome. A total of 7,000 patients will be randomly allocated to treatment, and the trial is expected to be completed in December 2019 (ClinicalTrials.gov Identifier: NCT01594333) (81).

The second aim of this thesis was to improve the understanding of the aetiology and pathophysiology of cardio-renal complications, and by applying novel and advanced cardiovascular imaging modalities together with flow cytometry of peripheral blood, important knowledge of early complications in asymptomatic type 2 diabetic patients has been gained. The prevalence of reduced CFR and increased CAC was significantly higher in patients with microalbuminuria, and novel inflammatory signatures in peripheral blood that were associated with impaired CFR were identified. Further, a strong correlation between several measures of
cardiac autonomic neuropathy and CFR was demonstrated. The findings highlight the presence of pathophysiological alterations in type 2 diabetes patients prior to development of symptoms and/or disease. Whether treatment options exist with the capability to modify these alterations and/or to improve prognosis for these patients remain to be further investigated. However, a recent study demonstrated that treatment with the mineralocorticoid receptor antagonist spironolactone improved the CFR in type 2 diabetes patients without clinical ischemic heart disease (139). Currently, we are awaiting the completion of the EU-funded prospective randomized, double-blind, placebo-controlled multicenter study PRIORITY including 1,811 normoalbuminuric type 2 diabetes patients (ClinicalTrials.gov Identifier: NCT02040441). While the primary aim is to test the clinical utility of urinary proteomics, the study drug is spironolactone and the hypothesis is that treatment will improve the renal and cardiovascular prognosis in high-risk (stratified by urinary proteomics), normoalbuminuric and asymptomatic type 2 diabetic patients. Also, the effect of finerenone, a novel mineralocorticoid receptor antagonist, is currently being investigated in a phase 3 programme in diabetic kidney disease, where two studies are being carried out. FIGARO-DKD will investigate finerenone vs. placebo in 6,400 patients, and the primary endpoint is the composite of cardiovascular death and non-fatal cardiovascular events. The study is expected to be completed in February 2020 (ClinicalTrials.gov Identifier: NCT02545049).

Concurrently, FIDELIO-DKD will investigate finerenone in comparison with placebo in 4,800 patients with the primary composite endpoint of onset of kidney failure, a sustained decrease of eGFR ≥ 40% from baseline over at least 4 weeks and renal death. The study is expected to be completed in October 2019 (ClinicalTrials.gov Identifier: NCT02540993).

The third aim of this thesis was to assess the cardio-renal effects of liraglutide treatment. Following the LEADER trial (34), treatment with liraglutide has gained much attention, since physicians all of the sudden are in possession of a glucose-lowering therapy with significant cardiovascular benefit in type 2 diabetic patients at high risk of cardiovascular disease. The effect of liraglutide on ambulatory blood pressure, measured kidney function and UAER in type 2 diabetes patients was investigated and an anti-albuminuric effect was shown. It was speculated that liraglutide had renoprotective effects, which subsequently were confirmed in the LEADER trial (34). However, the LEADER trial was a cardiovascular outcome study and therefore not designed or powered to detect long-term renal effects. Also, the effect of liraglutide on microvascular outcomes was mainly driven by a reduced risk of developing new and persistent macroalbuminuria. Whether liraglutide treatment is associated with beneficial effects on hard renal outcomes (i.e. need for renal replacement therapy or renal death) in type 2 diabetes needs to be determined in dedicated diabetic nephropathy studies.

Liraglutide treatment reduced cardiovascular biomarkers, and anti-inflammatory effects were proposed, which may to some extent explain the cardioprotection observed in the LEADER trial. To directly assess the anti-inflammatory potential of liraglutide, we have de-
signed a randomized, placebo-controlled, double-blind, parallel clinical PET/CT trial to investigate the effect on vascular inflammation in type 2 diabetes. The primary endpoint is change from baseline to 6 months of liraglutide treatment in vascular inflammation, assessed by FDG-PET/CT. This study will provide novel and important information to improve the understanding of the cardiovascular mode of action of liraglutide. First patient first visit is expected in August, 2017.

The final aim of this thesis was to test different methods in the assessment of renal function following weight changes, and it was concluded that weight reductions (of different magnitude) obtained by pharmaceutical intervention and counselling on lifestyle modification are apparently not associated with reductions in creatinine. For weight management programmes, it was therefore suggested that creatinine-based estimations of kidney function can be applied to monitor safety. However, following a fast and large weight reduction obtained by bariatric surgery, creatinine levels are reduced leading to increases in estimations of GFR, which are not reflected by measured GFR, and which are likely explained by reduction in skeletal muscle mass. Taken together, the results provide noteworthy implications for both clinicians and researchers aiding to improve the understanding of glomerular filtration rate physiology and highlight the restrictions of using creatinine-based equations in the setting of body weight changes. The literature on the long-term effect of a large and sustained weight reduction on different measures of kidney function is very sparse. We have included a follow-up visit ≈ 18 months after the bariatric surgery (still on-going), and we are planning to include an additional follow-up visit after 5 years to assess the long-term effect of bariatric surgery on measured and estimated kidney function.
<table>
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<tr>
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<td>4-6</td>
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<td>11</td>
<td>12</td>
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<td>Cross-sectional</td>
<td>Intervention study</td>
<td>Randomized clinical trial</td>
<td>Post hoc analysis</td>
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<td><strong>Number of participants</strong></td>
<td>200</td>
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<td>32</td>
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<td>20</td>
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<td>T2DM and normo/micro/macro-albuminuria; and healthy controls</td>
<td>T2DM and hypertension</td>
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T2DM, type 2 diabetes mellitus; LEAD, Liraglutide Effect and Action in Diabete; SCALE, Satiety and Clinical Adiposity – Liraglutide Evidence
REFERENCES


Paper I
Additive prognostic value of plasma N-terminal pro-brain natriuretic peptide and coronary artery calcification for cardiovascular events and mortality in asymptomatic patients with type 2 diabetes

Bernt Johan von Scholten 1*, Henrik Reinhard 1, Tine Willum Hansen 1, Morten Lindhardt 1, Claus Leth Petersen 2, Niels Wiinberg 3, Peter Riis Hansen 4, Hans-Henrik Parving 5,6, Peter Karl Jacobsen 7 and Peter Rossing 1,6,8

Abstract

Background: In patients with type 2 diabetes, cardiovascular disease (CVD) is the major cause of morbidity and mortality. We evaluated the combination of NT-proBNP and coronary artery calcium score (CAC) for prediction of combined fatal and non-fatal CVD and mortality in patients with type 2 diabetes and microalbuminuria (>30 mg/24-h), but without known coronary artery disease. Moreover, we assessed the predictive value of a predefined categorisation of patients into a high- and low-risk group at baseline.

Methods: Prospective study including 200 patients. All received intensive multifactorial treatment. Patients with baseline NT-proBNP >45.2 ng/L and/or CAC ≥400 were stratified as high-risk patients (n = 133). Occurrence of fatal- and nonfatal CVD (n = 40) and mortality (n = 26), was traced after 6.1 years (median).

Results: High-risk patients had a higher risk of the composite CVD endpoint (adjusted hazard ratio [HR] 10.6 (95 % confidence interval [CI] 2.4-46.3); p = 0.002) and mortality (adjusted HR 5.3 (95 % CI 1.2-24.0); p = 0.032) compared to low-risk patients. In adjusted continuous analysis, both higher NT-proBNP and CAC were strong predictors of the composite CVD endpoint and mortality (p ≤ 0.0001). In fully adjusted models mutually including NT-proBNP and CAC, both risk factors remained associated with risk of CVD and mortality (p ≤ 0.022). There was no interaction between NT-proBNP and CAC for the examined endpoints (p ≥ 0.31).

Conclusions: In patients with type 2 diabetes and microalbuminuria but without known coronary artery disease, NT-proBNP and CAC were strongly associated with fatal and nonfatal CVD, as well as with mortality. Their additive prognostic capability holds promise for identification of patients at high risk.

Introduction

In patients with type 2 diabetes, cardiovascular disease (CVD) is the major cause of morbidity and mortality. Compared with subjects without diabetes, the risk of cardiovascular complications is two to four times increased, and is even higher in patients with diabetes and established albuminuria [1, 2].

Several risk scores have been developed to estimate the cardiovascular risk in asymptomatic subjects. The Framingham Risk Score is the most commonly applied global risk score [3]. While proven to be useful to identify subjects at risk, these scoring models fail to identify up to 35 % of future CVD. Moreover, as risk scoring programs are not as predictive in diabetic patients compared to the general population better screening tools are needed for the latter large patient population [4]. The UKPDS Risk Engine is a type 2 diabetes specific risk calculator that is probably the most widely used and which estimates absolute risk of coronary heart disease or stroke using traditional risk factors plus diabetes-specific factors including duration of diabetes and HbA1c [5]. Several studies have examined the validity of the
UKPDS risk engine with inconsistent results and revised risk equations have been suggested [6, 7].

Brain natriuretic peptide (BNP) and its cleavage product N-terminal (NT)-proBNP are secreted in response to cardiac haemodynamic stress mediated by volume and pressure overload [8]. BNP exhibits biological activity while NT-proBNP has none, and when it comes to analytical methods the in-vitro stability of BNP is assay-dependent, whereas NT-proBNP is very stable at room temperature and measurement of NT-proBNP is therefore most often used in clinical practice [9].

In a type 2 diabetic population followed for 15 years, we previously identified plasma NT-proBNP levels as a powerful predictor of mortality, independent of urinary albumin excretion rate (UAER) and other risk factors [10]. Eighty percent of patients in the upper NT-proBNP tertile (>103 ng/L) died compared to 30% in the lower tertile (<41 ng/L; p < 0.001). Other studies have also shown a strong association of NT-proBNP with vascular outcomes [11, 12] and mortality [13] in patients with type 2 diabetes.

The coronary artery calcium score (CAC) is a non-invasive screening tool derived from a computed tomography (CT) scanning [14] that reliably identifies patients at high risk of future adverse cardiac events [15]. CAC reflects the global coronary atherosclerotic burden, and the scoring can be quantified by the Agatston score [16]. Diabetic patients with CAC of 0 have the same risk of CVD events as people without diabetes [17], and CAC > 400 has been defined as representative of severe coronary artery disease (CAD) with high risk of anatomic coronary stenosis as determined by coronary angiography (CAG) [18, 19].

To the best of our knowledge, the prognostic value of a combination of NT-proBNP and CAC has never been examined in patients with type 2 diabetes. To address this issue, we evaluate the joint predictive value of NT-proBNP and CAC for combined fatal and non-fatal CVD, and all-cause mortality, respectively, in patients with type 2 diabetes and microalbuminuria, but without known CAD. Moreover, we evaluate the predictive value of our predefined categorisation of patients into a high- and low-risk group at baseline. The pre-specified study hypothesis was that a screening algorithm based on NT-proBNP and CAC was able to identify asymptomatic patients at high risk. Furthermore, analyses of NT-proBNP and CAC as continuous variables were performed to support the results of the categorical analyses.

Materials and methods

Participants and study procedure

At Steno Diabetes Center, we identified from January 2007 to February 2008 a cohort of 200 outpatients with type 2 diabetes treated in a secondary care setting. All patients received treatment with intensive multifactorial intervention constituting of strict glycaemic, lipid and blood pressure control, as well as antithrombotic therapy and lifestyle modification according to the Steno-2 study [20]. Patients were aged between 20 and 70 years, with the ability to understand and give informed consent. Patients were included if they met the following inclusion criteria: 1) outpatients with type 2 diabetes defined according to WHO criteria; 2) no history of CAD or other cardiac diseases and without any symptoms from the heart, assessed from patient files and thorough interviews and questionnaires; and 3) persistent (two out of three consecutive measurements) UAER > 30 mg/24 h.

Written invitation was sent to 613 consecutive patients (69 % males and a mean (standard deviation [SD]) age of 47 (8) years). A total of 72 patients refused to participate. Furthermore, patients (n = 341) were excluded (either by phone interview or after examination in the outpatient clinic) if one or more of the following characteristics were present: 1) normal UAER or non-persistent elevated UAER (n = 52); 2) symptoms/signs or history of heart disease including Q waves in 12-lead ECG (n = 180); 3) relative contraindications to CT angiography (CTA) or CAG, including abnormal plasma creatinine levels (n = 86); 4) physical or mental disability (n = 10); or 5) malignancy (n = 13). Thus, the final study population included 200 patients. A detailed flow chart of the selection of the study population is shown in Fig. 1. The baseline data has previously been presented [8].

The a priori sample size calculation was based on the assumption that 20-30 % of the patients would experience a cardiovascular event during 5 years of follow-up. The treating physicians were blinded to the predictor variables NT-proBNP and CAC during the follow-up period. This study complies with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all patients gave written informed consent.

Biochemical analyses and other

NT-proBNP was measured in all patients and analysed by an immunoassay as previously described [10]. UAER was measured in 24 h urine collections by an enzyme immunoassay [21]. Current smoking was defined as one or more cigarettes/cigars/pipes a day. The estimated Glomerular Filtration Rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [22]. Transthoracic echocardiography was performed using a Philips IE 33 machine (Philips Medical Systems, Best, The Netherlands). Simpson’s apical biplane method was used to evaluate left ventricle ejection fraction (LVEF), as previously described [23].
Coronary artery calcium score

Determination of CAC was performed during a single breath hold using a 16 multidetector-row CT scanner with 3-mm-slice thickness (Philips Precedence MX 8000 IDT 16 slice; Philips Medical Systems, Best, The Netherlands). Quantification of Agatston CAC [16], including intimal and medial calcification in the left main, left anterior descending artery, circumflex artery and right coronary artery, was performed on a separate workstation with dedicated software (Heartbeat-CS, EBW; Philips Medical Systems) and summed to provide a total CAC for each participant.

Stratification into risk groups

Patients were stratified into high-risk and low-risk groups according to CAC and the median NT-proBNP of the first 50 patients examined in the study [8]; (i) NT-proBNP ≥45.2 ng/L = high-risk patients (n = 104); (ii) NT-proBNP ≤45.2 ng/L and CAC ≥400 = high-risk patients (n = 29) and (iii) NT-proBNP ≤45.2 ng/L and CAC < 400 = low-risk patients (n = 67). High-risk patients (n = 133) were further examined by myocardial perfusion scintigraphy imaging (MPI), CTA or CAG according to the algorithm shown in Fig. 1, which has previously been described in details [8]. Based on these additional examinations, 70 high-risk patients without any cardiovascular symptoms was exposed to have significant CAD, defined as the presence of one or more significant myocardial perfusion defects on myocardial perfusion imaging, and/or one or more significant major epicardial coronary artery stenosis at coronary angiography.

Follow-up

At 1st of January 2014, we traced all patients through the Danish National Death Register and the Danish National Health Register. For deceased patients, we obtained information on the date and cause of death. All deaths were classified as CVD unless an unequivocal non-CVD cause was established. Information about
hospital admission including non-fatal CVD was obtained from the Danish National Health Register.

The predefined primary endpoint was the combination of cardiovascular mortality, non-fatal myocardial infarction (ICD-10 codes 120 to I25), stroke (ICD-10 codes I61 or I63), ischaemic cardiovascular disease (ICD-10 codes I70), and heart failure (ICD-10 code I50). For participants who experienced multiple endpoints, the analysis included only the first event.

The secondary endpoint was all-cause mortality. No participants were lost to follow-up.

**Statistical analyses**

Logarithmic transformation was performed to achieve normal distribution for NT-proBNP, CAC and UAER. All continuous variables are given as medians with interquartile range (IQR) and the categorical variables are reported as total numbers with corresponding percentages. Differences between groups were calculated using Mann–Whitney U Test for continuous and \( \chi^2 \) for categorical variables.

First, we used Kaplan-Meier survival function estimates and the log-rank test to estimate and compare incidence rates by risk group. Next, we used Cox proportional hazards analyses to compute hazard ratios (HRs) and 95% confidence intervals (CIs) for 1 SD increase of the log transformed values. We adjusted for sex, age, LDL and HDL cholesterol, smoking, HbA1c, eGFR, systolic blood pressure and UAER. In fully adjusted models with known risk factors for CVD, we additionally included NT-proBNP and/or CAC. We evaluated the additive vs. synergistic effects of NT-proBNP and CAC on the endpoints by using appropriate interaction terms and likelihood-ratio tests. The additive predictive value of both NT-proBNP and CAC were evaluated with the use of relative integrated discrimination improvement (rIDI) based on the Cox models. In addition, the continuous net reclassification index (cNRI), with 5% risk reclassification as cut-off, was calculated based on logistic models. To facilitate interpretation of the results, we also computed 5-year absolute risk of the composite CVD endpoint and all-cause mortality associated with CAC at different levels of NT-proBNP from the adjusted Cox models.

A two-tailed \( p \)-value ≤ 0.05 was considered significant. Statistical analyses were performed using SPSS for Windows, version 20.0 (SPSS, Chicago, IL) and SAS software (version 9.3 and Enterprise Guide version 5.1; SAS Institute, Cary, NC).

**Results**

**Patient characteristics and risk groups**

The study population (n = 200) included 76% men, median (IQR) age was 60 (54–65) years, and median (IQR) of NT-proBNP and CAC was 48.7 (18.6–95.0) ng/L and 183 (6–604), respectively. Table 1 lists clinical characteristics of all patients and the comparisons between the high-risk and low-risk group. High-risk patients were older, had longer known diabetes duration, lower eGFR and total cholesterol levels, and were more frequently on beta-blocker treatment than patients classified as low-risk (\( p \leq 0.048 \)).

In addition to oral antidiabetic medications and insulin most patients were treated with cardiovascular medications: statins (95%), aspirin (90%), renin-angiotensin-aldosterone system blockade (98%), diuretics (64%), calcium channel blockers (40%) and beta-blockers (14%).

**Incidence of endpoints**

Median follow-up was 6.4 (range: 5.8–7.2) years for the survivors, 6.2 (0.8–7.2) years for the patients reaching the CVD endpoint, and 3.8 (0.3–6.8) years for the non-survivors. During this period, 40 patients experienced at least one CVD endpoint and 26 patients died.

Eleven CVD events were fatal and 29 were non-fatal events leading to hospital admission, including 2 fatal and 3 non-fatal cases of acute myocardial infarction, 3 non-fatal strokes, 1 fatal and 19 non-fatal cases of ischaemic cardiovascular disease, 6 sudden and otherwise unexplained deaths, and 2 fatal and 4 non-fatal cases of heart failure. Of the 26 deaths, 10 were CVD-related, 9 were cancer-related and 7 were due to other causes.

**Risk prediction in high-risk versus low-risk group**

Of the 40 patients with a CVD endpoint, 38 were in the high-risk and two in the low-risk group. The 26 deceased patients comprised 24 in the high-risk and two in the low-risk group.

In a Cox regression analysis, patients classified at high-risk at baseline had a significantly higher risk of the composite CVD endpoint compared to low-risk patients (unadjusted HR 11.4 (95% CI 2.7–47.3); \( p = 0.001 \), Fig. 2a; adjusted HR 11.8 (95% CI 2.7–52.8); \( p = 0.002 \)). Similarly, high-risk patients had a significantly higher risk of all-cause mortality in both unadjusted (HR 6.4 (95% CI 1.5–27.1); \( p = 0.012 \), Fig. 2b) and adjusted (HR 5.9 (95% CI 1.3–27.0); \( p = 0.022 \)) models.

**Risk associated with NT-proBNP and CAC in continuous analyses**

In adjusted continuous analysis, higher NT-proBNP was a strong predictor of the composite CVD endpoint and all-cause mortality (\( p \leq 0.002 \); Table 2). In the adjusted model including NT-proBNP, higher age and LDL were also predictors of the composite CVD endpoint (\( p \leq 0.047 \)), while only smoking was a predictor of all-cause mortality (\( p = 0.021 \)).
Table 1 Baseline clinical characteristics of all patients, and low- versus high-risk patients

<table>
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<tr>
<th></th>
<th>All patients (n = 200)</th>
<th>Low-risk patients (n = 67)</th>
<th>High-risk patients (n = 133)</th>
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<td>Male, n (%)</td>
<td>152 (76)</td>
<td>50 (75)</td>
<td>102 (77)</td>
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<tr>
<td>Age (years)</td>
<td>60 (54–65)</td>
<td>55 (47–61)</td>
<td>62 (59–67)</td>
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<td>Duration of diabetes (years)</td>
<td>12 (7–18)</td>
<td>8 (4–14)</td>
<td>14 (9–19)</td>
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<td>Body mass index (kg/m²)</td>
<td>31.4 (28.5–35.6)</td>
<td>31.7 (28.7–35.9)</td>
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<td>HbA₁c (%)</td>
<td>7.6 (6.9–8.8)</td>
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<td>HbA₁c (mmol/mol)</td>
<td>60 (52–73)</td>
<td>63 (53–75)</td>
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<td>UAER (mg/24 h)</td>
<td>103 (99–230)</td>
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<td>eGFR (CKD-EPI)</td>
<td>91.0 (76.0–102.0)</td>
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<td>86.0 (740–97.5)</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>130 (116–140)</td>
<td>129 (119–142)</td>
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<td>Total cholesterol (mmol/L)</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>1.7 (1.3–2.2)</td>
<td>1.8 (1.2–2.4)</td>
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<td>HDL cholesterol (mmol/L)</td>
<td>1.1 (0.9–1.4)</td>
<td>1.1 (1.0–1.3)</td>
<td>1.1 (0.9–1.4)</td>
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<td>Current smoker, n (%)</td>
<td>59 (30)</td>
<td>18 (27)</td>
<td>41 (31)</td>
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<td>Left ventricle ejection fraction (%)</td>
<td>60 (57–63)</td>
<td>60 (57–62)</td>
<td>60 (57–63)</td>
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<td>History of stroke, n (%)</td>
<td>19 (10)</td>
<td>4 (6)</td>
<td>15 (11)</td>
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<td>NT-proBNP (ng/L)</td>
<td>48.7 (18.6–95.0)</td>
<td>15.3 (9.3–26.3)</td>
<td>77.1 (48.7–141.7)</td>
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<td>Coronary artery calcium score</td>
<td>183 (6–604)</td>
<td>7 (0–104)</td>
<td>417 (80–963)</td>
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<td>Treatment with</td>
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<td>Oral antidiabetic, n (%)</td>
<td>170 (85)</td>
<td>57 (85)</td>
<td>113 (85)</td>
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<td>Insulin, n (%)</td>
<td>124 (62)</td>
<td>38 (57)</td>
<td>86 (65)</td>
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<td>RAAS blockade, n (%)</td>
<td>196 (98)</td>
<td>65 (97)</td>
<td>131 (98)</td>
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<td>Statin, n (%)</td>
<td>189 (95)</td>
<td>62 (93)</td>
<td>127 (95)</td>
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<td>Aspirin, n (%)</td>
<td>183 (92)</td>
<td>58 (87)</td>
<td>125 (94)</td>
<td>0.08</td>
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<td>Beta-blocker, n (%)</td>
<td>27 (14)</td>
<td>2 (3)</td>
<td>25 (19)</td>
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<tr>
<td>Calcium channel blockers, n (%)</td>
<td>80 (40)</td>
<td>21 (31)</td>
<td>59 (44)</td>
<td>0.08</td>
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<tr>
<td>Diuretics, n (%)</td>
<td>128 (64)</td>
<td>35 (52)</td>
<td>93 (70)</td>
<td>0.014</td>
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</tbody>
</table>

High-risk patients = patients with plasma NT-proBNP levels ≥45.2 ng/L, or plasma NT-proBNP levels ≥45.2 ng/L, and coronary artery calcium score ≥400, all other low-risk patients

P-values reflect comparison between high- and low-risk patients

Data are expressed as median (interquartile range) or number of patients (%)

UAER: urinary albumin excretion rate; RAAS: renin-angiotensin-aldosterone system

Also, adjusted continuous analysis of CAC revealed a strong independent prediction for risk of both the composite CVD endpoint and all-cause mortality (p ≤ 0.006; Table 2). In the adjusted model including CAC, no other variables were predictive of the composite CVD endpoint or all-cause mortality (p ≥ 0.12).

Both NT-proBNP and CAC remained significantly associated with risk of CVD and all-cause mortality in fully adjusted models mutually including both risk factors (p ≤ 0.022; Table 2). Addition of the interaction term between NT-proBNP and CAC was non-significant in relation to both CVD events (p = 0.90) and all-cause mortality (p = 0.31).

In adjusted analysis the rIDI for NT-proBNP in relation to the CVD endpoints was 28.7 % (p = 0.005), and 62.1 % (p = 0.01) for all-cause mortality. In similar analyses for CAC, the rIDI was 94.4 % (p < 0.0001) for the CVD endpoints, and 142.5 % (p < 0.0001) for all-cause mortality. Addition of NT-proBNP to the adjusted model had significant impact on cNRI for both CVD endpoints and all-cause mortality (0.36, p = 0.008 and 0.37, p = 0.02; respectively). Addition of CAC to the adjusted model had significant impact on CVD endpoints (0.57, p < 0.0001) and borderline impact on all-cause mortality (0.29, p = 0.055).

Figure 3 shows the fully adjusted 5-year absolute risk for CVD endpoints and all-cause mortality according to NT-proBNP and CAC.

Additional analyses
At baseline, high-risk patients were subdivided into two groups based on further examinations (Fig. 1): High-risk...
Fig. 2  

a Kaplan-Meier survival function estimates for risk of combined cardiovascular events by categorisation into low- and high-risk at baseline. Hazard ratio 11.4 (95% confidence interval 2.7-47.3); p < 0.0001. 

b Kaplan-Meier survival function estimates for risk of all-cause mortality by categorisation into low- and high-risk at baseline. Hazard ratio 6.4 (95% confidence interval 1.5-27.1); p = 0.004

Table 2  Hazard ratios for a 1 SD increase of the log transformed values of NT-proBNP and coronary artery calcium score for fatal and nonfatal cardiovascular events and all-cause mortality

<table>
<thead>
<tr>
<th>Label</th>
<th>Cardiovascular events</th>
<th>All-cause mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of events (%)</td>
<td></td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>40 (20)</td>
<td>26 (13)</td>
</tr>
<tr>
<td>Unadjusted</td>
<td>1.9 (1.4–2.7)</td>
<td>2.2 (1.4–3.4)</td>
</tr>
<tr>
<td>Adjusted</td>
<td>1.9 (1.3–2.7)</td>
<td>2.2 (1.4–3.6)</td>
</tr>
<tr>
<td>Fully adjusted</td>
<td>1.7 (1.1–2.5)</td>
<td>1.9 (1.2–3.2)</td>
</tr>
<tr>
<td>Coronary artery calcium score</td>
<td>3.6 (2.0–6.4)</td>
<td>3.4 (1.7–6.8)</td>
</tr>
<tr>
<td>Unadjusted</td>
<td>3.7 (1.9–7.4)</td>
<td>3.9 (1.4–6.3)</td>
</tr>
<tr>
<td>Adjusted</td>
<td>3.7 (1.7–6.7)</td>
<td>3.9 (1.4–6.3)</td>
</tr>
<tr>
<td>Fully adjusted</td>
<td>3.4 (1.7–6.7)</td>
<td>2.6 (1.2–5.6)</td>
</tr>
</tbody>
</table>

Values are hazard ratios (95% confidence intervals) and represent a 1 SD increase of the log transformed values of NT-proBNP and coronary artery calcium score. Adjusted models include sex, age, LDL and HDL cholesterol, smoking, HbA1c, eGFR, systolic blood pressure and urinary albumin excretion rate. Fully adjusted models additionally include coronary artery calcium score and NT-proBNP mutually.

Significance of the hazard ratios: *P < 0.05, **P <0.01, ***P <0.0001
patients with significant CAD (n = 70) and high-risk patients without CAD (n = 63). In additional analyses comparing each of these two high-risk groups to the low-risk group, both high-risk groups had an elevated risk of the composite CVD endpoint (adjusted HR ≥4.0; p ≤ 0.025), while risk of all-cause mortality was only elevated for the high-risk patients without CAD compared to low-risk patients (adjusted HR 7.7; p = 0.022).

When comparing the two high-risk groups, the high-risk patients with CAD at baseline had an elevated risk of the composite CVD endpoint compared with the high-risk patients without CAD (adjusted HR 2.8; p = 0.012). However, the risk of all-cause mortality was comparable between the two high-risk groups (p = 0.55).

In analyses including all 200 patients, additionally inclusion of LVEF in the adjusted models revealed that both NT-proBNP (adjusted HR 1.6; p = 0.027) and CAC (adjusted HR 3.3; p = 0.001) remained predictive of CVD endpoints. Similarly, both NT-proBNP (adjusted HR 1.8; p = 0.026) and CAC remained predictors of all-cause mortality (adjusted HR 2.9; p = 0.013). In neither of these models, LVEF was a significant predictor (p ≥ 0.08).

Similar models including history of stroke (n = 19) in the multivariate adjustment, revealed that NT-proBNP (adjusted HR 1.7; p = 0.017) and CAC (adjusted HR 2.9; p = 0.02) remained predictive of CVD endpoints. Also for all-cause mortality, both NT-proBNP (adjusted HR 2.2; p = 0.001) and CAC (adjusted HR 3.0; p = 0.009) remained predictive. History of stroke was a predictor of CVD endpoints (HR 3.3; p = 0.017), but not of all-cause mortality (p > 0.27).

Discussion

In this prospective study of asymptomatic patients with type 2 diabetes and microalbuminuria, without known CAD, risk stratification with NT-proBNP and CAC, alone and in combination, predicted CVD and all-cause mortality on top of established risk factors. According to our previously defined risk criteria based on NT-proBNP and CAC, 24 of 26 deaths occurred in the high-risk group during 6.1 years of follow-up and in these patients the adjusted HR for fatal or nonfatal CVD was 10.6 (95 % CI 2.4-46.3). Both risk markers added independent predictive value in the assessment of risk for future events. Identifying diabetic subjects at risk of CVD and death at an early stage and initiating more aggressive therapy in these individuals could improve outcome.

We demonstrated that patients stratified into a high-risk group (NT-proBNP ≥45.2 ng/L and/or CAC ≥400) had a significantly higher risk of CVD events and mortality compared to low-risk patients. Findings were similar when comparing each of the high-risk groups with the low-risk group. When comparing the high-risk patients with significant CAD with the high-risk group without CAD, the former had a higher risk of CVD endpoints, while there was no difference in all-cause mortality between these groups (presumably explained by the high fraction of cancer-related deaths). Patients stratified as low-risk had a strikingly low rate of CVD and fatalities.

In patients with type 2 diabetes, elevated UAER is considered to be the most consistent independent predictor of adverse outcomes [24]. Indeed, in more recent prospective studies in patients with type 2 diabetes, NT-proBNP has been reported to be a strong and independent
predictor of mortality [13] and cardiac events [11, 25]. In addition, NT-proBNP has been shown to be a strong risk predictor of CVD events in patients with stable CVD [26]. In agreement with this contention, our study showed that higher NT-proBNP was predictive of CVD and mortality even after adjustment for CAC and traditional CVD risk factors.

With regard to CAC, several studies in patients with type 2 diabetes have demonstrated CAC to be a useful risk marker for CVD events and mortality [15, 27–31]. An extensive high CAC (>600) has been shown to be related to high risk of developing chronic CAD-related events; whereas acute CAD-related events mainly occurred in subjects with mild and moderate CAC score (1–600) [32]. These findings were confirmed in our study in patients with albuminuria, where a higher CAC was associated with increased risk of CVD events and mortality even in fully adjusted models.

CAC is strongly correlated with a patient's underlying coronary atherosclerotic plaque burden, high levels of NT-proBNP are associated with depressed systolic function and diastolic dysfunction. Thus, NT-proBNP may provide unique risk information beyond the extent of coronary atherosclerosis defined by CAC, which indeed was the rationale of our risk stratification model performed at baseline. After 6 years of follow-up, we found this model to be very potent for prediction of CVD and mortality. The combination of the two measures has previously been investigated in two large cohorts of non-diabetic subjects and here demonstrated improved risk prediction for CVD and mortality in comparison to traditional cardiovascular risk factors [33, 34]. However, to our knowledge, we are the first to investigate the combined prognostic effect of NT-proBNP and CAC in patients with type 2 diabetes and we demonstrated an additive effect of these two risk factors. Therefore, we consider the combination of NT-proBNP and CAC to provide unique risk information in asymptomatic subjects with type 2 diabetes. The usual risk stratification measures in symptomatic patients consists of a combination of echocardiography and CAG, while our stratification model provided prognostic information for asymptomatic patients, but non-invasively and at a much lower cost. Although the identification of asymptomatic patients with type 2 diabetes at high risk of adverse outcomes may be accomplished by our simple screening algorithm, a major concern is how to eliminate or at least minimize the high risk burden. Accordingly, it remains to be proven that implementation of more aggressive medical management in these patients with elevated NT-proBNP and/or CAC improves clinical outcome. However, a randomized controlled study in 300 patients with type 2 diabetes and elevated NT-proBNP (>125 pg/ml) demonstrated that accelerated up-titration of renin-angiotensin-aldosterone system- and beta-blockers to maximum tolerated dosages was an effective and safe intervention for the primary prevention of CVD events. The study did not document a decrease in NT-proBNP concentrations in the treatment group [35]. Treatment of patients with increased CAC scores with lipid-lowering drugs has also been shown to reduce subsequent CVD [36], while another study did not find intensive lipid-lowering treatment to prevent progression of CAC [37]. Thus, more intervention studies are needed in order to clarify therapies with best impact to decrease/prevent CVD. While the optimal medical strategy is not yet fully elucidated in these high-risk patients, it is tempting to speculate that early invasive therapy with CAG and coronary revascularization would be useful. However, the effect of coronary revascularization in asymptomatic patients is controversial and such procedures are currently not recommended [38].

It should be acknowledged that assessment of NT-proBNP is feasible using a simple blood test, while quantification of CAC requires CT imaging, which includes radiation exposure to the patient and is of much higher cost. Therefore, selection of subjects for CT imaging should be performed carefully, albeit that this examination is rapid (<5 min) and the radiation dose is low (1.5 mSv) compared to, e.g. CTA (12 mSv). These cost and safety issues underscore the need for further investigations to maximize potential benefits of screening algorithms and aggressive therapy in high-risk patients. Given that multifactorial treatment is already applied, this algorithm provides prognostic information, but cannot guide additional treatment decisions. For a patient where multifactorial intervention is not initiated the algorithm would strengthen the recommendation for such intervention. New intervention studies are needed to clarify additional treatment options to mitigate the increased risk of CVD and mortality in type 2 diabetes. Considerations might include new therapeutic options, lower targets or increased doses of already applied treatments.

**Strengths and limitations**

Even though a limitation of our study is the relatively low number of patients and consequent limited amount of study endpoints making the conclusions not very robust, we found our risk stratification model to be very efficient for identification of subjects at risk of CVD. Out of 40 fatal and nonfatal CVD endpoints, only two low-risk patients experienced an event, which was nonfatal in both cases, and only two low-risk patients died during six years of follow-up, both from non-cardiovascular causes. Our initial division of the study cohort into high- or low risk patients might have provided changes in treatment and intervention, and therefore the estimate of risk in the
high-risk group is likely to be conservative. However, despi
the risk was still elevated in the high-risk group. The
strength of the study is the prospective design and inclu
dion of asymptomatic patients with type 2 diabetes and
microalbuminuria, which is an established predictor of ad
verse outcome.

Conclusion

In asymptomatic patients with type 2 diabetes and micro-
albuminuria without known CAD, risk stratification with
NT-proBNP and CAC was strongly associated with fatal and
nonfatal CVD, and all-cause mortality, respectively. While
both NT-proBNP and CAC were strong risk fac-
tors, their additive prognostic effect holds promise for
identification of patients at high-risk.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BIvA conceived and designed the research, acquired the data, performed statistical
analysis and drafted the manuscript. CR conceived and designed the research, acquired the
research, acquired the data, handled funding and supervision and made critical revision of the
manuscript for key intellectual content. CFP conceived and designed the research, acquired the
data, performed statistical analysis and made critical revision of the manuscript for key
intellectual content. HNF conceived and designed the research, acquired the data, performed statistical
analysis and made critical revision of the manuscript for key intellectual content. IR conceived and
designed the research, acquired the data, performed statistical analysis and made critical revision of the
manuscript for key intellectual content. HPL conceived and designed the research, acquired the
data, performed statistical analysis and made critical revision of the manuscript for key
intellectual content. NW conceived and designed the research, acquired the data, performed statistical
analysis and made critical revision of the manuscript for key intellectual content. PR conceived and
designed the research, acquired the data, performed statistical analysis and made critical revision of the
manuscript for key intellectual content. TWH conceived and designed the research, acquired the
data, performed statistical analysis and made critical revision of the manuscript for key
intellectual content. All authors read and approved the final manuscript.

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Markers of inflammation and endothelial dysfunction are associated with incident cardiovascular disease, all-cause mortality, and progression of coronary calcification in type 2 diabetic patients with microalbuminuria

Bernt Johan von Scholten a,⁎, Henrik Reinhard b, Tine Willum Hansen a, Casper G. Schalkwijk b, Coen Stehouwer b, Hans-Henrik Parving c,d, Peter Karl Jacobsen e, Peter Rossing a,d,f

a Steno Diabetes Center, Gentofte, Denmark
b Department of Internal Medicine and Cardiovascular Research Institute (CARIM), Maastricht University Medical Centre, Maastricht, the Netherlands
c Rigshospitalet, Copenhagen, Denmark
d University of Copenhagen, Copenhagen, Denmark
e The Heart Center, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
f Aarhus University, Aarhus, Denmark

Abstract

Background: We evaluated markers of inflammation and endothelial dysfunction and their associations with incident cardiovascular disease (CVD), all-cause mortality and progression of coronary artery calcium (CAC) in patients with type 2 diabetes (T2D) and microalbuminuria but without known coronary artery disease (CAD).

Methods: Prospective study including 200 patients receiving multifactorial treatment. Markers of inflammation (TNF-ɑ, sICAM-1, sICAM-3, hsCRP, SAA, IL-1β, IL-6, IL-8) and endothelial dysfunction (thrombomodulin, sVCAM-1, sICAM-1, sICAM-3, sE-selectin, sP-selectin) were measured at baseline. Adjustment included traditional CVD risk factors, and full adjustment additionally NT-proBNP and CAC. The "SQRT method" assessed CAC progression after 5.8 years, and cut-point was an annualised difference ≤-2.5.

Results: Occurrence of CVD (n = 40) and all-cause mortality (n = 26) was traced after 6.1 years. In adjusted and fully adjusted Cox models, TNF-ɑ was a determinant of CVD and all-cause mortality (p ≤ 0.007). Further, in adjusted and fully adjusted logistic regression, TNF-ɑ was related to CAC progression (p ≤ 0.042). Of the other biomarkers, sICAM-3 and thrombomodulin were also associated with both endpoints (p ≤ 0.046, IL-1β) and CVD endpoints (p = 0.021), and sICAM-1 and sICAM-3 with all-cause mortality (p ≤ 0.005). Higher composite z-scores including all markers of inflammation and endothelial dysfunction were associated with CVD and all-cause mortality (p ≤ 0.008).

Conclusions: In patients with T2D and microalbuminuria without known CAD and receiving multifactorial treatment, biomarkers of inflammation and endothelial dysfunction were independently associated with CVD, all-cause mortality and CAC progression. Especially TNF-ɑ was a robust determinant, even after adjusting for NT-proBNP and CAC.

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1. Introduction

Individuals with type 2 diabetes, and especially those with concomitant albuminuria, are at high risk of cardiovascular disease (CVD) (Carg & Bakris, 2002; Haffner, Lehto, Ronnemaa, Pyorala, & Laakso, 1998). Endothelial dysfunction is considered one of the earliest markers of atherosclerosis, and it is well documented that inflammatory processes play an important role in the causation of atherosclerotic CVD (Libby, 2002). Inflammatory mediators play a paramount role in the initiation, progression and rupture of atherosclerotic plaques. Thus, markers of inflammation and endothelial dysfunction may provide additional information about the risk of developing CVD and may become new targets for treatment. In previous studies, we have shown markers of endothelial dysfunction and inflammation to be strongly and independently associated with
CVD and mortality (de Jager, Dekker, Kooy, et al., 2006; Stenhauer et al., 2002). Intensified, targeted, multifactorial intervention in type 2 diabetes has subsequently been shown to reduce risk of CVD compared to conventional intervention (Garde et al., 2003). However, it is not known whether such treatment affects endothelial dysfunction and inflammation. If multifactorial treatment does have an effect, then markers of endothelial dysfunction and inflammation would be expected to be less associated with CVD and all-cause mortality in these patients.

The per-protocol pre-specified aim of this prospective study was to evaluate established and novel markers of low-grade inflammation and endothelial dysfunction as determinants of combined fatal and non-fatal CVD and all-cause mortality in patients with type 2 diabetes and microalbuminuria, but without known CAD and receiving multifactorial treatment. Moreover, as tertiary endpoint it was also pre-specified to evaluate the relation between markers of endothelial dysfunction and inflammation and the progression in coronary artery calcium score (CAC) in patients without fatalities.

2. Methods

2.1. Participants and study procedure

At Steno Diabetes Center, we identified, from January 2007 to February 2008, a consecutive cohort of 200 outpatients with type 2 diabetes treated in a secondary care setting. All patients received treatment with multifactorial intervention constituting of glycemic, lipid and blood pressure control, as well as antithrombotic therapy and lifestyle modification according to the Steno-2 study (Garde et al., 2003). Patients were included if they met the following inclusion criteria: 1) outpatients with type 2 diabetes defined according to WHO criteria; 2) no history of CAD or other cardiac disease including abnormal creatinine levels (n=86); 4) physical or mental disability (n=10); or 5) malignancy (n=13). Thus, the final study population included 200 patients. A detailed flow chart of the selection of the study population is shown in Fig. 1. The a priori sample size calculation was based on the assumption that 20%-30% of the patients would experience a cardiovascular event during 5 years of follow-up (Adler et al., 2003). This study complies with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all patients gave written informed consent.

2.2. Biochemical analyses and other

Measuring of the following biomarkers was pre-specified: tumor necrosis factor alpha (TNF-α), soluble intercellular adhesion molecule 1 (sICAM-1), soluble intercellular adhesion molecule 3 (sICAM-3), high-sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), soluble thrombomodulin, soluble vascular cell adhesion protein 1 (sVCAM-1), soluble E-selectin and soluble P-selectin. MSD multipanel measurements or enzyme-linked immunosorbent assay (ELISA) were applied. NT-proBNP was measured in all patients and analyzed by an immunoassay as previously described (Tarnow, Gall, Hansen, Hovind, & Parving, 2006). UAER was measured in 24-h urine collections by an enzyme immunoassay (Reinhard, Hansen, Persson, et al., 2011). Current smoking was defined as one or more cigarettes/cigars/pipes a day.

A written invitation was sent to 613 consecutive patients (58% males and a mean (standard deviation [SD]) age of 47 (8) years). A total of 72 patients refused to participate. Furthermore, patients (n = 341) were excluded (either by phone interview or after examination in the outpatient clinic) if one or more of the following characteristics were present: 1) normal UAER or non-persistently elevated UAER (n = 52); 2) symptoms/signs or history of heart disease including Q waves in 12-lead ECG (n = 180); 3) relative contraindications to CT angiography (CTA) or CAG, including abnormal plasma creatinine levels (n = 86); 4) physical or mental disability (n = 10); or 5) malignancy (n = 13). Thus, the final study population included 200 patients. A detailed flow chart of the selection of the study population is shown in Fig. 1. The a priori sample size calculation was based on the assumption that 20%-30% of the patients would experience a cardiovascular event during 5 years of follow-up (Adler et al., 2003). This study complies with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all patients gave written informed consent.

![Flow chart of the selection of the study population](image-url)

Selection of the final study population.
The CAC scan was performed as previously described (Reinhard et al., 2011). Quantification of Agatston CAC (Agatston et al., 1990), including intimal and medial calcification in the left main, left anterior descending artery, circumflex artery and right coronary artery were summed to provide a total CAC for each participant.

2.3. Follow-up

From 2012 to 2013, all patients still alive (n = 174) were invited for a 5-year CAC reassessment. The invitation was sent by mail and was followed by a telephone call to patients not responding to the letter. The CAC assessment was completed in 145 patients. Twenty patients refused to participate in the follow-up program; five were hospitalized, three were suffering from mental illness and one emigrated to a foreign country.

The reassessment of CAC was performed as at baseline. We evaluated progression of CAC from baseline to follow-up examination. The a priori method for assessing progression was “the SQRT method” (Hokanson, MacKenzie, Kinney, et al., 2004): The square root transformed difference $\sqrt{\text{CAC}(\text{follow-up})} - \sqrt{\text{CAC}(\text{baseline})}$. This method accounts for interscan variability in CAC. The predefined cut point was a square root transformed difference $\geq 2.5$, shown to be the strongest predictor of mortality in a cohort of 4609 asymptomatic patients (Budoff, Hokanson, Nasir, et al., 2010).

At 1st of January 2014, we traced all patients through the Danish National Health Register and the Danish National Health Register. No patients were lost to follow-up.

For deceased patients, we obtained information on the date and cause of death. All deaths were classified as CVD unless an unequivocal non-CVD cause was established. Information about hospital admission including non-fatal CVD was obtained from the Danish National Health Register.

The predefined primary endpoint was the combination of cardiovascular mortality, non-fatal myocardial infarction (ICD-10 codes I20 to I25), stroke (ICD-10 codes I61 or I63), ischemic cardiovascular disease (ICD-10 codes I20 to I25), heart failure (ICD-10 code I50). For patients who experienced multiple endpoints, the analysis included only the first.

The secondary endpoint was all-cause mortality and the tertiary endpoint was CAC progression.

2.4. Statistical analyses

Logarithmic transformation was performed to achieve normal distribution for TNF-$\alpha$, sICAM-1, sICAM-3, sVCAM-1, SAA, hsCRP, IL-1$\beta$, IL-6, IL-8, thrombomodulin, sE-selectin, sP-selectin, NT-proBNP, CAC and UAER. These variables are given as medians with interquartile range (IQR). All other continuous variables are given as means $\pm$ standard derivation (SD) and the categorical variables are reported as total numbers with corresponding percentages. Cox proportional hazards analyses were applied to compute hazard ratios (HR) with 95% confidence intervals (CI) for risk of CVD endpoints and all-cause mortality per SD increment of log-transformed values of the biomarkers. Logistic regression models were applied to compute odds ratios (OR) with 95% CI of CAC progression per SD increment of log-transformed values of the biomarkers. First, we used unadjusted models to determine if any association existed between the analyzed markers and our pre-determined endpoints. The subsequent adjustment in all analyses included traditional CVD risk factors, based on prior evidence: sex, age, total cholesterol, smoking, HBA1c, plasma creatinine, systolic blood pressure and UAER. Fully adjusted models additionally encompassed NT-proBNP and CAC.

For reasons of statistical efficiency and to reduce the influence of the biological variability of each measure, an overall z-score was determined for both low-grade inflammation and endothelial dysfunction. The composite z-score for low-grade inflammation included: TNF-$\alpha$, sICAM-1, sICAM-3, hsCRP, SAA, IL-1$\beta$, IL-6, IL-8; and the composite z-score for endothelial dysfunction included: thrombomodulin, sVCAM-1, sICAM-1, ICAM-3, sE-selectin, sP-selectin. The calculation was made by averaging the individual biomarkers signed z-scores. We included sICAM-1 and sICAM-3 in both scores because they are markers of both low-grade inflammation and endothelial dysfunction.

A two-tailed p-value < 0.05 was considered significant. Statistical analyses were performed using SPSS for Windows, version 20.0 (SPSS, Chicago, IL).

3. Results

3.1. Patient characteristics

Table 1 lists baseline characteristics of the participants divided according to occurrence or absence of the 3 predefined endpoints. The total population (n = 200) included 152 (76%) men, age averaged (± SD) 59 ± 9 years and diabetes duration was 13 ± 7 years. In addition to oral anti-diabetic medications and insulin most patients were treated with cardiovascular medications: antihypertensive drugs (98%), statins (95%) and aspirin (93%).

3.2. Incidence of CVD endpoints and all-cause mortality

Median follow-up was 6.1 (5th to 95th percentile 2.9–7.1) years. During this period, 40 patients experienced at least one CVD endpoint and 26 patients died.

Eleven CVD events were fatal and 29 were non-fatal events leading to hospital admission, including 2 fatal and 3 non-fatal cases of acute myocardial infarction, 3 non-fatal strokes, 1 fatal and 19 non-fatal cases of ischemic cardiovascular disease, 6 sudden and otherwise unexplained deaths, and 2 fatal and 4 non-fatal cases of heart failure. Of the 26 deaths, 10 were classified as CVD-related, 9 as cancer-related, and 7 as related to other causes.

3.3. CAC progression

Reassessment of CAC was performed in available patients after a median of 5.8 (5th to 95th percentile 5.1–6.1) years. CAC progressed from median [IQR] 107 [3; 547] to 782 [108;1807] (p < 0.001). Number of patients with CAC > 400 increased from 44 (30%) at baseline to 88 (61%) at follow-up; and number of patients with CAC > 1000 increased from 16 (11%) to 66 (46%). In patients with a baseline CAC > 0 completing the CAC reassessment (n = 118), the annual percent change in CAC was (median [IQR]) 51% (26%–126%).

A total of 62 patients reached the defined CAC progression endpoint, of which 18 had experienced a CVD event prior to the scan.

3.4. Markers of low-grade inflammation

Table 2 lists levels of all analyzed biomarkers of low-grade inflammation and their individual as well as combined association to CVD endpoints, all-cause mortality and CAC progression.

In adjusted analysis, higher TNF-$\alpha$ was the strongest determinant of the composite CVD endpoint and all-cause mortality (p ≤ 0.002). Besides TNF-$\alpha$, higher age and male sex were associated with the composite CVD endpoint (p ≤ 0.035), while higher age and smoking were associated with all-cause mortality (p ≤ 0.042).

In the fully adjusted model (additionally including NT-proBNP and CAC), higher TNF-$\alpha$ remained associated with both the composite CVD endpoint and all-cause mortality (p ≤ 0.007). In these models, higher CAC, NT-proBNP and cholesterol were also associated with the composite CVD endpoint (p ≤ 0.046), and higher CAC and NT-proBNP with all-cause mortality (p ≤ 0.031).
Higher TNF-α was related to the predefined CAC progression endpoint in both adjusted and fully adjusted models (p = 0.040). Besides TNF-α, higher sICAM-3 was also an independent determinant of both the composite CVD endpoint and all-cause mortality (p < 0.050). In the models including sICAM-3, risk of the composite CVD endpoint was also increased with higher age (p = 0.001), and all-cause mortality was increased in smokers (p = 0.006). In the fully adjusted models, sICAM-3 remained associated with all-cause mortality (p = 0.001), but not with the composite CVD endpoint (p = 0.082).

Of the other markers, higher IL-1β was associated with the composite CVD endpoint (p = 0.021), and higher sICAM-3 with all-cause mortality (p = 0.005) in adjusted analysis. After full adjustment, higher sICAM-1 remained associated with all-cause mortality (p = 0.007). A higher composite z-score for all markers of low-grade inflammation was associated with a higher risk of both the composite CVD endpoint and all-cause mortality (p = 0.003). In these models, higher age was also associated with the composite CVD endpoint (p = 0.001), while smoking was related to higher risk of all-cause mortality (p = 0.018). In fully adjusted models, the composite z-score remained associated with the composite CVD endpoint (p = 0.015) and all-cause mortality (p = 0.002).

### 3.5 Markers of endothelial dysfunction

Table 3 lists levels of all analyzed biomarkers of endothelial dysfunction and their individual as well as combined association to CVD endpoints, all-cause mortality and CAC progression.

Higher thrombomodulin and sVCAM-1 were associated with increased risk of all-cause mortality both in adjusted (p = 0.002) and fully adjusted (p ≤ 0.005) models. Further, in adjusted models higher thrombomodulin was associated with CVD endpoints (p = 0.046), while sVCAM-1 was not associated with the composite CVD endpoint (p = 0.10). sE-selectin and sP-selectin were not associated with CVD endpoints (p ≥ 0.06) or all-cause mortality (p ≥ 0.33).

A higher value of the composite z-score for all markers of endothelial dysfunction was associated with both the composite CVD endpoint and all-cause mortality (p ≤ 0.008). In these models, higher age was also a determinant of the CVD endpoints (p = 0.001) and smoking of all-cause mortality (p = 0.017). In the fully adjusted models, higher composite z-score remained associated with all-cause mortality (p = 0.001) in addition to higher CAC, NT-proBNP and smoking (p = 0.038), but was not associated with CVD endpoints (p = 0.087). None of the markers were related to CAC progression.

In an exploratory analysis, we computed a composite z-score of TNF-α and thrombomodulin, as these two markers were associated with both CVD endpoints and all-cause mortality, and represent inflammation and endothelial dysfunction, respectively. This combined measure was a determinant of both endpoints even in fully adjusted models (p ≤ 0.018). In these models, higher CAC and cholesterol were also associated with CVD endpoints, while only higher CAC was related to higher risk of all-cause mortality (p ≤ 0.047). NT-proBNP was not associated with CVD endpoints or all-cause mortality in these models (p ≥ 0.053). Further, this composite z-score was related to CAC progression in both adjusted and fully adjusted models (p ≤ 0.030).

### Discussion

In this prospective study of patients with type 2 diabetes and microalbuminuria, but without known CAD and receiving multifactorial treatment, we have demonstrated several biomarkers of inflammation and endothelial dysfunction to be associated with CVD events and all-cause mortality after 6 years of follow-up. Thus, a novel and important finding of this study is that multifactorial treatment apparently does not stop the effects of inflammation and endothelial dysfunction over this period of follow-up.

Even when accounting for levels of NT-proBNP and CAC, higher TNF-α was associated with both endpoints and was related to our predefined CAC progression endpoint. Of the other biomarkers, higher thrombomodulin and sICAM-3 were also associated with both endpoints, higher IL-1β with CVD endpoints, and higher sVCAM-1 and sICAM-1 with all-cause mortality. Moreover, in the model including the traditional CVD risk factors, the composite z-scores for all biomarkers of inflammation as well as endothelial dysfunction were associated with both CVD endpoints and all-cause mortality. The composite z-score including the strongest markers of inflammation

---

**Table 3**

Clinical characteristics of the study population at baseline according to event status.

<table>
<thead>
<tr>
<th>Patients without a cardiovascular event (n = 160)</th>
<th>Patients with a cardiovascular event (n = 40)</th>
<th>Survived (n = 174)</th>
<th>Died (n = 26)</th>
<th>Non-progressors in CAC (n = 83)</th>
<th>Progressors in CAC (n = 62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>115 (72)</td>
<td>36 (90)</td>
<td>128 (74)</td>
<td>22 (85)</td>
<td>62 (75)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58 (9)</td>
<td>63 (6)</td>
<td>58 (9)</td>
<td>62 (8)</td>
<td>57 (9)</td>
</tr>
<tr>
<td>Known duration of diabetes (years)</td>
<td>12 (7)</td>
<td>16 (8)</td>
<td>12 (7)</td>
<td>15 (8)</td>
<td>11 (7)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32.7 (5.8)</td>
<td>31.9 (5.8)</td>
<td>32.6 (5.7)</td>
<td>32.4 (6.4)</td>
<td>32.5 (5.6)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.8 (1.3)</td>
<td>8.0 (1.4)</td>
<td>7.9 (1.4)</td>
<td>7.5 (1.2)</td>
<td>7.9 (1.3)</td>
</tr>
<tr>
<td>Urinary albumin excretion rate (mg/24-h)</td>
<td>100 (38-209)</td>
<td>119 (44-509)</td>
<td>96 (38-200)</td>
<td>196 (52-510)</td>
<td>104 (44-194)</td>
</tr>
<tr>
<td>P-creatinine (μmol/L)</td>
<td>75 (18)</td>
<td>83 (13)</td>
<td>76 (18)</td>
<td>78 (20)</td>
<td>76 (17)</td>
</tr>
<tr>
<td>Symmetric blood pressure (mm Hg)</td>
<td>130 (16)</td>
<td>130 (15)</td>
<td>130 (16)</td>
<td>130 (18)</td>
<td>127 (16)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>31.9 (0.9)</td>
<td>4.1 (1.1)</td>
<td>3.9 (0.9)</td>
<td>3.9 (1.0)</td>
<td>3.9 (0.9)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>45 (28)</td>
<td>14 (35)</td>
<td>45 (26)</td>
<td>14 (54)</td>
<td>21 (28)</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>42.4 (16.0-34.3)</td>
<td>83.7 (44-212.0)</td>
<td>43.2 (16.0-85.0)</td>
<td>89.7 (50-246.6)</td>
<td>31.4 (12-83.5)</td>
</tr>
<tr>
<td>Coronary artery calcium score (Agatston units)</td>
<td>110 (–770)</td>
<td>125 (3-541)</td>
<td>569 (243-1766)</td>
<td>13 (0-246)</td>
<td>316 (103-770)</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD), median (interquartile range) or number of patients (%). NT-proBNP: N-terminal pro-brain natriuretic peptide.
and endothelial dysfunction (TNF-α and thrombomodulin) was associated with both endpoints and was related to CAC progression, even after adjustment for NT-proBNP and CAC, but these findings need confirmation in future studies.

Biomarkers of well-accepted pathophysiological pathways are potentially important in the prediction of diabetic complications as well as providing targets for therapy. The knowledge of the etiology of type 2 diabetes and the vascular complications has widened considerably over recent years. Therefore, the concept of interplay between inflammatory and metabolic abnormalities leading to tissue damage in diabetes has arisen. In both small and large vessels, the earliest indicator of these effects is endothelial dysfunction accompanied by the development of a prothrombotic state. This has led to the identification of an expanded array of circulating biomarkers of these processes offering new opportunities for pre-emption, early diagnosis and targeted therapy (de Jager, Kooy, Schalkwijk, et al., 2014; de Jager et al., 2006; van Sloten, Henry, Dekker, et al., 2014).

In the current cohort, we have recently demonstrated that a simple screening algorithm combining NT-proBNP and CAC added substantially to the risk information provided by traditional risk factors (von Scholten, Reinhard, Hansen, et al., 2015). However, even after adjustment for these powerful risk markers, TNF-α remained strongly associated with the composite CVD endpoint, while TNF-α, sICAM-1, sVCAM-1, and thrombomodulin all were associated with all-cause mortality.

TNF-α is a pro-inflammatory cytokine implicated in auto-immune diseases and has a pivotal role in development of insulin resistance (Cheung et al., 1998). There is growing evidence that TNF-α is associated with development of diabetic nephropathy and neuropathy (Navarro-Gonzalez & Mora-Fernandez, 2008; Shi, Chen, Nadeem, & Xu, 2013). However, to the best of our knowledge, no previous studies have investigated the association of TNF-α with risk of CVD and mortality in type 2 diabetic patients. In a study of 4609 asymptomatic patients progression of CAC was shown to be a stronger predictor of mortality than traditional CVD risk factors including baseline CAC (Budoff et al., 2010). In our cohort, higher sVCAM-1 and up-regulation of sICAM-1 on vascular endothelium in vitro (Littler et al., 1997).

The sICAM-3 is primarily expressed on resting leukocytes and not on the endothelium and may be particularly involved in initiating immune responses. In line with this, previous studies have found elevated

Table 2

<table>
<thead>
<tr>
<th>Marker (median [IQR])</th>
<th>Model</th>
<th>Cardiovascular events (n = 40)</th>
<th>All-cause mortality (n = 26)</th>
<th>Coronary artery calcium score progression (n = 62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sICAM-1 (1.1 [0.9-1.4]) ng/mL</td>
<td>Unadjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td>sVCAM-1 (264 [212-310]) ng/mL</td>
<td>Unadjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td>hsCRP (2.4 [0.9-5.0]) μg/L</td>
<td>Unadjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td>TNF-α (7.9 [6.6-9.7]) pg/mL</td>
<td>Unadjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
</tbody>
</table>

Values are hazard ratios (risk of cardiovascular events or all-cause mortality) or odds ratios (coronary artery calcium score progression) with 95% confidence intervals, and represent an SD increment of log-transformed values of the biomarkers.

* Significance of the hazard ratios: p < 0.05.
** Significance of the hazard ratios: p < 0.01.
*** Significance of the hazard ratios: p < 0.001.
levels of sICAM-3 in patients with auto-immune diseases, although not in type 1 diabetes (Martin, Beckmann, Melchers, et al., 1995). Interestingly, we found higher levels of sICAM-3 to be associated with both the composite CVD endpoint and all-cause mortality.

Thrombomodulin is a glycoprotein that binds to thrombin and activates protein C, thus mitigating the effects of cytokines produced by inflammatory and immunological processes (Califano, Giovannielli, Pantone, et al., 2000). Thrombomodulin is increased in various diseases with elevated systemic or local levels of inflammatory cytokines, including TNF-α (Chong, Blann, & Lip, 2003). Levels of thrombomodulin are elevated in both type 1 and type 2 diabetes and in atheromatous arterial disease, are higher with increased vascular inflammatory disease, and human studies using TNF-α blockade, primarily etanercept, have failed to demonstrate beneficial effects on insulin sensitivity or glucose metabolism (Esser, Paquette, & Scheen, 2015). However, these pilot studies comprised a limited number of individuals and were conducted only for a short-term period.

Agents with anti-inflammatory properties may reduce the risk of mortality and drugs exerting anti-inflammatory and vascular effects might have potential to reduce the significant CVD burden associated with type 2 diabetes. The Cardiovascular Inflammation Reduction Trial (CIRT) has been designed to test the inflammatory hypothesis of atherothrombosis by evaluating if low-dose methotrexate will reduce rates of myocardial infarction, stroke, and cardiovascular death among stable coronary artery disease patients with type 2 diabetes or metabolic syndrome, conditions associated with an enhanced pro-inflammatory response. The trial will randomly allocate 7,000 patients (Everett, Pradhan, Solomon, et al., 2013). Also, the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), will evaluate whether IL-1β inhibition as compared to placebo can reduce rates of recurrent myocardial infarction, stroke, and cardiovascular death among stable coronary artery disease patients who remain at high vascular risk due to persistent elevations of hsCRP despite contemporary secondary prevention strategies (Ridker, Thuren, Zalewski, & Libby, 2011).

### Table 3

<table>
<thead>
<tr>
<th>Marker (median [IQR])</th>
<th>Model</th>
<th>Cardiovascular events (n = 40)</th>
<th>All-cause mortality (n = 26)</th>
<th>Composite coronary artery calcium score progression (n = 62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombomodulin (3.1 [2.9–3.6]) ng/mL</td>
<td>Unadjusted</td>
<td>1.5 (1.1–2.1)</td>
<td>1.5 (1.0–2.5)</td>
<td>1.8 (1.4–2.5)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.5 (1.0–2.6)</td>
<td>1.5 (1.0–2.6)</td>
<td>1.8 (1.4–2.5)</td>
</tr>
<tr>
<td>sICAM-1 (348–479) ng/mL</td>
<td>Unadjusted</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
<tr>
<td></td>
<td>Fully</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
<tr>
<td>sVCAM-1 (17.9 [13.5–24.0] ng/mL</td>
<td>Unadjusted</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
<tr>
<td></td>
<td>Adjusted</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
<tr>
<td></td>
<td>Fully</td>
<td>1.2 (0.8–1.7)</td>
<td>1.1 (0.8–1.6)</td>
<td>1.9 (1.2–2.9)</td>
</tr>
</tbody>
</table>

Values are hazard ratios (risk of cardiovascular events or all-cause mortality) or odds ratios (coronary artery calcium score progression) with 95% confidence intervals, and represent an SD increment of log-transformed values of the biomarkers.

Adjusted models include sex, age, total cholesterol, smoking, HbA1c, plasma creatinine, systolic blood pressure and urinal albumin excretion rate. Fully adjusted models additionally include coronary artery calcium score and N-terminal pro-brain natriuretic peptide.

As sICAM-1 and sICAM-3 are considered biomarkers of both inflammation and endothelial dysfunction, they are also included in the composite z-score for endothelial dysfunction.

* As suggested by individual biomarker z-scores into one composite z-score for endothelial dysfunction.

** Significance of the hazard ratios: p < 0.05.

*** Significance of the hazard ratios: p < 0.001.

As highlighted above, TNF-α has the ability to regulate other important processes and biomarkers. Therefore, anti-TNF-α therapies, using the TNF receptor:Fc fusion protein (etanercept) or specific monoclonal antibodies (infliximab, adalimumab), are widely used with success in various inflammatory diseases such as rheumatoid arthritis (Gonzalez-Gay, Gonzalez-Juanatey, Vazquez-Rodriguez, Miranda-Filloy, & Llorca, 2010), psoriasis (Channan, Wu, & Dann, 2009) and Crohn’s disease (Parmentier-Decruyq, Duhamel, Ernst, et al., 2009). Of potential interest, in all these disorders, insulin resistance is present and in several studies it has been shown to be improved by drugs targeting TNF-α. However, the possible cardiovascular benefit of drugs targeting TNF-α should be weighed against known adverse effects associated with this pharmacological approach (risk of infection and tumor development). Furthermore, cost of therapy may be a barrier for the long-term clinical use. Moreover, in various populations with insulin resistance but without overt inflammatory disease, human studies using TNF-α blockade, primarily etanercept, have failed to demonstrate beneficial effects on insulin sensitivity or glucose metabolism (Esser, Paquette, & Scheen, 2015).
4.2. Clinical implications

Despite multifactorial treatment, our novel findings illustrate inflammation and endothelial dysfunction to be important in risk assessment of type 2 diabetic patients. Measuring biomarkers of inflammation and endothelial dysfunction is uncomplicated and at a lower cost as compared to assessment of CAC. As previously described, the prognostic value of NT-proBNP is strong, however when including the risk information provided by the composite z-score comprising the prognostic value of NT-proBNP is no longer a significant determinant of CVD or all-cause mortality. Further, no designated treatment toward elevated NT-proBNP exists, whereas, e.g., TNF-α is a potential target of treatment. Future studies will elucidate if explicit treatment of inflammation and endothelial dysfunction is of clinical benefit in terms of risk reduction for the individual patient.

5. Conclusion

In patients with type 2 diabetes and microalbuminuria without known CAD and receiving multifactorial treatment, biomarkers of inflammation and endothelial dysfunction were independently associated with CVD, all-cause mortality and CAC progression. Especially TNF-α was a robust determinant, even after adjusting for NT-proBNP and CAC.

Acknowledgments

We thank all participants and acknowledge the work of study nurse Lone Jørlsbjørg and lab technicians Anne G. Lundgaard, Berit R. Jensen, Tina R. Juhl, and Jessie A. Hermann, employees at Steno Diabetes Center A/S.

References


Paper III
Urinary biomarkers are associated with incident cardiovascular disease, all-cause mortality and deterioration of kidney function in type 2 diabetic patients with microalbuminuria

Bernt Johan von Scholten1 · Henrik Reinhard1 · Tine W. Hansen1 · Jens Oellgaard1,2,3 · Hans-Henrik Parving4 · Peter K. Jacobsen5 · Peter Rossing1,6,7

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Abstract
Aims/hypothesis We evaluated two urinary biomarkers reflecting different aspects of renal pathophysiology as potential determinants of incident cardiovascular disease (CVD), all-cause mortality and a reduced estimated GFR (eGFR) in patients with type 2 diabetes and microalbuminuria but without clinical features of coronary artery disease.

Methods In a prospective study of 200 patients, all received multifactorial treatment. Baseline measurements of urinary hepatocyte growth factor (HGF) and adiponectin were available for 191 patients. Cox models were adjusted for sex, age, LDL-cholesterol, smoking, HbA1c, plasma creatinine, systolic BP and urinary AER (UAER). The pre-defined endpoint of chronic kidney disease progression was a decline in the eGFR of >30% during follow-up. HRs per 1 SD increment of log-transformed values are presented.

Results Patients had a mean ± SD age of 59 ± 9 years with a median (interquartile range) UAER of 103 (39–230) mg/24 h. During a median 6.1 years of follow-up, there were 40 incident CVD events, 26 deaths and 42 patients reached the pre-defined chronic kidney disease progression endpoint after 4.9 years (median). Higher urinary HGF was a determinant of CVD in unadjusted (HR 1.9 [95% CI 1.3, 2.8], p = 0.001) and adjusted (HR 2.0 [95% CI 1.2, 3.2], p = 0.004) models, and of all-cause mortality in unadjusted (HR 2.3 [95% CI 1.3, 3.9], p = 0.003) and adjusted (HR 2.5 [95% CI 1.3, 4.8], p = 0.005) models. A higher adiponectin level was associated with CVD in unadjusted (HR 1.4 [95% CI 1.0, 1.9], p = 0.04) and adjusted (HR 1.4 [95% CI 1.1, 2.3], p = 0.013) models, and with a decline in the eGFR of >30% in unadjusted (HR 1.6 [95% CI 1.2, 2.2], p = 0.008) and adjusted (HR 1.5 [95% CI 1.1, 2.2], p = 0.007) models.

Conclusions/interpretation In patients with type 2 diabetes and microalbuminuria receiving multifactorial treatment, higher urinary HGF was associated with incident CVD and all-cause mortality, and higher adiponectin was associated with CVD and deterioration in renal function.

Keywords Adiponectin · Cardiovascular disease · Hepatocyte growth factor · Macrovascular disease · Microalbuminuria · Type 2 diabetes · Urinary biomarkers

Abbreviations
CAC Coronary artery calcium
CAD Coronary artery disease
CKD Chronic kidney disease
CVD Cardiovascular disease
eGFR Estimated GFR
HGF Hepatocyte growth factor
vascular modulator in humans [8]. In a cross-sectional study, with end-stage renal disease, and may be considered a novel biomarker. Studies have reported that HGF is markedly elevated in patients with type 2 diabetes; and (2) adiponectin, a marker of adipose tissue dysfunction associated with the development of end-stage renal disease [10, 11]. The association between these urinary markers, incident CVD and all-cause mortality, when adjusted for levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) and the renin–angiotensin–aldosterone system (RAAS) blocking treatment in type 2 diabetes has made a major contribution to slowing disease progression; however, patients with type 2 diabetes are still at a high risk of both renal and cardiovascular complications.

There is therefore an ongoing search for new biomarkers to provide additional prognostic information beyond that provided by albuminuria and to potentially provide new therapeutic targets.

In this study, we pre-selected two urinary biomarkers representing different aspects of renal pathophysiology: (1) hepatocyte growth factor (HGF), a marker linked to renal tubular epithelial cell regeneration [6]; and (2) adiponectin, a marker of early glomerular vascular damage [7]. The role of HGF in the cardiovascular system has been investigated previously. Studies have reported that HGF is markedly elevated in patients with end-stage renal disease, and may be considered a novel vascular modulator in humans [8]. In a cross-sectional study, we previously found urinary adiponectin excretion to be positively related to albuminuria in type 1 diabetes [9]. Moreover, prospective studies have shown that high urinary adiponectin is associated with the development of end-stage renal disease [10, 11]. The association between these urinary markers, incident CVD and all-cause mortality, when adjusted for levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) and the coronary artery calcium (CAC) score is, however, unknown.

The aim of this prospective study was to evaluate two pre-selected urinary biomarkers as determinants of incident CVD and all-cause mortality in patients who had type 2 diabetes and microalbuminuria but no clinical features of coronary artery disease (CAD), and were receiving multifactorial treatment. Deterioration in the estimated GFR (eGFR), as a measure of chronic kidney disease (CKD) progression, was also assessed.

Methods

Participants and study procedure

At the Steno Diabetes Center, we identified a cohort of 200 outpatients with type 2 diabetes treated in a secondary care setting from January 2007 to February 2008 [12]. All patients received treatment with multifactorial intervention, consisting of glycaemic, lipid and BP control, as well as antithrombotic therapy and lifestyle modification in line with the findings of the Steno-2 Study [13]. Patients were included if they: (1) were outpatients with type 2 diabetes defined according to WHO criteria; (2) had no history of CAD or other cardiac disease and no symptoms suggestive of cardiac disease, as assessed from their medical records and patient interviews and questionnaires; and (3) had a persistent (two out of three consecutive measurements) urinary AER (UAER) of >30 mg/24 h.

A written invitation was sent to 613 consecutive patients (69% men; mean ± SD age, 47±8 years). A total of 72 patients declined participation. After telephone interview or examination in the outpatient clinic, 341 patients were excluded because one or more of the following characteristics were present: (1) normal or non-persistent elevated UAER (n = 52); (2) symptoms/signs or a history of heart 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). Thus, the final study population comprised 200 patients. A detailed flow chart of the selection of the study population is shown in Fig. 1.

In the absence of data from other investigations using the biomarkers, this was an exploratory study using the sample size available. The study complied with the Declaration of Helsinki, the research protocol was approved by the local ethics committee and all patients gave written informed consent.

Biochemical and other analyses

The urinary biomarkers HGF and adiponectin were measured by ELISA (Roche Diagnostics, Basel, Switzerland) in a single 24 h urine sample from each patient. Immediately after collection, the urine was clarified by centrifugation, frozen at −80°C and stored in a research biobank for analysis immediately after the last study patient was examined. The maximal storage time of the samples prior to analysis of both biomarkers was 13 months. The values were corrected for the urinary creatinine concentration. The biomarkers were not measured in duplicate. For HGF, the intra-assay and interassay CVs were <10% and <15%, respectively. The high molecular weight isoform of adiponectin, which is the most biologically active, was measured: the intra-assay and interassay CVs were <10% and <15%, respectively.

UAER was measured in 24 h collected urine samples using an enzyme immunoassay (Vitros, Raritan, NJ, USA). Current smoking was defined as one or more cigarettes, cigars or pipes per day. Plasma NT-proBNP was analysed by immunoassay and CAC scanning was performed as previously described [12]. The total Agatston CAC score [14], including intimal

| IDI | Integrated discrimination improvement |
| NT-proBNP | N-terminal pro-brain natriuretic peptide |
| RAAS | Renin–angiotensin–aldosterone system |
| rIDI | Relative IDI |
| ROC | Receiver operating characteristic |
| UAER | Urinary AER |

Introduction

Albuminuria is considered one of the best available risk factors of renal disease [1, 2] and cardiovascular disease (CVD) [3, 4]; reductions in albuminuria have been linked to both renal and cardiovascular protection [1, 5]. The wide implementation of the renin–angiotensin–aldosterone system (RAAS) blocking treatment in type 2 diabetes has made a major contribution to slowing disease progression; however, patients with type 2 diabetes are still at a high risk of both renal and cardiovascular complications. There is therefore an ongoing search for new biomarkers to provide additional prognostic information beyond that provided by albuminuria and to potentially provide new therapeutic targets.

In this study, we pre-selected two urinary biomarkers representing different aspects of renal pathophysiology: (1) hepatocyte growth factor (HGF), a marker linked to renal tubular epithelial cell regeneration [6]; and (2) adiponectin, a marker of early glomerular vascular damage [7]. The role of HGF in the cardiovascular system has been investigated previously. Studies have reported that HGF is markedly elevated in patients with end-stage renal disease, and may be considered a novel vascular modulator in humans [8]. In a cross-sectional study, we previously found urinary adiponectin excretion to be positively related to albuminuria in type 1 diabetes [9]. Moreover, prospective studies have shown that high urinary adiponectin is associated with the development of end-stage renal disease [10, 11]. The association between these urinary markers, incident CVD and all-cause mortality, when adjusted for levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) and the coronary artery calcium (CAC) score is, however, unknown.

The aim of this prospective study was to evaluate two pre-selected urinary biomarkers as determinants of incident CVD and all-cause mortality in patients who had type 2 diabetes and microalbuminuria but no clinical features of coronary artery disease (CAD), and were receiving multifactorial treatment. Deterioration in the estimated GFR (eGFR), as a measure of chronic kidney disease (CKD) progression, was also assessed.
and medial calcification of the left main, left anterior descending artery, circumflex artery and right coronary artery, was determined for each patient.

Follow-up

On 1 January 2014, we traced all patients through the Danish National Death Register and the Danish National Health Register. No patients were lost to follow-up. For deceased patients, we obtained information on the date and cause of death. All deaths were classified as CVD unless an unequivocal non-CVD cause was established. Information on hospital admission, including for non-fatal CVD, was obtained from the Danish National Health Register.

We pre-defined a broad primary endpoint consisting of cardiovascular mortality, non-fatal myocardial infarction (ICD-10 codes I20–I25), stroke (ICD-10 codes I61 or I63), ischaemic cardiovascular disease (ICD-10 code I70) and heart failure (ICD-10 code I50). None of the patients were classified according to the results of baseline investigations (i.e. elevated CAC or NT-proBNP). For patients who experienced multiple endpoints, the analysis included only the first endpoint. The secondary endpoint was all-cause mortality.

Of the 200 patients originally included in the study, 177 (88.5%) were followed for a median (interquartile range [IQR]) of 4.9 (3.8–5.4) years after baseline examination, with a yearly measurement of plasma creatinine levels for calculating the eGFR by applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [15]. Changes in the eGFR were evaluated from baseline until the last available measurement. The last patient had the latest measurement performed in March 2013. A decline in the eGFR of >30% at any time point was the pre-defined endpoint of CKD progression, as proposed by Coresh et al [16]. Baseline values of urinary HGF and adiponectin were available for 191 patients (96%).

Statistical analyses

The distribution of the urinary biomarkers is shown in electronic supplementary material (ESM) Fig. 1. Based on their graphical distribution and a significant result in the Shapiro–Wilk test (p ≤ 0.001; indicating non-normal distribution), both biomarkers were log-transformed using log_{10}.

All non-normally distributed variables are summarised as the median with IQR. All other continuous variables are summarised as means ± SD, and categorical variables are reported as total numbers with corresponding percentages. Differences in potential confounders between quartiles of the two urinary biomarkers were tested with the trend test for continuous variable, and the χ² test for categorical variables.

Cox proportional hazards analyses were applied to compute the HRs with 95% CIs for the CVD endpoint and all-cause mortality, per SD increment of log-transformed values of the urinary biomarkers.

First, we used unadjusted models (Model 1) to determine whether there was any association between the analysed biomarkers and our pre-defined endpoints. Subsequent adjustment in all analyses included traditional CVD/renal risk factors based on existing evidence: sex, age, LDL-cholesterol, smoking, Hba1c, plasma creatinine, systolic BP and UAER (Model 2). For the CVD endpoint and all-cause mortality, we used models including the previously listed factors plus the NT-proBNP level and CAC score (Model 3). This model was included...
because the NT-proBNP level and CAC score were previously shown to be powerful risk markers in this cohort [17].

To minimise the risk of instability in the models, we also used a model including only those covariates related to each of the endpoints. These covariates were selected based on backward elimination, using \( p < 0.05 \) as the threshold for retention. For the CVD endpoint, age and LDL-cholesterol level were included as covariates; for all-cause mortality, smoking was the only covariate included; and for the CKD progression endpoint, the UAER and plasma creatinine level were included.

Finally, we applied the Kaplan–Meier failure function to compare the risks of the CVD endpoint and all-cause mortality according to the median HGF level.

To examine the potential additional predictive ability of each urinary biomarker over traditional CVD risk factors, we plotted receiver operating characteristic (ROC) curves based on logistic regression models and calculated the AUC in a model including only the traditional CVD/renal risk factors and in the same model with each of the biomarkers added.

Next, we calculated the integrated discrimination improvement (IDI) statistics [18] to compare the overall improvement in sensitivity and specificity between two models: the model including traditional CVD/renal risk factors alone vs the model including the urinary biomarker. In other words, the IDI assesses the ability of the new biomarkers to improve the average sensitivity without sacrificing average specificity. The IDI may be a more powerful way to demonstrate improved diagnostic performance than the AUC [18]. The results of the IDI can be difficult to interpret, so we have also provided a relative IDI (rIDI) reported as a percentage than the IDI [18].

A two-tailed \( p \) value of \(<0.05\) was considered significant. Statistical analyses were performed using SPSS for Windows (version 20.0, Chicago, IL, USA), SAS software (version 9.3, SAS Institute, Cary, NC, USA) and Stata/IC version 14.0 for Windows (StataCorp, College Station, TX, USA).

Results

Patient characteristics

Table 1 lists the baseline characteristics of patients, categorised according to the occurrence (or not) of the three pre-defined endpoints. The total population (\( n = 200 \)) included 151 (76%) men, with a mean \( \pm \) SD age of 59 \( \pm \) 9 years, known diabetes duration of 13 \( \pm \) 7 years and median (interquartile range) UAER of 103 (39–230) mg/24 h. In addition to oral hypoglycaemic medications and insulin, most patients were treated with cardiovascular medication: antihypertensive drugs (99%), RAAS-blocking treatment (98%), statins (95%) and aspirin (90%).

ESM Tables 1 and 2 show the clinical characteristics of the study population according to quartiles of HGF and adiponectin, respectively (as potential confounders). For HGF, only NT-proBNP differed significantly (\( p = 0.04 \)) between quartiles (higher levels with increasing quartiles). For adiponectin, the levels of HbA1c, UAER and NT-proBNP were significantly higher with increasing quartiles (\( p \leq 0.04 \)).

Incidence of the CVD endpoint and mortality

The median follow-up was 6.1 years (IQR 5.9–6.6 years). During this period, 40 patients experienced at least one CVD endpoint and 26 patients died.

Eleven CVD events were fatal and 29 were non-fatal events leading to hospital admission, including two fatal and three non-fatal cases of acute myocardial infarction, three non-fatal strokes, one fatal and 19 non-fatal cases of ischaemic cardiovascular disease, six sudden and otherwise unexplained deaths, and two fatal and four non-fatal cases of heart failure. Of the 26 deaths, 11 were classified as CVD-related, nine as cancer-related, and six as related to other causes.

Decline in the eGFR

Patients with at least one annual eGFR measurement after baseline were included (\( n = 177 \)). The median (IQR) follow-up was 4.9 years (3.8–5.4 years). The eGFR declined from a mean \( \pm \) SD of 89.8 \( \pm \) 18.3 ml min\(^{-1}\) 1.73 m\(^{-2}\) at baseline to 79.1 \( \pm \) 21.4 ml min\(^{-1}\) 1.73 m\(^{-2}\) during follow-up (\( p < 0.001 \)). A total of 42 patients (24%) reached the pre-defined CKD progression endpoint. In 23 of these patients (55%), the eGFR had declined by >30% in at least two measurements during follow-up. However, for patients with a decline in eGFR in only one measurement (17 out of 19; 89%), this was seen for the first time at the final follow-up visit. Hence, it was not possible to confirm the eGFR decline of >30% in these patients in a second test. However, based on the natural history of kidney disease in diabetes (which includes an expected annual decline in the eGFR), we do not think our findings reflect a transient fluctuation in the eGFR.

No patients reached end-stage renal disease or dialysis during follow-up.

Urinary biomarkers

Table 2 shows the associations for the urinary biomarkers with the composite CVD endpoint, all-cause mortality and CKD progression in all three models.
Hepatocyte growth factor

In Model 1, higher HGF was associated with both the composite CVD endpoint (HR 1.9 [95% CI 1.3, 2.8]) and all-cause mortality (HR 2.3 [95% CI 1.3, 3.9]; Table 2). In Model 2, higher HGF was associated with the composite CVD endpoint (HR 2.0 [95% CI 1.2, 3.2]) and all-cause mortality (HR 2.5 [95% CI 1.3, 4.8]). In Model 3 (Model 2 plus NT-proBNP and CAC), higher HGF remained associated with both the composite CVD endpoint (HR 1.5 [95% CI 1.1, 2.3]) and all-cause mortality (HR 1.9 [95% CI 1.0, 3.5]).

Table 1 — Clinical characteristics of the study population at baseline according to event status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CVD event (n = 160)</th>
<th>All-cause mortality (n = 26)</th>
<th>CKD progression (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>115 (72)</td>
<td>129 (74)</td>
<td>125 (93)</td>
</tr>
<tr>
<td>Age, years</td>
<td>58 (9)</td>
<td>58 (9)</td>
<td>58 (8)</td>
</tr>
<tr>
<td>Known duration of diabetes, years</td>
<td>12 (7)</td>
<td>12 (7)</td>
<td>13 (8)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>32.7 (5.8)</td>
<td>32.6 (5.7)</td>
<td>32.5 (5.7)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.8 (1.3)</td>
<td>7.9 (1.4)</td>
<td>7.8 (1.3)</td>
</tr>
<tr>
<td>HbA1c, mmol/mol</td>
<td>62 (14)</td>
<td>63 (15)</td>
<td>63 (16)</td>
</tr>
<tr>
<td>UAER, mg/24 h</td>
<td>100 (38–209)</td>
<td>96 (38–200)</td>
<td>96 (36–192)</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/l</td>
<td>75 (18)</td>
<td>76 (18)</td>
<td>75 (18)</td>
</tr>
<tr>
<td>eGFR, ml min⁻¹ 1.73 m²⁻¹</td>
<td>91.5 (18.3)</td>
<td>90.2 (18.1)</td>
<td>90.9 (17.5)</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>130 (16)</td>
<td>130 (16)</td>
<td>129 (16)</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>1.8 (0.8)</td>
<td>1.8 (0.8)</td>
<td>1.9 (0.8)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>45 (28)</td>
<td>45 (28)</td>
<td>42 (31)</td>
</tr>
<tr>
<td>NT-proBNP, ng/l</td>
<td>42.4 (16.9–83.4)</td>
<td>43.2 (16.9–85.0)</td>
<td>34.7 (15.3–83.1)</td>
</tr>
<tr>
<td>CAC score, Agatston units</td>
<td>110 (2–418)</td>
<td>125 (3–541)</td>
<td>178 (3–614)</td>
</tr>
<tr>
<td>Treatment with Oral antidiabetic, n (%)</td>
<td>138 (86)</td>
<td>148 (85)</td>
<td>114 (84)</td>
</tr>
<tr>
<td>Insulin, n (%)</td>
<td>94 (59)</td>
<td>106 (61)</td>
<td>82 (61)</td>
</tr>
<tr>
<td>Antihypertensive drugs, n (%)</td>
<td>158 (99)</td>
<td>172 (99)</td>
<td>133 (99)</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>151 (94)</td>
<td>163 (94)</td>
<td>127 (94)</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>147 (92)</td>
<td>160 (92)</td>
<td>122 (90)</td>
</tr>
<tr>
<td>HGF, pg ml⁻¹ 1 g⁻¹ creatinine</td>
<td>13.4 (8.6–17.1)</td>
<td>13.3 (8.9–17.0)</td>
<td>13.0 (8.9–17.0)</td>
</tr>
<tr>
<td>Adiponectin, pg ml⁻¹ 1 g⁻¹ creatinine</td>
<td>2.2 (0.6–8.8)</td>
<td>2.3 (0.6–10.3)</td>
<td>2.0 (0.6–7.0)</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD), median (interquartile range) or number of patients (%)

* A total of 177 patients were available for the analyses of eGFR decline

**Hepatocyte growth factor** In Model 1, higher HGF was associated with both the composite CVD endpoint (HR 1.9 [95% CI 1.3, 2.8]) and all-cause mortality (HR 2.3 [95% CI 1.3, 3.9]; Table 2). In Model 2, higher HGF was associated with the composite CVD endpoint (HR 2.0 [95% CI 1.2, 3.2]) and all-cause mortality (HR 2.5 [95% CI 1.3, 4.8]). In Model 3 (Model 2 plus NT-proBNP and CAC), higher HGF remained associated with both the composite CVD endpoint (HR 1.5 [95% CI 1.1, 2.3]) and all-cause mortality (HR 1.9 [95% CI 1.0, 3.5]).

Table 2 — Urinary biomarkers related to risk of fatal and non-fatal CVD event, all-cause mortality and CKD progression

<table>
<thead>
<tr>
<th>Urinary biomarker</th>
<th>Model</th>
<th>CVD event (n = 40)</th>
<th>All-cause mortality (n = 26)</th>
<th>CKD progression (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF, log scale (1 SD = 0.22)</td>
<td>1</td>
<td>1.9 (1.3, 2.8)</td>
<td>2.3 (1.3, 3.9)</td>
<td>1.4 (0.9, 2.1)</td>
</tr>
<tr>
<td>Adiponectin, log scale (1 SD = 0.74)</td>
<td>1</td>
<td>1.4 (1.0, 1.9)</td>
<td>1.1 (0.7, 1.6)</td>
<td>1.6 (1.2, 2.2)</td>
</tr>
</tbody>
</table>

Values represent an SD increment of log-transformed values of the biomarkers

Model 1 is unadjusted

Model 2 is adjusted for sex, age, LDL-cholesterol, smoking, HbA1c, plasma creatinine, systolic BP and UAER

Model 3 (for cardiovascular events and all-cause mortality) is Model 2 plus adjustment for CAC score and NT-proBNP level
When dividing patients into two groups according to level of HGF (above or below the median [14.1 pg ml⁻¹ g⁻¹ creatinine]), the unadjusted risk of the composite CVD endpoint (HR 2.5 [95% CI 1.2, 4.8]) and all-cause mortality (HR 2.6 [95% CI 1.1, 6.2]) was higher in patients with levels above the median (Fig. 2).

As shown in ESM Fig. 2a,b, in relation to the composite CVD endpoint, the AUC changed from 0.738 (95% CI 0.660, 0.816) to 0.775 (95% CI 0.698, 0.853) after HGF was added to the model including traditional CVD/renal risk factors (change in AUC 0.0374 [95% CI −0.003, 0.078]; p = 0.072). In relation to all-cause mortality, the AUC changed from 0.746 (95% CI 0.645, 0.846) to 0.774 (95% CI 0.663, 0.884) after adding HGF to the model including traditional CVD/renal risk factors (change in AUC 0.0280 [95% CI −0.046, 0.102]; p = 0.46).

The rIDI for HGF was 29.5% (95% CI 13.7%, 45.3%; p = 0.005) in relation to the CVD endpoint and 87.1% (50.0%, 123.8%; p < 0.001) in relation to all-cause mortality.

Levels of HGF were not related to the CKD progression endpoint in Model 1 (HR 1.4 [95% CI 0.9, 2.1]) or Model 2 (HR 1.1 [95% CI 0.7, 1.7]).

Adiponectin

In Model 1, higher adiponectin was associated with the composite CVD endpoint (HR 1.4 [95% CI 1.0, 1.9]), but was not associated with all-cause mortality (HR 1.1 [95% CI 0.7, 1.6]). Higher levels of adiponectin were also associated with the composite CVD endpoint (HR 1.4 [95% CI 1.1, 2.3]) in Model 2. However, in Model 3, when NT-proBNP and CAC were included in the adjustment, adiponectin lost significance and was not associated with the composite CVD endpoint (HR 1.4 [95% CI 0.9, 2.2]).

As shown in ESM Fig. 2c,d, in relation to the composite CVD endpoint, the AUC changed from 0.738 (95% CI 0.660, 0.816) to 0.763 (95% CI 0.687, 0.839) after adding adiponectin to the model including traditional CVD/renal risk factors (change in AUC 0.025 [95% CI −0.014, 0.061]; p = 0.22).

In relation to all-cause mortality, the AUC changed from 0.746 (95% CI 0.645, 0.846) to 0.761 (95% CI 0.662, 0.859) after adding adiponectin to the model including traditional CVD/renal risk factors (change in AUC 0.015 [95% CI −0.022, 0.042]; p = 0.65).

The rIDI for adiponectin was 14.4% (95% CI −1.6%, 29.6%; p = 0.054) in relation to the CVD endpoint, and 8.9% (95% CI −21.1%, 40.2%; p = 0.26) in relation to all-cause mortality.

In Model 1, higher levels of adiponectin were associated with decline in the eGFR of >30% (HR 1.6 [95% CI 1.2, 2.2]). In Model 2, higher levels of adiponectin remained associated with a decline in the eGFR of >30% (HR 1.5 [95% CI 1.1, 2.2]).

Additional analyses

In the Cox models with adjustment based on backward selection, higher levels of HGF were associated with the composite CVD endpoint (HR 1.9 [95% CI 1.2, 2.9]; p = 0.003) and all-cause mortality (HR 2.4 [95% CI 1.3, 4.2]; p = 0.003); higher levels of adiponectin were associated with the composite CVD endpoint (HR 1.4 [95% CI 1.0, 2.0]; p = 0.037) and the CKD progression endpoint (HR 1.6 [95% CI 1.1, 2.2]; p = 0.010).

As novel biomarkers may not be as accessible to clinicians, we also sought to determine whether similar findings were obtained for risk indicators that are already available (i.e. albuminuria). Therefore, we evaluated the rate of change in degree of albuminuria prior to baseline in relation to risk of CVD, all-cause mortality and decline in the eGFR. A greater increase in albuminuria was related to all-cause mortality in
have investigated the relationship between serum levels of HGF and the presence of retinal arteriosclerotic lesions [24]. In patients with end-stage renal disease, studies have demonstrated that serum HGF levels correlate with carotid intima–media thickness and concentric left ventricular geometry [8]. However, to the best of our knowledge, we are the first to show an association between levels of urinary HGF and both incident CVD and all-cause mortality in type 2 diabetes. Importantly, the addition of HGF to traditional cardiovascular risk factors added predictive value for both endpoints.

Adiponectin has a wide range of well-known protective effects against insulin resistance, vascular dysfunction, atherosclerosis and inflammation [25], but has also been shown to be important for maintaining healthy podocytes [26]. A cross-sectional study by von Eynatten et al demonstrated that higher urinary adiponectin levels correlated significantly with increased intima–media thickness in type 2 diabetes [7]. In our cohort, we extended these findings to show that levels of urinary adiponectin were associated with incident CVD and CKD progression. In relation to serum adiponectin, high levels have been linked to cardiovascular mortality in men with type 2 diabetes [27]. Moreover, we and others have previously demonstrated that high serum adiponectin levels predict all-cause mortality, cardiovascular mortality and progression to end-stage renal disease in patients with type 1 diabetes [28, 29]. Of note, our pre-defined CKD progression endpoint was a decline in the eGFR of >30% at any time point. We considered that this is appropriate because our cohort included microalbuminuric patients, and decreases in the eGFR associated with less than a doubling of serum creatinine have recently been shown to be strongly and consistently associated with the risk of end-stage renal disease and mortality in a large meta-analysis [16]. Therefore, a smaller decline in the eGFR (such as a 30% reduction) has been suggested as an alternative endpoint for CKD progression [16]. Lastly, the European Medicines Agency has proposed a clinically relevant delay in milder renal function loss (e.g. loss of 30% in the eGFR) as a potential endpoint in clinical trials investigating new products [30].

Clinical implications
Measuring urinary HGF and adiponectin is relatively uncomplicated and inexpensive. Our findings illustrate that these biomarkers could improve risk assessment for type 2 diabetic patients. However, our findings need to be validated. Identifying patients at elevated risk of CVD and all-cause mortality is crucial because these patients may benefit from even tighter goal setting for traditional risk factors, such as BP or LDL-cholesterol. Moreover, intervention studies might show whether treating HGF and adiponectin is of clinical benefit in terms of risk reduction for individual patients.
Strengths and limitations

The study strengths were that no patients were lost to follow-up and our analysis allowed extensive adjustment for important risk factors. However, there were limitations. Although the sample size and number of events were limited, the CVD event rate was as expected based on our sample size calculation. In addition, although the statistical model might be vulnerable to instability due to overfitting, additional analyses to confirm the robustness of our findings indicated stability. We lacked information on how long the patients had received multifactorial treatment before the baseline blood sampling and to what extent they effectively received such treatment during follow-up. Nevertheless, attending physicians generally follow our local guidelines on intensive multifactorial treatment for individuals with type 2 diabetes and microalbuminuria, based on the Steno-2 study findings [13]. Finally, serum levels of HGF and adiponectin were not measured in this study.

Conclusion

In patients with type 2 diabetes and microalbuminuria but without clinical features of CAD who were receiving multifactorial treatment, levels of urinary HGF were associated with incident CVD and all-cause mortality, even after adjustment for albuminuria, NT-proBNP level and CAC score. Higher adiponectin was a determinant of the CVD endpoint and was associated with CKD progression, even when accounting for baseline creatinine and albuminuria.

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Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

References


Paper IV
Cardiac $^{82}$Rb PET/CT for fast and non-invasive assessment of microvascular function and structure in asymptomatic patients with type 2 diabetes

Bernt J. von Scholten¹ · Philip Hasbak² · Thomas E. Christensen² · Adam A. Ghotbi² · Andreas Kjaer² · Peter Rossing¹,³,⁴ · Tine W. Hansen¹

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Abstract
Aims/hypothesis Coronary flow reserve (CFR) and coronary artery calcium (CAC) represent functional and structural aspects of atherosclerosis. We examined the prevalence of reduced CFR and high CAC scores in three predefined groups of patients without known cardiovascular disease: (1) patients with type 2 diabetes and albuminuria; (2) patients with type 2 diabetes and normoalbuminuria; and (3) non-diabetic controls.

Methods In a cross-sectional design, cardiac $^{82}$Rb positron emission tomography/computed tomography was conducted in 60 patients with type 2 diabetes who were free of overt cardiovascular disease and who were stratified by normoalbuminuria (<30 mg/24 h) (n=30; age [mean±SD] 60.9±10.1 years) and albuminuria (≥30 mg/24 h) (n=30; age 65.6±4.8 years), and in 30 healthy, non-diabetic controls (age 59.8±9.9 years).

Results In controls, normoalbuminuric and albuminuric patients, CFR was 3.0±0.8, 2.6±0.8 and 2.0±0.5, respectively. Reduced CFR (<2.5) was observed in 16.7%, 40.0% and 83.3% of participants, respectively, and median (interquartile range) CAC scores were 0 (0–81), 36 (1–325) and 370 (152–1,025), respectively (p for trend <0.01). After adjustment, the difference in CFR and CAC between albuminuric patients and controls remained significant (p≤0.001). There were trends towards lower CFR and higher CAC scores in normoalbuminuric patients vs controls (p≤0.023) and towards higher CAC scores in albuminuric vs normoalbuminuric patients (p=0.026). In multivariate regression analysis, a higher urinary albumin excretion rate (UAER) tended to predict reduced CFR in the total population (p=0.045). When the CAC score was added, there was also a trend (p=0.032) towards an inverse association with reduced CFR.

Conclusions/interpretation Type 2 diabetic patients who were free of overt cardiovascular disease had a high prevalence of coronary microvascular dysfunction, especially with concomitant albuminuria, suggesting a common microvascular impairment occurring in multiple microvascular beds. Prospective studies are needed to show the prognostic significance of this finding.

Keywords Cardiovascular disease · Coronary artery calcium score · Coronary flow reserve · Coronary microcirculation · Microalbuminuria · Microvascular disease · PET/CT · Type 2 diabetes

Abbreviations
CAC Coronary artery calcium
CAD Coronary artery disease
CFR Coronary flow reserve
CT Computed tomography
eGFR Estimated GFR
IQR Interquartile range
LVEF Left ventricle ejection fraction
PET Positron emission tomography
UAER Urinary albumin excretion rate
Introduction

Type 2 diabetes is commonly associated with the early development of coronary artery disease (CAD) [1]. For diabetic patients with concomitant albuminuria, the risk of cardiovascular disease is further pronounced [2]. More research is needed to define potential subsets of patients with diabetes who might benefit from additional testing for asymptomatic CAD.

Abnormalities of the coronary circulatory function are well documented to occur before and to accelerate during the development of CAD in patients with diabetes [3]. However, these changes in coronary circulatory function, as well as mild structural variations in the arterial wall, might begin early as subclinical forms, while it presumably takes substantially more time for the clinical appearance of advanced stages of CAD [4].

Definitions of clinically significant CAD vary substantially, based on whether anatomical or functional criteria are used. It is important to note that the agreement between anatomical (e.g. degree of coronary stenosis) and functional (e.g. myocardial perfusion) criteria is often poor [5].

Quantitative cardiac positron emission tomography (PET) is a new and innovative method with which to detect patients with subclinical CAD. One of the major advantages of cardiac PET is that, uniquely, it can measure absolute myocardial blood flow (in ml/min/g of tissue) at rest and during pharmacologically induced hyperaemic conditions, from which a ratio can be calculated (termed the coronary flow reserve [CFR]) as an adjunct to the visual interpretation of myocardial perfusion [6].

CFR is an important physiological variable in the coronary circulation that reflects the function of large epicardial arteries and the microcirculation. Thus, reduced CFR can be caused by both stenosis in the epicardial coronary arteries and coronary microvascular dysfunction. In individuals without epicardial coronary stenosis, cardiac PET can assess the function of the microcirculation, including the combined function of cells in the vascular smooth muscle and the endothelial cells. Among individuals without indications of CAD, microvascular dysfunction is associated with cardiovascular risk factors, including hypercholesterolaemia, diabetes, hypertension and smoking [7]. Moreover, it is a predictor of cardiac mortality [8]. Most patients with type 2 diabetes have associated hypertension, dyslipidaemia and obesity, all of which can contribute to coronary microvascular damage.

In addition, a computed tomography (CT) scan can be used to estimate coronary artery calcium (CAC), and this imaging modality can be usefully combined with quantitative cardiac PET in a hybrid scanner. CAC is known to be highly correlated with the extent of coronary atherosclerosis, and can identify asymptomatic patients who are at higher risk for inducible ischaemia and mortality [9, 10]. The presence of calcium in the coronary arteries is a specific marker of atherosclerosis, independent of its aetiology.

We undertook a cross-sectional study of type 2 diabetic patients with or without albuminuria and age- and sex-matched healthy controls without known manifest cardiovascular disease. The aims were twofold. First, to examine the prevalence of impaired CFR and elevated CAC in the three predefined groups of participants without known cardiovascular disease: (1) patients with type 2 diabetes and albuminuria; (2) patients with type 2 diabetes and normoalbuminuria; and (3) healthy controls. And second, to determine predictors of impaired CFR and elevated CAC in the total population.

The prespecified study hypotheses were that: (1) patients with type 2 diabetes and albuminuria have an impaired coronary microcirculation (assessed using the CFR) and increased coronary calcification (assessed using CAC) when compared with normoalbuminuric patients; and (2) patients with type 2 diabetes and normoalbuminuria have impaired CFR and increased CAC when compared with healthy controls.

Methods

Study population A cohort of 60 consecutive outpatients with type 2 diabetes, defined according to the WHO criteria, was identified at Steno Diabetes Center. Participants were aged between 35 and 80 years, and had the ability to understand and give informed consent. Patients were stratified as normoalbuminuric if the urinary albumin excretion rate (UAER) was <30 mg/24 h in two of three consecutive urine collections (two of the samples were collected over 24 h in relation to the present study). A priori, we decided to include 30 patients with normoalbuminuria and 30 with persistent elevated albuminuria (UAER ≥30 mg/24 h). In addition, 30 non-diabetic controls were recruited from a newspaper advertisement and matched for age and sex to the 30 patients with type 2 diabetes, defined according to the WHO criteria, who most closely resembled and matched for age and sex to the 30 patients with type 2 diabetes and normoalbuminuria. Participants were excluded if one of the following characteristics were present: (1) history of CAD or other cardiovascular disease (including stroke) or heart symptoms, assessed from patient files and thorough interviews and questionnaires; (2) asthma or chronic obstructive pulmonary disease requiring treatment; (3) kidney disease other than diabetic nephropathy; (4) end-stage renal disease; (5) office BP >200/110 mmHg; (6) second- or third-degree atrioventricular block; or (7) pregnancy or lactating.

A power calculation was performed using the power statement implemented in the SAS software, version 9.3 (SAS Institute, Cary, NC, USA). In the assumption of a mean difference in CFR of 0.6 and an SD of 0.8, at least 27 participants in each group were needed to provide 80% power for a type I error of 5%. To account for technical difficulties and incomplete investigations, we included a total of 30 participants in each group.

The study was performed from April to December 2013 and was conducted in compliance with the Declaration of
Calculated as body weight (kg) divided by height (m$^2$). A standard 12-lead resting ECG was obtained.

Clinical measurements Three office BPs were measured in the sitting position after 5–10 min rest, with an oscillometric device (A&D Medical, San Jose, CA, USA) using an appropriate cuff size and averaged. HbA$_1c$ was measured by HPLC and plasma creatinine by an enzymatic method (Hitachi 912, Roche Diagnostics, Mannheim, Germany). Estimated (e)GFR was calculated using the CKD-EPI equation [11].

Measurement of 24 h BP was conducted using BPro (HealthStats, Singapore), a watch-like device that captures radial pulse wave reflection with tonometry and calculates brachial 24 h BP from the pulse wave after calibration to brachial BP. The device meets standards from the European Society of Hypertension and the Association for the Advancement of Medical Instrumentation [12]. The device was programmed to capture BP every 15 min for 24 h. Mean systolic and diastolic BP were calculated using all readings over the 24 h.

UAER was measured in two 24 h urine collections by an enzyme immunoassay and calculated as the geometric mean of the two collections.

A detailed medical history was obtained along with demographic and anthropometric variables, including smoking status. Current smoking was defined as one or more cigarettes, cigars or pipes per day. Information on medical treatment was obtained from questionnaires and cross-checked against medical records at the Steno Diabetes Center. The weight and height of each participant were measured, and BMI was calculated as body weight (kg) divided by height (m$^2$). A standard 12-lead resting ECG was obtained.

Hybrid cardiac PET/CT imaging A dynamic, gated cardiac PET study was performed using a hybrid PET/CT scanner in 3D mode (Siemens Biograph mCT 128, Siemens, Munich, Germany) following the administration of 1,100 MBq $^{82}$Rb (CardioGen-82, Bracco Diagnostics, Monroe Township, NJ, USA). The myocardium was covered in a single bed position, with ECG gating (8 frames/RR cycle; total acquisition time 7 min). Low-dose CT was acquired for attenuation correction. Images were reconstructed into 16 images ($12 \times 10$ s, $2 \times 30$ s, $1 \times 60$ s, $1 \times 120$ s) with attenuation, scatter and decay corrections using iterative 3D ordered subsets expectation–maximisation (two iterations; 24 subsets) and Gaussian filtering with 10 mm full width at half maximum. Semiquantitative data were processed using Cedars QPS/QGS software (version 2012, Cedars-Sinai, Los Angeles, CA, USA). Non-gated images were presented in the horizontal and vertical long axis and in the short axis, and displayed in a polar map format divided into 17 segments, as suggested by the American Heart Association [13], and normalised to peak myocardial activity (100%) [14]. Myocardial blood flow was calculated automatically using the Siemens Syngo MBF 2.3 (Siemens Medical Solutions, Malvern, PA, USA) with one-compartment tracer kinetic models for $^{82}$Rb, including regional uptake and clearance variables, blood to myocardium spill-over and partial volume corrections [15], and the extraction curve from Lortie et al [16]. Maximal hyperaemia was induced with adenosine infused at 140 μg kg$^{-1}$ min$^{-1}$ for 6 min. For semiquantitative assessment of myocardial perfusion abnormalities, 17-segment visual interpretation of gated myocardial perfusion images was performed by two experienced operators. Participants abstained from all caffeine- or methylxanthine-containing substances for at least 18 h prior to the cardiac PET scan.

CAC content was quantified using the method described by Agatston et al [17] and semiautomated commercially available software (Corridor4DM, INVIA, Ann Arbor, MI, USA). Coronary artery specific scores were calculated in the three main coronary arteries and then summed to provide a total CAC score for each participant.

Coronary microvascular dysfunction was defined as CFR <2.5, as suggested by Schindler et al [18]. Elevated CAC was a value >300, according to a previous study by Detrano et al [19].

Statistical analysis The distribution of CAC was skewed and was therefore log-transformed (log$_e$ [CAC + 1]) in all analyses, as was UAER and known duration of diabetes. These variables are given as medians with interquartile range (IQR). All other continuous variables are given as means ± SD and the categorical variables are given as total numbers with corresponding percentages. When analysing differences between two groups (controls vs normoalbuminuric patients, and normoalbuminuric vs albuminuric patients) we used independent samples $t$ test for continuous variables and $\chi^2$ for categorical variables. ANCOVA was applied when comparing levels of CFR and CAC between the three groups.

We ascertained that the four principal assumptions of linear regression were fulfilled. The proportion of the variability in the dependent variable explained by the model is presented as $R^2$. Because of the use of repeated independent $t$ tests increasing the probability of a type I error, results were considered to be significant at a two-tailed $p<0.01$, and $p<0.05$ to $p=0.01$ as trend. Statistical analyses were performed using SAS software (version 9.3; SAS Institute).

Rationale for selection of covariates Because there was a modest number of participants in each group, only a limited sum of covariates could be included due to the risk of overfitting. Therefore, we included traditional cardiovascular risk factors based on prior evidence in the multivariate analysis. Owing to bias by indication, we did not include variables for medical treatment. Moreover, total cholesterol...
was not included because patients had lower levels than controls, likely due to lipid-lowering treatment. In the analysis comparing levels of CFR and CAC between the groups, HbA\textsubscript{1c} and UAER were not included, since the groups were precategorised based on these variables. Thus, the final adjustment for the group comparisons consisted of sex, age, 24 h systolic BP, eGFR and smoking.

For the multivariate linear regression models for predictors of reduced CFR and increased CAC, we applied the enter method. This approach was chosen because we did not know which independent variables would be significantly correlated to the outcome variables (i.e. CFR and CAC). The model included sex, age, 24 h systolic BP, eGFR, smoking, HbA\textsubscript{1c} and UAER.

To determine the robustness of the findings for the differences between groups, we additionally performed a multivariate analysis including variables showing statistically significant ($p<0.05$) correlations in the sample; again excluding total cholesterol and medical treatment. This approach can, however, result in biased estimates of effect [20].

### Results

**Clinical characteristics** The total cohort ($n=90$) comprised 64.4% men and the mean±SD age was 62.1±9.2 years. The characteristics of the participants in the three groups are shown in Table 1. The normoalbuminuric patient group had a higher mean BMI and heart rate than controls, a higher frequency of antihypertensive or lipid-lowering treatment and lower total cholesterol ($p\leq0.001$). Patients with albuminuria had a lower eGFR than normoalbuminuric patients ($p<0.001$). There were no intergroup differences in the proportion of men, 24 h BP or the prevalence of smoking ($p\geq0.067$). Patients with microalbuminuria (UAER 30–299 mg/24 h; $n=23$) and macroalbuminuria (UAER >300 mg/24 h; $n=7$) did not differ in any of the examined characteristics ($p>0.40$), except for a trend towards lower eGFR in those with macroalbuminuria ($p=0.037$).

All patients were treated with dietary modifications and oral glucose-lowering medication, and 48% also received insulin. Moreover, most patients received lipid-lowering therapy (93%), aspirin (88%) and renin–angiotensin–aldosterone system blocking treatment (90%).

### Table 1  Clinical characteristics of participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls ($n=30$)</th>
<th>Normoalbuminuria ($n=30$)</th>
<th>Albuminuria ($n=30$)</th>
<th>$p$ controls vs normoalbuminuria</th>
<th>$p$ normoalbuminuria vs albuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>12 (40)</td>
<td>12 (40)</td>
<td>8 (27)</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.8±9.9</td>
<td>60.9±10.1</td>
<td>65.6±6.8</td>
<td>0.08</td>
<td>0.038</td>
</tr>
<tr>
<td>Known diabetes duration (years)</td>
<td>–</td>
<td>11.2 (4.1–14.7)</td>
<td>13.7 (8.2–23.0)</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>24.8±3.4</td>
<td>31.6±4.6</td>
<td>31.5±4.5</td>
<td>&lt;0.001</td>
<td>0.93</td>
</tr>
<tr>
<td>Office systolic BP (mmHg)</td>
<td>130±18</td>
<td>140±19</td>
<td>137±17</td>
<td>0.027</td>
<td>0.51</td>
</tr>
<tr>
<td>Office diastolic BP (mmHg)</td>
<td>77±10</td>
<td>83±9</td>
<td>78±10</td>
<td>0.030</td>
<td>0.069</td>
</tr>
<tr>
<td>24 h systolic BP (mmHg)</td>
<td>126±14</td>
<td>133±17</td>
<td>135±20</td>
<td>0.11</td>
<td>0.77</td>
</tr>
<tr>
<td>24 h diastolic BP (mmHg)</td>
<td>79±8</td>
<td>82±12</td>
<td>79±11</td>
<td>0.23</td>
<td>0.37</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>60.7±8.9</td>
<td>71.9±13.7</td>
<td>72.0±10.9</td>
<td>&lt;0.001</td>
<td>0.93</td>
</tr>
<tr>
<td>HbA\textsubscript{1c} (%)</td>
<td>5.4±0.2</td>
<td>7.3±1.1</td>
<td>7.1±1.0</td>
<td>&lt;0.001</td>
<td>0.44</td>
</tr>
<tr>
<td>HbA\textsubscript{1c} (mmol/mol)</td>
<td>35.8±1.9</td>
<td>56.7±12.1</td>
<td>54.4±10.8</td>
<td>&lt;0.001</td>
<td>0.44</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.5±0.7</td>
<td>4.3±0.9</td>
<td>4.2±0.9</td>
<td>&lt;0.001</td>
<td>0.58</td>
</tr>
<tr>
<td>eGFR (ml min\textsuperscript{-1} 1.73 m\textsuperscript{2})</td>
<td>82.8±13.1</td>
<td>86.6±23.4</td>
<td>67.5±24.7</td>
<td>0.68</td>
<td>0.005</td>
</tr>
<tr>
<td>Urinary albumin excretion rate (mg/24 h)</td>
<td>6.0 (5.0–10.5)</td>
<td>6.5 (5.5–13.5)</td>
<td>146 (51–298)</td>
<td>0.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>4 (13.3)</td>
<td>4 (13.3)</td>
<td>10 (33.3)</td>
<td>–</td>
<td>0.067</td>
</tr>
<tr>
<td>Alcohol (beverages/week)</td>
<td>9.2±6.7</td>
<td>7.3±7.3</td>
<td>5.9±6.4</td>
<td>0.36</td>
<td>0.50</td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>3 (10)</td>
<td>25 (83)</td>
<td>30 (100)</td>
<td>&lt;0.001</td>
<td>0.020</td>
</tr>
<tr>
<td>RAAS inhibition treatment</td>
<td>3 (10)</td>
<td>24 (80)</td>
<td>30 (100)</td>
<td>&lt;0.001</td>
<td>0.010</td>
</tr>
<tr>
<td>Beta-blocker treatment</td>
<td>0 (0)</td>
<td>3 (10)</td>
<td>5 (17)</td>
<td>0.076</td>
<td>0.45</td>
</tr>
<tr>
<td>Aspirin treatment</td>
<td>1 (3)</td>
<td>23 (77)</td>
<td>30 (100)</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Lipid-lowering treatment</td>
<td>0 (0)</td>
<td>27 (90)</td>
<td>29 (97)</td>
<td>&lt;0.001</td>
<td>0.30</td>
</tr>
<tr>
<td>Insulin treatment</td>
<td>–</td>
<td>13 (43)</td>
<td>16 (53)</td>
<td>–</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Data are n (%), mean±SD or geometric mean (IQR). RAAS, renin–angiotensin–aldosterone system.
Cardiac PET/CT scanning  Results from the cardiac PET/CT scanning are summarised in Table 2. There was a trend towards lower CFR and higher CAC in patients with normoalbuminuria compared with controls ($p\leq0.045$). CFR was significantly lower and CAC significantly higher in patients with albuminuria compared with normoalbuminuric patients ($p\leq0.002$). In trend tests across the three groups, CFR significantly decreased and CAC significantly increased ($p<0.001$). There was a trend towards a higher frequency of reduced CFR ($>2.5$) in normoalbuminuric patients compared with controls ($p=0.045$). The frequency of reduced CFR was significantly ($p<0.001$) higher in patients with albuminuria compared with normoalbuminuric patients, and there was a trend ($p=0.035$) towards a higher frequency of elevated CAC ($>300$).

After adjustment for sex, age, 24 h systolic BP, eGFR and smoking, the difference in CFR and CAC between albuminuric patients and controls remained significant ($p<0.001$). There was a trend towards lower CFR and higher CAC in normoalbuminuric patients vs controls ($p=0.023$), and towards higher CAC in albuminuric vs normoalbuminuric patients ($p=0.026$; Fig. 1).

Left ventricle ejection fraction (LVEF) was within the normal range ($>50\%$) both during rest and stress conditions in all groups, with no intergroup difference ($p\geq0.42$; Table 2). The LVEF increased (mean±SD: 6.0±4.7) during stress conditions in all except five participants.

Reversible ischaemia was observed in 12 participants (13%; one control, seven normoalbuminuric patients and four albuminuric patients), with a median (range) extent of 7.5% (2–23%). Three patients had fixed perfusion defects, two of whom had fixed defects with partial reversibility.

Variables correlated with CFR and CAC In the total population, CFR was significantly negatively correlated with CAC ($R^2=0.24; p<0.001$) and UAER ($R^2=0.20; p<0.001$), and positively correlated with eGFR ($R^2=0.11; p=0.001$) (Fig. 2). Moreover, there was a tendency towards a negative correlation between CAC and eGFR ($R^2=0.08; p=0.034$) and a significant positive correlation between CAC and UAER ($R^2=0.21; p<0.001$).

Predictors of reduced CFR in adjusted analyses In multivariate linear regression (model $R^2=0.32$), higher UAER tended to be a predictor of reduced CFR in the total population ($p=0.045$). When CAC was added (model $R^2=0.36$), there was also a trend ($p=0.032$) towards an inverse association with reduced CFR.

Predictors of increased CAC in adjusted analyses In multivariate linear regression (model $R^2=0.47$), the predictors of an increased CAC in the total population were older age (higher CAC in normoalbuminuric patients vs controls ($p<0.001$) and higher HbA1c ($p=0.004$), and a tendency for higher UAER ($p=0.046$) and male sex ($p=0.015$). When CFR was added (model $R^2=0.50$), there was also a trend ($p=0.032$) towards an inverse association with elevated CAC.

Additional analyses After adjustment for variables showing statistically significant correlations in the sample (i.e. age, BMI, eGFR and heart rate), the difference in CFR and CAC remained significant between albuminuric patients and controls ($p\leq0.008$) and between albuminuric and normoalbuminuric patients for CAC ($p=0.004$). There was a trend towards a difference in CFR in normoalbuminuric patients vs controls ($p=0.039$).

To avoid the potential confounding effect of epicardial stenosis on CFR, we performed a sensitivity analysis including only those participants without perfusion defects on semiquantitative analysis of the PET scan ($n=75$). The results were confirmatory; CFR was $3.0±0.8, 2.7±0.7$ and $2.1±0.5$ in controls, normoalbuminuric and albuminuric patients, respectively, and all intergroup differences were significant ($p<0.024$). Moreover, CFR was significantly negatively correlated with CAC ($R^2=0.21; p<0.001$) and UAER ($R^2=0.25; p<0.001$), and positively correlated with eGFR ($R^2=0.13; p=0.002$).

### Table 2  Cardiac PET/CT scanning

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=30)</th>
<th>Normoalbuminuria (n=30)</th>
<th>Albuminuria (n=30)</th>
<th>p controls vs normoalbuminuria</th>
<th>p normoalbuminuria vs albuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFR*</td>
<td>3.0±0.8</td>
<td>2.6±0.8</td>
<td>2.0±0.5</td>
<td>0.045</td>
<td>0.0023</td>
</tr>
<tr>
<td>CFR &lt;2.5*</td>
<td>5 (17)</td>
<td>12 (40)</td>
<td>25 (83)</td>
<td>0.045</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CAC score*</td>
<td>0 (0–81)</td>
<td>36 (1–325)</td>
<td>370 (152–1025)</td>
<td>0.044</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CAC score &gt;300*</td>
<td>2 (7)</td>
<td>8 (27)</td>
<td>16 (53)</td>
<td>0.095</td>
<td>0.035</td>
</tr>
<tr>
<td>LVEF at rest</td>
<td>60±7.6</td>
<td>62±8.7</td>
<td>61±9.5</td>
<td>0.42</td>
<td>0.87</td>
</tr>
<tr>
<td>LVEF at stress</td>
<td>67±6.5</td>
<td>68±8.2</td>
<td>67±10.1</td>
<td>0.94</td>
<td>0.77</td>
</tr>
<tr>
<td>Difference in LVEF</td>
<td>7.2±4.6</td>
<td>5.7±4.8</td>
<td>5.4±4.4</td>
<td>0.22</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Data are n (%), mean±SD or geometric mean (IQR)

*p<0.001 for trend across the three groups
In asymptomatic patients with type 2 diabetes, impaired CFR and elevated CAC were demonstrable with 82 Rb PET/CT, a new, fast and non-invasive hybrid imaging method for assessing these measures of vascular function and structure. The findings were further pronounced in patients with concomitant albuminuria. Our findings suggest that even in asymptomatic individuals, patients with type 2 diabetes and albuminuria, known to be at high risk of cardiovascular disease, have impaired coronary microcirculation when compared with normoalbuminuric patients. However, this was partly explained by other cardiovascular risk factors. Furthermore, asymptomatic normoalbuminuric type 2 diabetic patients showed a tendency towards impaired coronary microcirculation compared with age- and sex-matched controls, even after adjustment for cardiovascular risk factors.

Moreover, for CFR, we found a tendency towards a negative correlation with CAC and UAER. Older age and higher HbA1c were correlated with elevated CAC, and there was a tendency towards a correlation of higher UAER and male sex with elevated CAC.

Impaired CFR has previously been described as a powerful, independent correlate of cardiac mortality among patients with diabetes [8]. That study concluded that diabetic patients without known CAD and with impaired CFR experienced a rate of cardiac death comparable with that for non-diabetic patients with known CAD. Diabetic patients without known CAD and preserved CFR had very low annualised rate of cardiac mortality.

A CFR below 2.5 is considered reduced [18], and in our study 83% of patients with type 2 diabetes and albuminuria had impaired CFR according to this threshold, compared with 40% of normoalbuminuric patients. Previously, the degree of CFR restriction in diabetes has been associated with the magnitude of retinopathy [21] and with renal insufficiency [22]; altogether, suggestive of a common microvascular impairment occurring in multiple microvascular beds, which might be hypothesised to be part of the natural history of diabetic patients.

CAC is usually measured in asymptomatic patients with intermediate cardiovascular risk, and it has been consistently shown that a CAC level of 0 in this patient group is associated with a low risk of coronary events in 5–10 years [19]. A CAC level above 300, on the other hand, has been associated with an increased risk of cardiovascular events and mortality in both non-diabetic individuals and diabetic patients in 3–5 years of follow-up [9]. Furthermore, in a prospective study of 200 microalbuminuric patients with type 2 diabetes, we recently showed CAC to be the strongest predictor of cardiovascular events and mortality after 6 years of follow-up [23].
In the current study, we found that asymptomatic diabetic patients had higher CAC levels and a higher prevalence of increased CAC levels compared with healthy control participants. More than 50% of the albuminuria group had a CAC level above 300, indicative of a high risk of cardiovascular disease and mortality. Furthermore, both higher UAER and lower eGFR correlated with higher CAC. This could indicate that part of the increased cardiovascular risk in patients with impaired kidney function is mediated by metastatic calcification.

We found that CFR was negatively correlated with CAC. Recent cross-sectional studies have revealed a similar correlation between coronary microcirculation dysfunction and coronary artery calcification. Wang et al reported that CFR was negatively correlated with the presence and severity of CAC in a population of 222 individuals without known cardiovascular disease enrolled in a multiethnic study of atherosclerosis [24]. In contradiction to these findings, Pirich et al found no relationship between CAC and CFR in 22 asymptomatic individuals with a family history of premature CAD [25]. Curilova et al, however, documented a significant association between increasing CAC and declining CFR among patients suspected to have CAD [26]. Thus, although microvascular dysfunction is an important factor in the development of atherosclerosis, the association between functional and structural alterations in atherosclerosis might not be straightforward and might expose different pathophysiological processes and differences in time course.

This was also reflected in our population, where a CAC of 0 could not solely be used as a gatekeeper. In the 24 participants with a CAC of 0, the CFR range was 1.8–4.9, and three participants (3%) had coronary microvascular dysfunction (CFR <2.5).

Besides CFR and CAC, cardiac PET/CT imaging allows the identification of individuals with regional perfusion defects in the myocardium, induced by pharmacological stress. Interestingly, in our asymptomatic type 2 diabetic patients with a mean age of 63.2±8.8 years, the prevalence of myocardial regional perfusion defects was only 20%. This suggests that advanced and flow-limiting damage to the vessels might require a longer period to develop than previously estimated. However, the low prevalence could be biased by the inclusion of only asymptomatic patients. It is likely that patients with a more rapid development of advanced lesions were excluded because of previous cardiovascular disease or symptoms.

Clinical implications Cardiac PET imaging offers several advantages with regards to patient comfort, radiation safety and duration of image acquisition (<1 h, as compared with up to 2 days for classic cardiac single-photon emission CT). PET might be a promising imaging method to guide personalised care. However, every new technique must find its place in the clinical scenario through the demonstration of cost-effectiveness and superiority over established methodologies. Because CFR provides a quantitative assessment of the integrated effects of epicardial coronary stenosis, diffuse atherosclerosis and microvascular dysfunction [27], its role as an early and sensitive marker of myocardial tissue perfusion impairment is promising. By adding the known prognostic value of both CAC and LVEF to CFR, cardiac PET/CT imaging might be a potential screening tool with which to identify asymptomatic patients with type 2 diabetes who are at high risk of cardiovascular disease and mortality. However, since this technique is cumbersome and costly, the added predictive value compared with other predictors should be proven in larger, prospective studies.

Strengths and limitations The strength of this study is that, to our knowledge, it is the first to evaluate CFR in asymptomatic type 2 diabetic patients using the new, fast and non-invasive cardiac $^{82}$Rb PET/CT technique, while comparing these patients with healthy controls. Limitations of the study include the relatively small sample size and its cross-sectional nature. However, the findings were robust when applying two different statistical approaches for the multivariate models.

Prospective studies are needed to assess the relative impact on future cardiovascular events of the measures provided by cardiac $^{82}$Rb PET/CT imaging. Moreover, information on markers of inflammation and endothelial activation might have reinforced the concept that measuring CFR and CAC with the new technology works better than other approaches. In addition, evaluation of other parts of the vascular system (e.g. carotid arteries) would have strengthened our findings.

Conclusion In asymptomatic patients with type 2 diabetes, we found a high prevalence of impaired CFR and elevated CAC, especially in patients with concomitant albuminuria, suggesting a common microvascular impairment occurring in multiple microvascular beds.

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Some of these data were presented as an abstract at the EASD and ARTERY meetings in 2014.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

 Contribution statement BJvS conceived and designed the research, acquired data, performed statistical analysis and drafted the manuscript. PH, AK and PR conceived and designed the research, acquired data, handled funding and supervision, and critically revised the manuscript for key intellectual content. TEC and AAG acquired data and critically revised the manuscript for key intellectual content. TWH conceived and
References

Paper V
Impaired coronary microcirculation in type 2 diabetic patients is associated with elevated circulating regulatory T cells and reduced number of IL-21R+ T cells

Bernt Johan von Scholten1*, Alexander Rosendahl2,3,7†, Philip Hasbak4, Regine Bergholdt2, Andreas Kjaer4, Peter Rossing1,5,6 and Tine W. Hansen1

Abstract

Background: Low-grade systemic inflammation is considered to participate in the progression of type 2 diabetes (T2D) and in diabetic complications.

Methods: To determine if circulating leukocytes were abnormally regulated in T2D patients, 8-color flow-cytometry (FACS) analysis was performed in a cross-sectional study of 37 T2D patients and 16 controls. Data obtained from the FACS analysis were compared to coronary flow reserve (CFR), assessed by Rb82-PET-imaging, to uncover inflammatory signatures associated with impaired CFR.

Results: Presence of T2D was associated with T cell attenuation characterized by reduced overall T cell, Th17, IL-21R+ T cells, and TLR4+ T cells, while the monocyte population showed enhanced TLR4 expression. Further, our data revealed reduced M1-like CD11c expression in T2D which was associated with impaired CFR. In contrast, we show, for the first time in T2D, increased TLR4 expression on CD8 T cells, increased Treg cell number and Treg maturation and reduced IL-21R expression on CD8 T cells to be functionally associated with impaired CFR.

Conclusions: Our demonstration that HbA1c inversely correlates to several T cell populations suggests that T cells may play disease modulating roles in T2D. Further, the novel association between impaired CFR and regulatory T cells and IL-21R+ T cells imply an intricate balance in maintaining tissue homeostasis in vascular diabetic complications.

Keywords: Type 2 diabetes, Flow-cytometry (FACS) analysis, Coronary microcirculation, Coronary flow reserve, Inflammation, Cardiovascular disease, Peripheral blood, Monocyte sub-populations
Patients with T2D are at high risk of cardiovascular disease [6], and inflammatory processes play an important role in the development of cardiovascular disease [7]. Coronary flow reserve (CFR) is an important physiological parameter in the coronary circulation that reflects the function of large epicardial arteries and the microcirculation. Impaired CFR has previously been described as a powerful, independent correlate to higher cardiac mortality among patients with diabetes [8].

IL-21R positive T cells have been shown to exhibit non-redundant roles in several systemic inflammatory conditions e.g. rheumatoid arthritis [9, 10]. TLR4 is classically associated with myeloid cells and with function as signalling receptor through which the cells sense bacterial infections [11]. Recently, expression and activation of TLR4 on various lymphoid cells, e.g. CD4+ T cells, was demonstrated, and has been suggested to act as a negative regulator of the immune response limiting the excessive inflammation [12, 13].

The present study investigated if altered leukocyte sub-populations were present in peripheral blood in patients with T2D compared to healthy controls. Further, the association between leukocyte sub-populations and impaired coronary microcirculation (assessed by CFR) was determined to identify potential novel biomarkers or functional explanations so far not known in T2D subjects.

**Methods**

**Study population**

From April to December 2013, we conducted a cross-sectional study at Steno Diabetes Center with the aim to assess the value of cardiac positron emission tomography/computed tomography (PET/CT) for non-invasive estimation of microvascular function and structure [14]. A total of 60 consecutive patients with T2D (defined according to the WHO criteria) and 30 non-diabetic controls were included. Participants were aged between 35 and 80 years, with the ability to understand and give informed consent. Participants were excluded if one of the following characteristics were present: (1) history of coronary heart disease or other cardiovascular disease (including stroke) or symptoms from the heart, assessed from patient files and thorough interviews and questionnaires; (2) asthma or chronic obstructive pulmonary disease requiring treatment; (3) kidney disease other than diabetic nephropathy; (4) end-stage renal disease; (5) office blood pressure >200/110 mmHg; (6) second or third degree atrioventricular block; or (7) pregnancy or lactating.

In this per-protocol specified sub-study, 37 of the patients with T2D and 16 of the non-diabetic control persons were included. The inclusion of the participants was based on time of the day for the drawing of the fresh blood sample at Steno Diabetes Center, to allow the flow cytometric analyses to be completed on the same day. Hence, only participants with a drawing of the blood performed before 12 p.m. were included.

Power calculation was performed for the primary study [14] and not for this sub-study. Analyses presented in this manuscript were hypothesis generating, and based on existing literature we anticipated that our sample size was sufficient.

The study was conducted in compliance with the Declaration of Helsinki. All participants gave informed written consent and the study protocol was approved by The Research Ethics Committee, Capital Region of Denmark.

**Clinical measurements**

HbA1c was measured by high-performance liquid chromatography and plasma creatinine by an enzymatic method (Hitachi 912, Roche Diagnostics, Germany). Current smoking was defined as one or more cigarettes/cigars/pipes a day. Weight and height of each participant were measured, and body mass index was calculated as body weight in kilograms divided by height in meter squared.

**Hybrid cardiac positron emission tomography (PET)/computed tomography (CT) imaging**

A dynamic, gated cardiac PET study was performed using a hybrid PET/CT scanner in 3D mode (Siemens Biograph mCT 128, Siemens, Germany) following administration of 1100 MBq 82Rb (Cardiogen®Bracco Diagnostics Inc., USA). Myocardial Blood Flow was calculated automatically with Siemens Syngo MBF 2.3® (Siemens Medical Solutions, USA), using one-compartment tracer kinetic models for 82Rb, including regional uptake and clearance parameters, blood to myocardium spill-over and partial volume corrections, and the extraction curve from Lortie et al. [15]. Maximal hyperemia was induced with adenosine infused at 140 μg/kg/min for 6 min. Participants abstained from all caffeine or methylxanthine containing substances for at least 18 h prior to the cardiac PET scan.

**Flow cytometry analysis**

Flow cytometric analysis was performed according to standard procedures and samples acquired on a FACScalibur equipped with blue, red and violet laser followed by data analysis using FACSdiva software (BD Biosciences). Briefly, to inhibit unspecific binding cells were blocked with anti-CD16 (BD Biosciences) and anti-CD32 (BD Pharmingen). This was followed by surface
staining of 7-amino-actinomycin D (7-AAD) (Biolegend), CD45 (Biolegend), CD3 (eBioscience), CD4 (Biolegend), CD8 (BD), CD19 (eBioscience) IL-21R (BD Pharmingen), ICOS (eBioscience), CXC53 (BD), CCR6 (Biolegend), CD14 (BD), CD16 (Biolegend), CD16c (Biolegend), CD163 (Biolegend), TLR4 (Biolegend), CD68 (Biolegend), CD25 (Biolegend), CD127 (BD), CTLA-4 (Biosciences), FoxP3 (Biolegend), GITR (Biolegend).

To determine total cell number in the whole blood samples, total cell count analysis was performed using 123 count beads (eBioscience) according to manufactures description.

Mean fluorescence intensity (MFI) was determined using the geometric mean value after staining with the marker antibody. To verify specificity, a control with a threefold higher concentration of an isotype control antibody was used both in healthy control subjects and in the patients. The MFI after isotype control was similar between healthy and patient samples and hence the value used and shown is the uncorrected MFI value obtained after each marker antibody.

Th17 cells were determined by identifying CD3+ CD4+ CCR6+ cells that upon first 1 h activation with 50 ng/ml PMA and 1 mg/ml ionomycin in (RPMI+ 10 % FCS and P/S, Sigma) and then an additional 5 h in the presence of 10 mg/ml BrefeldinA were positive for IL-17 (eBio64DEC17, eBioscience) after fixation and permeabilization.

Statistical analysis

Based on their graphical distribution and the results of the Shapiro-Wilk test [p < 0.05; indicating a non-normal distribution], the skewed variables were log-transformed the Shapiro-Wilk test [(p < 0.05); indicating a non-normal distribution], the skewed variables were log-transformed prior to the t test and linear regression analysis. Differences between patients and controls were assessed by independent samples t test for age, sex, body mass index and smoking the statistical significance. Statistical analysis was performed using SAS software (version 9.3; SAS Institute, NC, USA).

Results

Cohort description

The total cohort (n = 53) included 67.9 % male and mean (±SD) age was 62.0 ± 9.3 years. Characteristics of participants in the two groups are shown in Table 1. Age and sex distribution did not differ significant between the groups (p ≥ 0.58). However, the patients had higher body mass index compared to the controls (p < 0.001).

The cohort (n = 53) was similar to the total population in the main study (n = 90) in relation to age (p = 0.96), sex (p = 0.50), body mass index (p = 0.20) and smoking (p = 0.54).

The number of Th17 lymphocytes is reduced, while the TfH cells are increased in T2D patients

The total number of circulating whole blood cells is known to be increased in obese subjects [16]. Detailed analysis in T2D patients is yet to be carefully evaluated and linked to risk parameters. Hence, we determined if the number of T and B cells was modulated in our T2D patient cohort using the gating strategy shown in Additional file 1: Figure S1 and Fig. 1.

The frequency of viable cells in the peripheral blood was similar in healthy and T2D patients determined as frequency of 7AAD exclusion (data not shown). On a cellular level, T2D patients was shown to have significantly (p = 0.008) lower total number of CD4+ T cells compared to healthy subjects (433 ± 24 and 579 ± 54 CD4+ T cells/µl blood respectively) (Fig. 2a). In contrast, no difference between the T2D patients and healthy subjects was discovered on B cells level (Fig. 2b). On a CD4 subset level, significant reduction of Th17 cells and a significant increase of TfH cells were observed (Fig. 2c, d). No difference between the T2D patients and healthy subjects was discovered on B cells level (Fig 2e). When adjusting for age, sex, body mass index and smoking the statistical difference between healthy and T2D patients on the CD4

<table>
<thead>
<tr>
<th>Table 1 Clinical characteristic of the participants</th>
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<tbody>
<tr>
<td>Controls (n = 16)</td>
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<td>-------------------</td>
</tr>
<tr>
<td>Female, n (%)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Diabetes duration (years)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
</tr>
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<td>Smokers, n (%)</td>
</tr>
</tbody>
</table>

Data represent percentage (%) or mean ± SD. P values denote differences between controls and patients and were assessed by t test. Diabetes duration was non-normal distributed and is represented as mean [IQR]
level and the reduction of Th17 cells remained significant ($p \leq 0.003$; Table 2).

Taken together, these results reveal that a reduction of the total number of CD4$^+$ T cells and of Th17 cells is present in T2D, and that the reduction in this T2D cohort is independent of age, sex, body mass index and smoking.

Circulating M1-like monocytes are reduced in T2D patients and lower CFR is associated with reduced expression of CD11c$^{\text{high}}$ on monocytes.

Low grade inflammation is characterized by an enhanced number of M1-like macrophages in adipose tissue and skeletal muscle. The total number of circulating monocytes is not significantly modulated in patients at risk to
develop T2D [17], while pre-clinical models of T2D have demonstrated a repolarization from an initial M1-like phenotype into a M2-like phenotype in established disease [18]. To address if patients with established T2D display an altered profile of circulating monocyte polarization profile compared to healthy subjects associated with CFR, we performed analysis of peripheral blood in our T2D patient cohort. Using the gating strategy in Additional file 1: Figure S1 and Fig. 3 monocyte subsets were identified.

Healthy subjects and T2D patients in our cohort both had approximately 300 monocytes/μl blood (Fig. 4a). Analysis of the monocyte compartment using the CD14 and CD16 expression profile as functional markers of M1- and M2-like polarization [19, 20] uncovered a disease specific regulation of the polarization signature (Fig. 4b–d). The undifferentiated CD14^+CD16^-M0-like monocytes, show a moderate reduction in T2D blood compared to healthy subjects (257 ± 9 and 294 ± 20/μl respectively), while the M2-like CD14^+CD16^+ cells show no difference between the groups (Fig. 4b, c). Most interestingly, the M1-like CD14^dimCD16^- monocytes, showed a strong and highly significant reduction (p < 0.001) in the T2D patients compared to the healthy...

**Fig. 2** Circulating lymphocyte populations in diabetic patients and healthy controls. The number of CD4 T cells (a), CD8 T cells (b), Th17 T cells (c), TfH T cells (d) and B cells (e) is shown. A total of 2 ml blood was analysed and the total number of each cell population was calculated as described in the “Methods” section. Each dot represents one individual and the horizontal line represents the mean value in each group. P values represent difference between groups assessed by t test.

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subjects (30 ± 2 vs 44 ± 3/µl respectively) (Fig. 4d). No
difference between groups was observed after adjusting
for age, sex, body mass index, and smoking (p ≥ 0.16; Table 2). In contrast, a significant difference remained
between healthy and T2D patients also after adjustment
in the M1-like subset of monocytes (p = 0.006; Table 2).
To further evaluate the reduction of M1-like monocytes,
expression of the M1-associated cell marker CD11c on
the monocyte subsets was performed. As expected, no
modulation of CD11c expression on the M2- and M0-like
monocyte subsets or on the total monocyte population
was identified (Fig. 5a–c). In sharp contrast, the remain-
ing CD14dimCD16+ M1-like monocytes showed a highly
significant increase of CD11c surface expression suggest-
ing that although the cell population as such is reduced
the remaining cells have augmented capacity to respond
in a pro-inflammatory manner (Fig. 5d). When perform-
ing adjustment for age, sex, body mass index and smok-
ing, this increase of CD11c surface expression remained
significant (p = 0.031; Table 2).

In adjusted models, the CFR was higher with higher
surface expression of CD11c both on total CD68+ mono-
cytes and on the M2-like CD14+CD16+ sub-population
(p ≤ 0.038; Table 4).

Taken together, the results demonstrate that no sig-
nificant change is noted on the overall circulating mono-
cyte count in peripheral blood in T2D patients although
a small reduction of M1-like monocytes is noted which
show an enhanced M1-like polarization. However, most
interestingly high expression of the M1-like marker
CD11c was correlated to high CFR indicating that
M1-like monocyte may be associated with improved car-
diac status in diabetic patients.

Circulating IL-21R+ T cells and monocytes are reduced
in T2D patients while high CFR is associated with high
numbers of IL-21R+ CD8 T cells

Cytokine receptor activation of leukocyte is essential
and determines polarization and effector cell function.
In obese subjects it has been demonstrated that IL-21

<table>
<thead>
<tr>
<th>Variable</th>
<th>Direction</th>
<th>Controls (n = 16) vs. patients (n = 37)</th>
<th>Hba1c (n = 53)</th>
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</tr>
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</tr>
<tr>
<td>M2 Mac number (CD14+CD16+)</td>
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<td>Mac CD11c_MFI (CD68)</td>
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<td>0.24</td>
</tr>
<tr>
<td>M0 CD14+CD16+CD11c</td>
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<td>0.20</td>
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<td>M1 CD14+CD16+CD11c</td>
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<td>0.88</td>
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<tr>
<td>M2 CD14+CD16+CD11c</td>
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<td>0.65</td>
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<td>0.003</td>
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<tr>
<td>B cell_IL21R_number</td>
<td>−</td>
<td>0.14</td>
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</tr>
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<td>CD4Tcell_IL21R_MFI</td>
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</tr>
<tr>
<td>CD8Tcells_IL21R_MFI</td>
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<td>0.004</td>
<td>0.006</td>
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<td>B cell_IL21R (MFI)</td>
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<td>0.023</td>
<td>0.076</td>
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<tr>
<td>CD68_IL21R APC-A Mean</td>
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<td>0.38</td>
<td>0.82</td>
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</table>

Values represent the p values after adjustment for age, sex, body mass index, and smoking. Significant values are highlighted in italics.

MFI = mean fluorescence intensity
+ = indicates higher values in patients and with increasing Hba1c; − = indicates higher values in controls and with decreasing Hba1c.
responsive cells are accumulated in adipose tissue where they are hypothesized to contribute to the metabolic disease progression by fuelling the inflammatory pathways [21]. To determine if a similar IL-21R modulation was present in T2D patients we evaluated the IL-21R surface expression levels (Fig. 6e) and the total number of IL21R+ cells and correlated that to the CFR.

The expression level of IL-21R on the circulating CD4+ T cells was highly significantly reduced (p < 0.0001) compared to healthy controls (Fig. 6a, e). A similar, but less pronounced reduction of IL-21R expression level was demonstrated on CD8+ T cells and B cells (p = 0.0017 and p = 0.0029 respectively) (Fig. 6b, c, e). In contrast, monocytes expressed low and non-modulated levels of IL-21R regardless disease (Fig. 6d, e). This reduced IL-21R expression level resulted in a twofold reduction of the number of IL-21R CD4+ and CD8+ T cells in the T2D patients (Additional file 1: Figure S1A, B). Interestingly,
no modulation was demonstrated on the number of IL-21R positive B cells in T2D patients, whereas a moderate reduction of IL-21R monocytes was determined (Additional file 2: Figure S2C, D).

The reduction of IL-21R expression level and total number of IL-21R positive cells in the T2D cohort remained highly significant in the CD4$^+$ and CD8$^+$ subsets also after adjustment for age, sex, body mass index and smoking ($p \leq 0.004$; Table 2). In fact, the reduction of IL-21R level on CD4$^+$ and CD8$^+$ T cells and the number of CD4$^+$-IL-21R positive cells was demonstrated to be a direct continuous variable for increased HbA1c ($p \leq 0.048$; Table 2).

The CFR was higher in patients that had a high number of IL-21R expressing CD8$^+$ T cells ($p = 0.0024$; Table 4). No association was noted on IL-21R$^+$ CD4$^+$ T cell or CD68$^+$ monocyte level (Table 4).

Taken together, with increasing HbA1c a strong reduction of IL-21R$^+$ T cells and IL-21R expression level was evident. Further the positive correlation between high CFR and elevated CD8$^+$ IL-21R T cells suggests that these IL-21R$^+$ T cells may be associated with better cardiac function in diabetes.

The number of TLR4 lymphocytes is reduced in T2D patients, but inversely correlated with high CFR TLR4 expression on B cells and T cells has been associated with immunosuppressive effect [22, 23]. Further, TLR4 expressing B cells are associated with increasing body mass index and an inverse correlation exists between the number of TLR4 T cells and fasting plasma glucose in pre-diabetic as risk [24]. To determine if TLR4 expression level is modulated in patients with T2D and associated with CFR, we performed flow cytometric analysis of the blood in our T2D cohort using the gating strategy from Additional file 1: Figure S1 and Fig. 7e.

The expression level of TLR4 on CD4$^+$ and CD8$^+$ T cell was not significantly modulated as a population (Fig. 7a, b). However, in both the CD4$^+$ and in the CD8$^+$ population, six individuals showed highly elevated expression of...
TLR4 compared to the remaining population in the T2D cohort which was not observed in the healthy subjects (Fig. 7a, b). No significant change in the TLR4 expression on B cells was demonstrated (Fig. 7c). However, the TLR4 expression level on monocytes in the blood was significantly increased in T2D patients compared to healthy subjects (Fig. 7d). In sharp contrast to the expression level of TLR4, the total number of circulating TLR4+CD4+ and CD8+ T cells and B cells was significantly reduced in the T2D cohort (Additional file 3: Figure S3A–C). The circulating monocytes expressing TLR4 was however not increased (Additional file 3: Figure S3D).

In fact, the reduced number of TLR4+CD4+ and CD8+ T cells remained even after adjustment for age, sex, body mass index and smoking ($p \leq 0.016$; Table 3).

The CFR was higher with reduced surface expression of TLR4 on CD8+ T cells ($p = 0.037$; Table 4). There was no correlation between CFR and TLR4 expression on CD4+ T cells or on monocytes (Table 4).

Taken together, although individual subjects showed enhanced expression level of TLR4 on the T cells, the number of TLR4+ T cells and B cells in the T2D cohort was significantly decreased. Further, the data demonstrate that the reduced expression of TLR4 on T cells was correlated with enhanced CFR suggesting that TLR4 lymphocytes may be related to cardiac functions in diabetes.

**Attenuated presence of regulatory T cells in T2D patients and inverse association between Treg presence and CFR**

Aberrant inflammation is present in several autoimmune diseases with enhanced activity in e.g. rheumatoid arthritis as well as reduced activity in certain tumours [25]. Recently this was demonstrated to relate to a reduced presence and activity of regulatory lymphocytes in autoimmune diseases and inversely an augmented activity of regulatory cells in tumours. To determine if regulatory T cells were modulated in our T2D cohort and associated with CFR, flow cytometric analysis of various Treg sub-populations in the peripheral blood was performed using gating strategy from Figs. 1 and 8e.

The mean number of regulatory T cells (CD3+CD4+CD25+/−CD127−/+) phenotype was 52 ± 4 cells per µl blood in healthy controls (Fig. 8a). A highly significant 26 ± 3 % reduction ($p < 0.001$) of the number of CD25+/−CD127−/+ Tregs was present in the T2D patients (Fig. 8a). While on average 8.3 ± 0.9 cells/µl blood co-expressed the regulatory transcription factor FoxP3 in healthy subjects, a significant ($p = 0.016$) 33 % reduction to 5.6 ± 0.4 cells/µl blood was evident in T2D patients.
Fig. 6: IL-21R expression level on leukocyte populations in diabetic patients and healthy controls. The IL-21R expression on CD4+ T cells (a), CD8+ T cells (b), B cells (c) and CD68+ monocytes (d) is shown. Representative histogram analysis of IL-21R expression on CD19+, CD68+, CD4+ and CD8+ cells compared to the isotype antibody signal (e) that were first identified using the gating strategy from Figs. 1, 2 and Additional file 1: Figure S1. A total of 2 ml blood was analysed and the total number of each cell population was calculated as described in the “Methods” section. Each dot represents one individual and the horizontal line represents the mean value in each group. P values represent difference between groups assessed by t test.
Further detailed analysis of the FoxP3+ cells revealed that 4.4 ± 0.4 cells/µl blood in healthy subjects also co-expressing CTLA4 while only 2.7 ± 0.3 cells/µl (p = 0.006; 38 % reduction) were CTLT4+ in T2D patients (Fig. 8c). To evaluate if the Tregs present in the blood had different transcriptional machinery associated with regulatory functions in healthy and diabetic subjects, the expression level of the transcription factor
FoxP3 was determined. The results clearly demonstrate that T cells in healthy subjects have a significantly higher expression level of FoxP3 than in T2D subjects in our cohort (Fig. 8d).

When adjusted for age, sex, body mass index and smoking the results remained significant (p ≤ 0.039; Table 3), except for the expression level of FoxP3 on the CD25$^+$CD127$^-$ cells (p = 0.29; Table 3).

Intriguingly and in sharp contrast, in adjusted analyses, the CFR was higher with reduced number of regulatory T cells (p = 0.029; Table 4). This inverse correlation was even more pronounced with highly significantly lower expression of FoxP3 in the regulatory T cells in patients with high CFR (p = 0.001; Table 4).

Taken together, this demonstrates that the number of regulatory cells is highly significantly reduced in T2D patients and that the remaining T cells show an attenuated differentiation towards a regulatory phenotype. Most importantly, high CFR was associated with low presence of regulatory T cells, suggesting that regulatory T cells might be linked to progression of cardiac complications in patients with T2D.

**Discussion**

In the present study we show that presence of diabetes is associated with a general T cell attenuation characterized by reduced overall T cell, Th17, IL-21R+$^+$, Treg$s^+$ and TLR4$^+$ T cell count, while the monocyte population shows enhanced TLR4 expression. Further, our data revealed a reduced M1-like CD11c expression on our T2D cohort which was functionally associated with lower CFR. In contrast, we show for the first time in a T2D cohort increased TLR4 expression on CD8 T cells, increased Treg cell number and Treg maturation and reduced IL-21R expression on CD8 T cells to be functionally associated with impaired CFR. Even with early and multifactorial treatment, T2D patients show an enhanced incidence of cardiovascular complications. Presence of low grade systemic and local tissue inflammation is now a well-established characteristic and strong evidence exists linking vascular diabetic complications to inflammatory pathways [26]. However, to the best of our knowledge, we are the first to evaluate the relation between systemic inflammatory signature and CFR in T2D patients. These findings might provide important novel information potentially guiding future therapeutic approaches to reduce the burden of cardiovascular complications in T2D patients.

Signalling through the IL-21 receptor promotes proliferation and contributes to effector mechanisms like viral elimination and antibody switch [27]. Interestingly, our T2D cohort demonstrated a significantly reduced expression level of IL-21R on circulating T cells compared to healthy subjects. Fabrizi et al. recently demonstrated an increase of IL-21R mRNA transcripts in adipose tissue in obese compared to healthy subjects [21]. Through local regulation of IRF4, these adipose immigrating IL-21R$^+$ T cells performed immunoregulation which reduced the activity of Treg cells in the adipose tissue fuelling the low grade inflammation [21]. These IL-21R$^+$

### Table 3  Circulating biomarkers in T2D patients vs. controls and in relation to Hba1c as continuous variable in adjusted analyses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Direction</th>
<th>Controls (n = 16) vs. patients (n = 37)</th>
<th>Hba1c (n = 53)</th>
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<tbody>
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<td>TLR4 on leukocytes in peripheral blood</td>
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<td>CD4 T cell TLR4 (MFI)</td>
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<td>0.016</td>
</tr>
<tr>
<td>Treg CD25$^+$CD127$^+$FoxP3 (number)</td>
<td>−</td>
<td>0.039</td>
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</tr>
<tr>
<td>Treg CD25$^+$CD127$^+$GITR$^+$ (number)</td>
<td>−</td>
<td>0.005</td>
<td>0.012</td>
</tr>
<tr>
<td>TregCD25CD127FoxP3_MFI</td>
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<td>0.29</td>
<td>0.076</td>
</tr>
</tbody>
</table>

Values represent the p-values after adjustment for age, sex, body mass index, and smoking. Significant values are highlighted in italics. MFI = Mean fluorescence intensity

+ indicates higher values in patients and with increasing Hba1c; − indicates higher values in controls and with decreasing Hba1c.
and strengthen our novel observations. Furthermore, we

TLR4 on leukocytes in peripheral blood

Regulatory T cells and expression of FoxP3 in T cells

Thus, our novel findings in established T2D extend our previous observations in patients at risk of developing T2D that the systemic presence of IL-21R+ CD4 T cells is reduced in obesity, pre-diabetes and T2D. Based on the Fabrizi and the McGuire data this may be a direct consequence of altered migratory patterns [21, 28]. Hence, circulating IL-21R+ T cells might act as a novel biomarker allowing identification of patients with metabolic syndromes.

Interestingly, on a CD4 subset level, a significant increase of Tfh cells was observed in T2D patients with Hba1c. As these cells migrate through CXCR5, the accumulation in the systemic compartment may indicate absence of or reduced levels of local chemokine production of CXCL13. Interaction with CXCR5/CXCL13 is essential for the organization and establishment of lymphoid tissues [29]. In human visceral leishmaniasis infections reduce lymphoid tissue production of CXCL13 results in impaired B/T cell interaction leading to suboptimal adaptive immunity [30]. Hence it is tempting to speculate that the reduced numbers of circulating T cells in our cohort may be associated with a similar mechanism.

Patients with a reduced number of systemic IL-21R+ CD8 T cells presented with a significantly lower CFR, indicative of impaired coronary microcirculation and hence a worse cardiovascular prognosis. This phenomenon was not observed on CD4+ T cell level in the cohort. Discrepancies in tissue accumulation have previously been described in viral pre-clinical models and in human HIV patients [31, 32]. With the distinct expression profile of IL-21R+ on CD4 and CD8 cells it is intriguing to speculate that IL-21R+ effector CD8 T cells rather than contributing to disease progression delivers yet to be identified signals protecting the vasculature in T2D. IL-21 and IL-21R inhibition has been frequently debated in the literature, where both higher and lower levels have been linked to atherosclerosis [33]. Our novel finding with impaired CFR in patients with a reduced number of circulating CD8 T cells add additional important knowledge currently lacking in the understanding of the cardiovascular complications associated with T2D potentially aiding in the contradictory data around IL-21 in vasculature complications. Consequently, low IL-21R on circulating CD8 T cells may be considered to be a good biomarker to identify patients with increased risk of having impaired CFR.

M1 macrophages have been linked to low-grade inflammation, insulin resistance and weight gain [34]. Tissue recruited monocytes differentiate into an M1

Table 4 Circulating biomarkers in T2D patients in relation to coronary flow reserve in adjusted analyses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Direction</th>
<th>Coronary flow reserve (n = 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating monocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac_number (CD68)</td>
<td>+</td>
<td>0.23</td>
</tr>
<tr>
<td>M0 Mac_number (CD14+CD16-)</td>
<td>+</td>
<td>0.75</td>
</tr>
<tr>
<td>M1 Mac_number (CD14+CD16+)</td>
<td>+</td>
<td>0.42</td>
</tr>
<tr>
<td>M2 Mac_number (CD14+CD16-)</td>
<td>+</td>
<td>0.32</td>
</tr>
<tr>
<td>MacCD11c_MFI (CD68)</td>
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</tr>
<tr>
<td>M0CD14+CD16+CD11c</td>
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</tr>
<tr>
<td>M1CD14+CD16+CD11c</td>
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<td>0.13</td>
</tr>
<tr>
<td>M2CD14+CD16+CD11c</td>
<td>+</td>
<td>0.037</td>
</tr>
<tr>
<td>IL-21R on leukocytes in peripheral blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4Tcell_IL21R_number</td>
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</tr>
<tr>
<td>CD8Tcell_IL21R_number</td>
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</tr>
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<td>CD8Tcell_IL21R_MFI</td>
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<tr>
<td>B cell_IL21R_MFI</td>
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<td>0.83</td>
</tr>
<tr>
<td>Mac_IL21R_APC_A mean</td>
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<td>0.68</td>
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<tr>
<td>ILR4 on leukocytes in peripheral blood</td>
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<td></td>
</tr>
<tr>
<td>CD4T cell ILR4 (MFI)</td>
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</tr>
<tr>
<td>CD8T cell TL4 (MFI)</td>
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</tr>
<tr>
<td>B cell TL4 (MFI)</td>
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<tr>
<td>Mac TL4 (MFI)</td>
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<tr>
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<td>B cellTL4_number</td>
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<td>MacTLR4_number</td>
<td>+</td>
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<tr>
<td>Regulatory T cells and expression of FoxP3 in T cells</td>
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<td>TregCD25*CD127− (number)</td>
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<td>TregCD25*CD127+FoxP3 (number)</td>
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<tr>
<td>TregCD25*CD127+GITR+ (number)</td>
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<td>0.31</td>
</tr>
<tr>
<td>TregCD25CD127FoxP3_MFI</td>
<td>−</td>
<td>0.001</td>
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Values represent the p values after adjustment for age, sex, body mass index, smoking and diabetes duration. Significant values are highlighted in italics. MFI Mean fluorescence intensity

+ indicates higher values with increasing coronary flow reserve; − indicates lower values with increasing coronary flow reserve

T cells migrated to the obesity adipose tissue through specific chemokine patterns not identified in the study by Fabrizi et al. [21]. A similar tissue homing of IL-21R+ T cells in diabetic patients has previously been shown to be dependent on the activity of CCR9 for gut-homing [28]. Due to both the gut-homing (CCR9 dependent) and adipose tissue homing (undefined) the net-result in the systemic compartment will be a reciprocal decrease of IL-21R+ circulating cells which is in accordance with and strengthen our novel observations. Furthermore, we recently demonstrated in a cohort of 20 subjects at risk to develop diabetes an inverse correlation of the frequency of IL-21R+ T cells with increased body mass index [17]. Thus, our novel findings in established T2D extend our previous observations in patients at risk of developing T2D that the systemic presence of IL-21R+ CD4 T cells is reduced in obesity, pre-diabetes and T2D. Based on the Fabrizi and the McGuire data this may be a direct consequence of altered migratory patterns [21, 28]. Hence, circulating IL-21R+ T cells might act as a novel biomarker allowing identification of patients with metabolic syndromes.

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M1 macrophages have been linked to low-grade inflammation, insulin resistance and weight gain [34]. Tissue recruited monocytes differentiate into an M1
macrophage phenotype and their accumulation leads to an imbalance between M1 and M2 macrophages locally in the tissue [34]. Increased pro-inflammatory cytokine production from M1 macrophages and/or reduced anti-inflammatory signals from the M2 macrophages promote adipose tissue dysfunction and impairs glucose tolerance in the early stages of diabetes [1]. In contrast, in late-stage established diabetes the macrophages...
exhibit a polarization profile closely associated with remodelling M2-like macrophages in mice [18, 20]. This disease-dependent maturation and polarization of the inflammatory response may directly contribute to the excessive tissue fibrosis which is the hallmark of diabetic complications [18, 35]. Herein we now extend our previous pre-clinical observations and demonstrate that macrophages associated with pro-inflammation (M1-like) are significantly reduced in circulation also in clinical samples from T2D patients. The pro-inflammatory M1-like cells may have accumulated in adipose and skeletal muscle contributing to the insulin resistance resulting in a reciprocal loss in the systemic compartment [36]. The observation that the remaining peripheral monocytes in patients with reduced CFR show lower expression of CD11c, i.e. classical M1-like monocytes, is intriguing. These cells are associated with production of pro-inflammatory cytokines but not with repair and remodelling mechanisms [37]. Further, CD11c on monocytes has been shown essential for monocyte adherence and migration into atherosclerotic plaques in pre-clinical models [38]. The discrepancies between the pre-clinical macrovascular diseases and the clinical microvascular disorder could be due to analyses in different disease segments and at different locations. With our demonstration that the overall M1-like monocytes are reduced in number, but the remaining monocytes express elevated CD11c indicates that low CD11c expression could be used as a functional biomarker to identify patients with impaired CFR.

Regulatory T cells control adaptive immune responses by suppressing T cells, NK cells, NKT cells, B cells and dendritic cells [39]. Regulatory T cells and subsets of CD4\(^+\) Th2 cells are known to secrete anti-inflammatory and homeostatic cytokines with the ability to inhibit and modulate macrophage recruitment and activation state [40]. Previous studies have shown that in obese adipose tissue, the regulatory T cells were diminished leaving these patients at risk to progress into diabetes [41]. In line with this, we demonstrate that the number of circulating regulatory cells is reduced in patients with T2D compared to healthy controls. Further, the remaining T cells showed an attenuated differentiation towards a regulatory phenotype. Similar reductions have been described in other chronic diseases often associated with immune deficiency (e.g. HIV) where it is strongly associated with a poor prognosis and active progression of disease development [42].

Transforming growth factor beta (TGF-β) is a crucial pleiotropic cytokine associated with the development of Tregs and Th17 cells [43]. In our cohort of patients with T2D, we showed a reduction of both Tregs and Th17. This suggests that neither the pro-inflammatory Th17 nor the regulatory Tregs may be suitable functional biomarkers of T2D as they are both down-regulated in the same disease stage. In sharp contrast, they may be good disease and functional biomarkers for coronary diabetic complications as they both are strongly associated with impaired coronary microcirculation. Most interestingly, enhanced numbers of Tregs as well as the elevated expression level of FoxP3 on the regulatory T cells was associated with impaired coronary microcirculation in our cohort. TGF-β promotes tissue remodelling and is highly associated with the development of Tregs [44]. In healthy subjects this is in balance, and accordingly, injury induces a pro-inflammatory response which after a short period is turned down by locally produced TGF-β that promotes Tregs leading to repair [44]. In contrast, in patients with chronic disease excessive TGF-β is produced in the “termination phase”. This leads to polarization towards M2-like macrophages, tissue remodelling, stiffening of tissues and in the end of this cascade to atherosclerosis [45]. In our T2D cohort, patients with increased Tregs had a lower CFR, indicative of impaired coronary microcirculation. Our data might suggest that augmented down-regulation is associated with excessive locally produced TGF-β leading to stiffening of the tissue. Hence, the balance is disturbed which may favour remodelling rather than normal tissue repair.

Along the same line, TLR4 expression on CD8 T cells was lower in patients with reduced CFR. This is highly interesting as TLR4 expressing lymphocytes has been described as regulators of the immune responses by providing signals that terminate pro-inflammation, while TLR4 expressing macrophages classically is known as potentiators of pro-inflammation [46]. The detrimental role of TLR4 in atherosclerosis through monocytes and macrophages is well established [47]. Our novel finding that reduced TLR4 on CD8 lymphocytes correlates to an impaired coronary microcirculation in T2D patients is a novel and potentially important finding placing immune-modulating CD8 T cells in a novel tissue protective role. Thus, caution should be taken when considering therapies inhibiting TLR4 in vascular diabetic complications as this might risk to further worsen the coronary microcirculation by inhibiting the TLR4+CD8 T cells.

Matrougui et al. [48] elegantly showed that injections with Tregs in hypotensive mice lead to reduced macrophage activation and infiltration, lower TNF\(\alpha\) levels and improved coronary arteriolar endothelium-dependent relaxation. Hence while Tregs may play a protective role in the development of coronary arteriolar endothelial dysfunction in hypertension, our data suggest that
in chronic diabetic induced disease increased Tregs may actually correlate to impaired coronary microcirculation.

Recent findings also depict the role of inflammation and immune dysregulation on coronary microvascular function. A study, including patients with non-obstructive coronary artery disease, demonstrated an independent relationship between higher levels of soluble urokinase-type plasminogen activator receptor (suPAR), a surrogate of systemic inflammation and immune function, and lower CFR [49].

Multiple factors can be involved in impairment of CFR including hyperglycemia, insulin resistance, endothelial dysfunction and increased cardiac sympathetic activity [50]. Moreover, recent cross sectional studies have shown lower CFR to be associated with decreased aortic distensibility [51] and left ventricular diastolic dysfunction [52] in asymptomatic patients with type 2 diabetes. Treatment of diabetic hyperglycemia may improve CFR [50]. However, in a short-term (10 weeks) study, the GLP-1 analogue liraglutide did not have any significant effect on CFR (assessed by Doppler flow echocardiography) in patients with type 2 diabetes [53]. These conflicting results might be explained by short observation times and selection criteria.

Strengths and limitations

A major strength of this study is the use and combination of two advanced procedures; FACS analyses and assessment of CFR by cardiac Rb82 PET/CT (a sensitive marker of coronary microcirculation (currently considered the gold standard)) [50] in a clinical relevant setting including both T2D patients and healthy controls. Despite these strengths, limitations merit considerations. As the aim of this study was hypothesis generating and thought provoking, we investigated a long series of markers. Further, due to the demanding procedures applied, the number of included participants is relatively low leading to limited power.

Conclusions

Herein we describe for the first time novel insights linking inflammatory signatures to the CFR. A pattern emerged suggesting low presence of Tregs, FoxP3 expression and TLR4+CD8+ T cells together with enhanced of IL21R+ CD8+ T cells and CD11c M1-like monocytes in peripheral blood to be associated with high CFR. Our data imply that low CFR seems to be associated with an attenuated inflammatory signature in combination with elevated regulatory activity. Our data provide important clues about inflammation-diabetes-CFR that may be useful when designing novel therapeutic agents filling the current unmet medical need as well as providing novel non-invasive functional biomarkers useful to identify patients at risk to develop severe T2D complications.

Additional files

Additional file 1: Figure S1. Gating strategy to identify living singlet leukocytes.

Additional file 2: Figure S2. Total number of IL-21R+ leukocyte populations in diabetic patients and healthy controls. The number of IL-21R+ CD4+ T cells (A), CD8+ T cells (B), B cells (C) and CD68+ monocytes (D) is shown. A total of 2 ml blood was analysed and the total number of each cell population was calculated as described in the Materials and Methods section. Each dot represents one individual and the horizontal line represents the mean value in each group. P values represent difference between groups assessed by t test.

Additional file 3: Figure S3. Total number of TLR4+ leukocyte populations in diabetic patients and healthy controls. The number of TLR4+ CD4+ T cells (A), CD8+ T cells (B), B cells (C) and CD68+ monocytes (D) is shown. A total of 2 ml blood was analysed and the total number of each cell population was calculated as described in the Materials and Methods section. Each dot represents one individual and the horizontal line represents the mean value in each group. P values represent difference between groups assessed by t test.

Authors’ contributions

BvS conceived and designed the research, acquired the data, performed statistical analysis and wrote the first draft of the manuscript. AR conceived and designed the research, acquired the data, handled funding and supervision and made critical revision of the manuscript for key intellectual content. PH and AK contributed to design of the study, acquired and analysed the CFR data, made critical revision of the manuscript. TWH conceived and designed the research, acquired the data, performed statistical analysis and made critical revision of the manuscript for key intellectual content. All authors read and approved the final manuscript.

Author details

1 Department of Diabetic Complications, Steno Diabetes Center, Niels Steensens Vej 1, 2820 Gentofte, Denmark. 2 Diabetes Complications Research, Novo Nordisk A/S, Måleje, Denmark. 3 Department of New Haemophilia, Novo Nordisk A/S, Gentofte, Denmark. 4 Department of Clinical Physiology, Nuclear Medicine & PET and Cluster for Molecular Imaging, Rigshospitalet, Copenhagen, Denmark. 5 University of Copenhagen, Copenhagen, Denmark. 6 Aarhus University Denmark, Aarhus, Denmark. 7 Baxter Inc, Medical Affairs, Tobalsvej 2, 2860 Søborg, Denmark.

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Competing interests

Steno Diabetes Center, where BvS, PR and TWH are employed, receives part of its core funding from unrestricted grants from Novo Nordisk Foundation and Novo Nordisk, and is owned by Novo Nordisk. BvS’s reports having given lectures for Novo Nordisk, all fees given to Steno Diabetes Center. AR and RB are employees of Novo Nordisk A/S. PR reports having given lectures for Novo Nordisk, all fees given to Steno Diabetes Center, and has equity interest in Novo Nordisk.

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References
Paper VI
Cardiac Autonomic Function Is Associated With the Coronary Microcirculatory Function in Patients With Type 2 Diabetes

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Cardiac autonomic dysfunction and cardiac microvascular dysfunction are diabetic complications associated with increased mortality, but the association between these has been difficult to assess. We applied new and sensitive methods to assess this in patients with type 2 diabetes mellitus (T2DM). In a cross-sectional design, coronary flow reserve (CFR) assessed by cardiac 82Rb-positron emission tomography/computed tomography, cardiac autonomic reflex tests, and heart rate variability indices were performed in 55 patients with T2DM, without cardiovascular disease, and in 28 control subjects. Cardiac 123I-metaiodobenzylguanidine scintigraphy was conducted in a subgroup of 29 patients and 14 control subjects and evaluated as the late heart-to-mediastinum ratio and washout rate. Impaired function of all the cardiac autonomic measures (except the washout rate) was associated with reduced CFR. A heart rate variability index, reflecting sympathetic and parasympathetic function (low-frequency power), and the late heart-to-mediastinum ratio, reflecting the function of adrenergic receptors and sympathetic activity, were positively correlated with CFR after adjustment for age and heart rate. The late heart-to-mediastinum ratio remained correlated with CFR after further adjustment. In patients with T2DM without cardiovascular disease, we demonstrate an independent association between cardiac autonomic function and CFR. We suggest that a reduced cardiac autonomic function and damage to the adrenergic receptors may contribute to the development of cardiac microvascular dysfunction.

Cardiovascular autonomic neuropathy (CAN) is an often overlooked and severe complication of type 2 diabetes mellitus (T2DM). CAN results from damage to the autonomic nerve fibers that innervate the heart and blood vessels and results in abnormalities in heart rate control and vascular dynamics (1). Early signs of CAN, characterized by increased sympathetic activity and/or decreased parasympathetic activity at rest, are reflected in detrimental changes in indices of heart rate variability (HRV). Failure in autoregulation in response to physical stimuli (e.g., cardiovascular autonomic reflex tests [CARTs]) is seen in later more severe stages of CAN. CAN is strongly associated with cardiovascular morbidity and mortality (1,2). CARTs, indices of HRV, and cardiac 123I-metaiodobenzylguanidine (123I-MIBG) scintigraphy have all been reported to be valid measures of cardiac autonomic function in patients with diabetes (3). 123I-MIBG scintigraphy allows a direct assessment of the integrity of the adrenergic cardiac innervation in contrast to CAN assessment by HRV and CART analyses, which are indirect measures of nervous dysfunction. Thus, 123I-MIBG scintigraphy may be more reliable to evaluate cardiac autonomic function (4). Cardiac 123I-MIBG scintigraphy may also diagnose CAN in early clinical stages before it can be detected by tests that indirectly show the autonomic function of the heart (5).

Impaired cardiac autonomic function has been suggested to promote cardiovascular disease by inducing ventricular arrhythmias and sudden death and by impairing circadian blood pressure fluctuations (6,7). Coronary flow reserve (CFR) is an important physiological variable in the cardiac circulation that reflects the function of large epicardial arteries and the microcirculation. Impaired CFR has been described as a powerful, independent predictor of cardiac
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mortality among patients with diabetes (8). The coronary artery calcium (CAC) score is known to be highly correlated with the extent of coronary atherosclerosis and can identify asymptomatic patients who are at higher risk for cardiac events and death. The presence of calcium in the coronary arteries is a specific marker of atherosclerosis, independent of its etiology (9).

We undertook a cross-sectional study of patients with T2DM, with or without albuminuria, and age- and sex-matched healthy control subjects without clinical cardiovascular disease. The aims were to determine cardiac autonomic function and the potential association between different measures of cardiac autonomic function and cardiac vascular function assessed by CFR and coronary atherosclerosis assessed by CAC; both measured by cardiac $^{82}$Rb-positron emission tomography/computed tomography (PET/CT).

Further, we determined the correlation between cardiac autonomic function assessed by HRV analyses and CARTs and by cardiac $^{123}$I-MIBG scintigraphy. We hypothesized that impaired cardiac autonomic function would be associated with lower CFR and higher CAC.

**RESEARCH DESIGN AND METHODS**

**Study Population**

The study population has previously been described (10). In brief, a cohort of 60 consecutive outpatients with T2DM, defined according to the World Health Organization criteria, was identified at Steno Diabetes Center. Participants were aged between 35 and 80 years and had the ability to understand and give informed consent. Patients were stratified as normoalbuminuric if the urinary albumin excretion rate (UAER) was <30 mg/24 h in two of three consecutive urine collections (two of the samples were collected over 24 h in relation to the current study). A priori we included 30 patients with normoalbuminuria and 30 with persistent elevated albuminuria (UAER \( \geq \)30 mg/24 h). In addition, 30 control subjects without diabetes were recruited from a newspaper advertisement and matched for age and sex to the 30 patients with normoalbuminuria. We divided participants in these three groups because one of the aims of the overall study was to examine the prevalence of impaired CFR and elevated CAC in 1) patients with T2DM and albuminuria, 2) patients with T2DM and normoalbuminuria, and 3) healthy control subjects (10). Keeping with the original study design, we considered it most appropriate to present this division also in the present report. Participants were excluded if one of the following characteristics was present:

1. history of coronary artery disease or other cardiovascular disease (including stroke) or heart symptoms, assessed from medical records and patient interviews and questionnaires;
2. asthma or chronic obstructive pulmonary disease requiring treatment;
3. history of kidney disease other than diabetic nephropathy;
4. end-stage renal disease;
5. office blood pressure >200/110 mmHg;
6. second- or third-degree atrioventricular block; or
7. pregnancy or lactating.

For the analyses presented in this report, 83 participants were included, with 7 participants (2 control subjects, 2 normoalbuminuric patients, and 3 albuminuric patients) excluded because of incomplete data on the HRV indices (n = 6) or atrial fibrillation (n = 1).

The first 15 participants randomly enrolled in each group were invited to a cardiac $^{123}$I-MIBG scintigraphy. All accepted initially, but 1 patient in the albuminuric group was unable to undergo the examination because of a newly diagnosed breast cancer, and 1 control person declined, thus a total of 43 participants were analyzed. The 43 participants with data for cardiac $^{123}$I-MIBG scintigraphy and HRV indices did not differ from the 83 patients in the overall population in relation to age (P = 0.89), sex (P = 0.47), heart rate (P = 0.52), HbA₁c (P = 0.75), or any of the CARTs (P \( \geq \) 0.10). Treatment with insulin was similar in participants with (n = 43) and without (n = 40) data on cardiac $^{123}$I-MIBG scintigraphy (15 vs. 14 patients, P = 0.86).

Power calculation was performed for the primary study (10) and not for the present substudy. On the basis of existing literature, we anticipated that our sample size was sufficient (11,12).

The study was performed in compliance with the Declaration of Helsinki. All participants gave informed written consent, and the study protocol was approved by the Capital Region of Denmark Research Ethics Committee (ref. no. H-3-2013-015).

**Clinical Measurements**

HbA₁c was measured by high-performance liquid chromatography and plasma creatinine by an enzymatic method (Hitachi 912; Roche Diagnostics, Mannheim, Germany). The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (13).

Measurement of 24-h blood pressure was conducted using BPro (HealthStats, Singapore), a watch-like device that captures radial pulse wave reflection with tonometry and calculates brachial 24-h blood pressure from the pulse wave after calibration to the brachial blood pressure. The device meets the standards from European Society of Hypertension and the Association for the Advancement of Medical Instrumentation (14). The device was programmed to capture blood pressure every 15 min for 24 h. Mean blood pressure was calculated using all readings over the 24-h period. To evaluate the circadian blood pressure fluctuation, we calculated the night-to-day blood pressure ratio.

UAER was measured in two 24-h urine collections by an enzyme immunoassay and calculated as the geometric mean of the two collections. A detailed medical history was obtained along with demographic and anthropometric variables, including smoking...
status. Current smoking was defined as one or more cigarettes, cigars, or pipes per day. Information on medical treatment was obtained from questionnaires and cross checked against medical records at the Steno Diabetes Center.

Hybrid Cardiac PET/CT Imaging
All participants underwent a dynamic, electrocardiogram-gated cardiac PET scan using a hybrid PET/CT scanner in three-dimensional mode (Biograph mCT 128; Siemens, Munich, Germany) after the administration of $1100$ MBq $^{82}$Rb (Cardiogen; Bracco Diagnostics Inc., Monroe Township, NJ). Myocardial blood flow was calculated automatically with the Siemens Syngo MBB 2.3 (Siemens Medical Solutions, Malvern, PA), using one-compartment tracer kinetic models for $^{82}$Rb, including regional uptake and clearance parameters, blood to myocardium spillover and partial volume corrections, and the extraction curve from Lortie et al. (15). Maximal hyperemia was induced with adenosine infused at 140 mg/kg/min for 6 min. The CAC score was quantified using the method described by Agatston et al. (16) and semiautomated Corridor4DM software (INVIA, Ann Arbor, MI). CAC scores specific to the three main coronary arteries were calculated and then summed to provide a total CAC score for each participant.

Measurements of Cardiac Autonomic Function by Heart Rate Analyses
All electrocardiographic signals used for HRV analyses and CARTs were measured by laboratory technicians using the Vagus (Medicus Engineering, Aarhus, Denmark) device, with a sampling frequency of 1,000 Hz. After the subject rested supine for 5 min, 5-min resting heart rate measures for HRV analyses were obtained. From the 5-min resting heart rate recordings, time-domain HRV indices were derived: the SD of normal-to-normal (SDNN) inter-rate from early to late images was calculated according to recently published guidelines (21). The mean count within each ROI was reported. The myocardial washout rate from early to late images was calculated according to guidelines of the European Association of Nuclear Medicine Cardiovascular Committee and the European Council of Nuclear Cardiology (21).

Evidence supports the use of the late heart-to-mediastinum ratio for assessment of symptomatic CAN (22,23). An abnormal late heart-to-mediastinum ratio was defined as $<1.6$ (24).

Statistical Analysis
The distribution of the time- and frequency-domain HRV indices, CAC, UAER, and known duration of diabetes was skewed, and these variables were log2-transformed (CAC+1). The CAC test was not performed in patients with laser-treated retinopathy ($n=6$).

The CARTs were evaluated according to age-related reference intervals (18), and CAN was defined using the American Diabetes Association criteria (19) as no CAN: no pathological CARTs; borderline CAN: one abnormal CART; definite CAN: two or three abnormal CARTs.

All tests were performed between 8:00 A.M. and 2:00 P.M. in a quiet examination room. A standard protocol was applied in accordance with recommendations (20). Participants were advised to abstain from hard physical activity 24 h before the examination.

Cardiac $^{123}$I-MIBG Scintigraphy
On a separate day, within 3 weeks after the cardiac PET/CT scan, a planar cardiac $^{123}$I-MIBG scintigraphy was performed. Patients were given 130 mg potassium iodine 1 h before tracer injection and 20 mg potassium iodine 24 h after tracer injection to block thyroid iodine uptake. Approximately 200 MBq of $^{123}$I-MIBG was injected intravenously, and planar anterior-posterior images of the chest were obtained 15 min (early) and 240 min (late) after the tracer injection using a Philips SKYLight gamma camera with JETStream software (Philips Medical Systems, Best, the Netherlands), with medium energy collimator, $256 \times 256$ matrix, acquisition time of 600 s. $^{123}$I was imaged with a 15% energy window set symmetrically over the 159-keV photo peak. Image interpretation was done using Extended Brilliance Workspace NM Application Suite V4.5.3.40140 (Philips Medical Systems). One experienced observer assessed the images. A region of interest (ROI) was drawn above the heart, following the epicardial contour, and a rectangular ROI was drawn above the mediastinum on early and late anterior images in accordance with recently published guidelines (21). The mean count within each ROI was reported. The myocardial washout rate from early to late images was calculated according to guidelines of the European Association of Nuclear Medicine Cardiovascular Committee and the European Council of Nuclear Cardiology (21).
Analysis of covariance was applied when levels of the continuous variables among three groups were compared. We ascertained that the four principal assumptions of linear regression were fulfilled. The proportion of the variability in the dependent variable explained by the model is presented as the $R^2$.

All patients were pooled in the linear regression analyses, where we applied stepwise adjustment. First, we used unadjusted models (model 1) to determine whether any association existed among the measures of cardiac autonomic function and CFR as well as CAC. The subsequent adjustments included age (model 2), age and heart rate (model 3), and age, heart rate, and risk factors based on prior evidence (sex, 24-h systolic blood pressure, HbA1c, UAER, and smoking; model 4). For the measures of CARTs, model 3 was omitted, and model 4 did not include heart rate, because these tests are slightly influenced by the resting heart rate (20).

Owing to bias by indication, we did not include variables for medical treatment. Moreover, total cholesterol was not included because patients had lower levels than control subjects, likely due to lipid-lowering treatment. Standardized regression coefficients were reported. A two-tailed $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SAS 9.3 software (SAS Institute, Inc., Cary, NC).

**RESULTS**

**Clinical Characteristics**

The total cohort (n = 83) comprised 35% women, and the mean age was 62.1 ± 9.3 years. The characteristics of the participants in the three groups are reported in Table 1. The normoalbuminuric patients had higher mean heart rate, were more frequently receiving renin-angiotensin-aldosterone (RAAS) system inhibition and lipid-lowering treatment, and had lower total cholesterol compared with the control subjects ($P = 0.005$). All time- and frequency-domain HRV indices, except the LF-to-HF ratio, were lower in normoalbuminuric patients than in control subjects ($P < 0.005$). The E-to-I ratio and the late heart-to-mediastinum ratio were also lower in normoalbuminuric patients than in control subjects ($P < 0.004$). Patients with albuminuria had a lower eGFR, 30-to-15 ratio, and Valsalva test than the normoalbuminuric patients ($P = 0.004$).

All patients were treated with oral glucose-lowering medication, 49% received insulin, and none received glucagon-like peptide 1 receptor agonists. Most patients received lipid-lowering (93%) and RAAS-blocking (89%) treatment. Treatment with calcium channel blockers was prescribed in 57%, diuretics in 49%, and $\beta$-blockers in 11% of the patients; none were treated with $\alpha$-blockers. Three patients were treated with allopurinol and one with levothyroxine, and no other medications were prescribed.

**Prevalence of CAN**

The late heart-to-mediastinum ratio in the total population was 2.6 ± 0.5. Two participants (5%) had CAN according to a late heart-to-mediastinum ratio of <1.6. On the basis of the CARTs, 6 participants (8%) had definitive CAN, 24 (31%) had borderline CAN, and 47 (61%) had no signs of CAN. One of the two participants with CAN according to late heart-to-mediastinum ratio had CAN based on the CARTs.

**Correlations Between Cardiac Autonomic Function and CFR**

In unadjusted analyses (model 1), all measures of cardiac autonomic function, except the LF-to-HF ratio, correlated positively with CFR ($P = 0.005$) (Table 2). In age-adjusted analyses (model 2), all measures, except the LF-to-HF ratio and the 30-to-15 ratio, remained positively associated with CFR ($P = 0.04$). In model 3 (adjusted for age and heart rate), the late heart-to-mediastinum ratio and LF power were positively associated with CFR ($P = 0.01$). After adjustment for additional risk factors (model 4), the late heart-to-mediastinum ratio remained positively associated with CFR ($P = 0.01$). In model 4, all measures of cardiac autonomic function were associated with CFR ($P = 0.001$).

The unadjusted correlation of CFR to the late heart-to-mediastinum ratio is illustrated in Fig 1A, total power in Fig 1B, and Valsalva test in Fig 1C. The levels of CFR, according to tertiles of the late heart-to-mediastinum ratio, are illustrated in Fig 2A and absence of CAN, borderline CAN, or definitive CAN, based on the CARTs, is illustrated in Fig 2B.

**Correlations Between Cardiac Autonomic Function and CAC**

In unadjusted analyses (model 1), all measures of cardiac autonomic function, except the late heart-to-mediastinum ratio, were negatively associated with CAC ($P = 0.043$) (Table 3). In model 2 (age adjusted) all measures, except the late heart-to-mediastinum ratio, HF power, 30-to-15 ratio, and the Valsalva test, were negatively associated with CAC ($P = 0.027$). Further adjustment for heart rate (model 3) of the time- and frequency-domain HRV indices did not alter significance. However, after adjustment for additional risk factors (model 4), none of the measures of cardiac autonomic function were associated with CAC ($P = 0.072$).

**Agreement Between the Late Heart-to-Mediastinum Ratio and the HRV Measures and CARTs**

In unadjusted analyses, the late heart-to-mediastinum ratio correlated positively with the time- and frequency-domain HRV indices ($P = 0.004$), except for the LF-to-HF ratio ($P = 0.17$). For the CARTs, the late heart-to-mediastinum ratio correlated positively with the 30-to-15 ratio ($P = 0.04$) but not with the E-to-I ratio or the Valsalva test ($P = 0.43$). After adjustment for age, heart rate (only for the time- and frequency-domain HRV indices), sex, 24-h systolic blood pressure, HbA1c, UAER, and smoking, the late heart-to-mediastinum ratio correlated positively with LF power ($P = 0.002$), SDNN ($P = 0.049$), and RMSSD ($P = 0.037$), but not with any of the other HRV measures or the CARTs ($P = 0.07$).
Table 1—Clinical characteristics and measures of cardiac autonomic function

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Normoalbuminuria</th>
<th>Elevated albuminuria</th>
<th>P control subjects vs. normoalbuminuria</th>
<th>P normoalbuminuria vs. albuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>10 (36)</td>
<td>11 (39)</td>
<td>8 (30)</td>
<td>0.78</td>
<td>0.45</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>59.6 ± 10.2</td>
<td>60.6 ± 10.4</td>
<td>65.3 ± 7.1</td>
<td>0.71</td>
<td>0.06</td>
</tr>
<tr>
<td>Known diabetes</td>
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<tr>
<td>duration (years)</td>
<td></td>
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<tr>
<td>24-h systolic blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pressure (mmHg)*</td>
<td>128 ± 13</td>
<td>135 ± 16</td>
<td>137 ± 17</td>
<td>0.06</td>
<td>0.71</td>
</tr>
<tr>
<td>Night-to-day systolic</td>
<td>0.88 ± 0.06</td>
<td>0.90 ± 0.07</td>
<td>0.93 ± 0.07</td>
<td>0.22</td>
<td>0.04</td>
</tr>
<tr>
<td>blood pressure ratio*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)*</td>
<td>58.4 ± 10.8</td>
<td>70.1 ± 14.2</td>
<td>71.2 ± 9.5</td>
<td>0.003</td>
<td>0.76</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 ± 0.2</td>
<td>7.3 ± 1.3</td>
<td>8.0 ± 0.9</td>
<td>&lt;0.001</td>
<td>0.30</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)*</td>
<td>35.7 ± 1.8</td>
<td>56.8 ± 12.5</td>
<td>53.6 ± 10.0</td>
<td>&lt;0.001</td>
<td>0.30</td>
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<tr>
<td>Total cholesterol</td>
<td>5.5 ± 0.07</td>
<td>4.4 ± 0.9</td>
<td>4.3 ± 0.9</td>
<td>&lt;0.001</td>
<td>0.87</td>
</tr>
<tr>
<td>(mmol/L)*</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)*</td>
<td>83.1 ± 13.4</td>
<td>86.1 ± 20.1</td>
<td>67.8 ± 24.5</td>
<td>0.52</td>
<td>0.004</td>
</tr>
<tr>
<td>UAER (mg/24 h)*</td>
<td>6 (5–11)</td>
<td>7 (5–14)</td>
<td>146 (58–298)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Smokers</td>
<td>4 (14)</td>
<td>4 (14)</td>
<td>10 (37)</td>
<td>1.0</td>
<td>0.53</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensive*</td>
<td>3 (11)</td>
<td>23 (82)</td>
<td>27 (100)</td>
<td>&lt;0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>RAAS inhibition*</td>
<td>3 (11)</td>
<td>22 (79)</td>
<td>27 (100)</td>
<td>&lt;0.001</td>
<td>0.11</td>
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<tr>
<td>Calcium channel blocker*</td>
<td>1 (4)</td>
<td>9 (32)</td>
<td>14 (58)</td>
<td>0.005</td>
<td>0.11</td>
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<td>β-Blocker*</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>4 (15)</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>Diuretic*</td>
<td>1 (4)</td>
<td>11 (39)</td>
<td>17 (63)</td>
<td>0.001</td>
<td>0.08</td>
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<tr>
<td>Aldosterone antagonist*</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>6 (22)</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Lipid-lowering*</td>
<td>0 (0)</td>
<td>25 (89)</td>
<td>26 (96)</td>
<td>0.32</td>
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</tr>
<tr>
<td>Aspirin*</td>
<td>1 (4)</td>
<td>23 (82)</td>
<td>30 (100)</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Insulin*</td>
<td>13 (46)</td>
<td>14 (62)</td>
<td>8.9</td>
<td></td>
<td></td>
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<tr>
<td>CFR*</td>
<td>2.9 (0.7)</td>
<td>2.6 (0.8)</td>
<td>2.1 (0.5)</td>
<td>0.10</td>
<td>0.007</td>
</tr>
<tr>
<td>CAC score*</td>
<td>7 (0–97)</td>
<td>58 (2–423)</td>
<td>352 (151–1,025)</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Heart rate variability measures</td>
<td></td>
<td></td>
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<tr>
<td>Time and frequency domains</td>
<td></td>
<td></td>
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<tr>
<td>SDNN (ms)*</td>
<td>39.4 (28.6–53.0)</td>
<td>21.5 (13.2–25.9)</td>
<td>21.5 (16.2–29.3)</td>
<td>&lt;0.001</td>
<td>0.71</td>
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<tr>
<td>RMSSD (ms)*</td>
<td>25.0 (20.4–39.5)</td>
<td>13.8 (7.9–18.3)</td>
<td>13.7 (8.40–20.5)</td>
<td>&lt;0.001</td>
<td>0.96</td>
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<tr>
<td>LF power (ms²)*</td>
<td>196.7 (78.3–308.7)</td>
<td>38.6 (16.4–73.0)</td>
<td>30.4 (16.9–53.4)</td>
<td>&lt;0.001</td>
<td>0.56</td>
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<tr>
<td>HF power (ms²)*</td>
<td>70.9 (45.9–131.5)</td>
<td>23.9 (11.9–63.3)</td>
<td>20.7 (8.8–38.2)</td>
<td>0.005</td>
<td>0.44</td>
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<tr>
<td>HF-to-LF ratio*</td>
<td>2.05 (1.38–4.24)</td>
<td>1.36 (0.92–3.21)</td>
<td>2.05 (0.82–3.57)</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>Total power (ms²)*</td>
<td>606.1 (253.6–1106)</td>
<td>156.4 (63.2–281.5)</td>
<td>143.2 (96.2–207.7)</td>
<td>&lt;0.001</td>
<td>0.81</td>
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<td>CARTs</td>
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<tr>
<td>30-to-15 ratio</td>
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<tr>
<td>(response to standing)*</td>
<td>1.24 ± 0.17</td>
<td>1.20 ± 0.15</td>
<td>1.09 ± 0.09</td>
<td>0.38</td>
<td>0.003</td>
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<td>l-to-E ratio</td>
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<tr>
<td>(deep breathing)*</td>
<td>1.24 ± 0.15</td>
<td>1.12 ± 0.07</td>
<td>1.11 ± 0.08</td>
<td>&lt;0.001</td>
<td>0.70</td>
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<tr>
<td>Valsalva test ratio#</td>
<td>1.77 ± 0.41</td>
<td>1.62 ± 0.29</td>
<td>1.38 ± 0.23</td>
<td>0.17</td>
<td>0.004</td>
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<tr>
<td>CAN#</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>None</td>
<td>18 (72)</td>
<td>17 (61)</td>
<td>12 (50)</td>
<td>0.35</td>
<td>0.52</td>
</tr>
<tr>
<td>Borderline</td>
<td>7 (28)</td>
<td>9 (32)</td>
<td>8 (33)</td>
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<td>Definite</td>
<td>0 (0)</td>
<td>2 (1)</td>
<td>4 (17)</td>
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<td>123I-MIBG imaging</td>
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<tr>
<td>n</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td></td>
<td></td>
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<tr>
<td>Late heart-to-</td>
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<td></td>
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</tr>
<tr>
<td>mediastinum ratio</td>
<td>2.89 ± 0.39</td>
<td>2.38 ± 0.47</td>
<td>2.52 ± 0.60</td>
<td>0.004</td>
<td>0.49</td>
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<tr>
<td>Late heart-to-</td>
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<tr>
<td>mediastinum ratio &lt;1.6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (14)</td>
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<td></td>
</tr>
</tbody>
</table>

Data are presented as n (%), mean ± SD, or median (interquartile range). eGFR, estimated glomerular filtration rate. *P < 0.05 for trend across the three groups. #Not available in all participants.
The washout rate was not correlated with CFR in unadjusted \( P = 0.92 \) or adjusted analyses \( P = 0.29 \). A lower late heart-to-mediastinum ratio correlated significantly with lower early heart-to-mediastinum ratio \( P < 0.001 \) but not with a higher washout rate \( P = 0.08 \). The systolic night-to-day blood pressure ratio was not correlated with CFR in unadjusted \( P = 0.08 \) or adjusted analyses \( P = 0.69 \).

### DISCUSSION

In this cross-sectional study of patients with T2DM without clinical cardiovascular disease, we demonstrate that impaired function of the cardiac autonomic system correlated with lower CFR measured with cardiac \( 82\text{Rb-PET/CT} \). Especially, the late heart-to-mediastinum ratio, assessed by cardiac \( 123\text{I-MIBG} \) scintigraphy, and LF power were associated with CFR. We further found agreement between the measures of cardiac autonomic function. In this cohort of asymptomatic patients with T2DM, CAN was present in 7%, defined by a late heart-to-mediastinum ratio <1.6 \( P < 0.001 \) and in 11% according to the American Diabetes Association criteria based on CARTs \( P = 0.92 \). We demonstrated cardiac autonomic function, assessed by cardiac \( 123\text{I-MIBG} \) scintigraphy (the late heart-to-mediastinum ratio) and by HRV indices, was lower in patients with T2DM compared with control subjects without diabetes. However, only the 30-to-15 ratio and the Valsalva test were lower in albuminuric patients compared with normoalbuminuric patients.

CAN is an overlooked and serious complication associated with increased risk of cardiovascular morbidity and mortality, including cardiac arrhythmias and sudden death. In the Detection of Ischemia in Asymptomatic Diabetics study of 1,123 patients with T2DM, CAN, assessed by the 30-to-15 ratio, was a strong predictor of silent ischemia (evaluated with adenosine-stress myocardial perfusion imaging) and cardiovascular events \( P = 0.01 \).

The CARTs are validated and recommended by American Diabetes Association for the diagnosis of CAN. These tests are simple and can be performed in the practitioner’s office. In this study, we additionally included time
and frequency HRV indices to acquire more comprehensive information on the tone of the autonomic nervous system (26). The Atherosclerosis Risk in Communities Study found lower HRV was associated with an increased risk of incident coronary heart disease during an average follow-up of 8 years among patients with T2DM but not in individuals without diabetes at baseline (27).

Cardiac radionuclide imaging enables a direct quantification of cardiac sympathetic innervation in various diseases, including CAN (6). Cardiac $^{82}$Rb PET/CT is a promising technique providing CFR, a quantitative measure of the coronary microcirculation and the function of the large epicardial arteries, and CAC, quantifying the overall atherosclerotic burden of the heart. In individuals without epicardial coronary stenosis, reduced CFR cannot be entirely attributed to structural microvascular disease but can be functional and reversible. PET imaging is considered the gold standard for quantification of myocardial blood flow and CFR (28). Lower CFR is strongly associated with the risk of cardiovascular disease and death in patients with diabetes (8).

We are, to the best of our knowledge, the first to investigate the association between a comprehensive panel of cardiac autonomic function measures and CFR in asymptomatic patients with T2DM. We demonstrate a positive correlation between all measures of cardiac autonomic function (except the washout rate) and CFR; however, of particular interest, the late heart-to-mediastinum ratio, assessed by cardiac $^{123}$I-MIBG scintigraphy, was strongly associated with CFR, even after adjustment for appropriate risk factors. In age- and heart rate–adjusted models, LF power was also associated with CFR. However, our limited sample size implies a higher sampling variability increasing the risk of a type II error.

Few studies have investigated similar associations in patients with diabetes. In a study including 28 patients with type 1 or T2DM, patients with evidence of sympathetic nerve dysfunction, as assessed by the norepinephrine analog $^{11}$C-hydroxyephedrine, had impaired sympathetically mediated dilation of coronary resistance vessels (11). A study in 28 patients with type 1 diabetes concluded that subjects with preclinical microangiopathy had wide-ranging abnormalities of cardiac sympathetic innervation and blood flow regulation (12).
An impaired late heart-to-mediastinum ratio might reflect damage in adrenergic receptors and also be a result of enhanced washout. The washout rate in our study population was not related to CFR or to the late heart-to-mediastinum ratio, indicating that damage in adrenergic receptors might be the impelling cause of the impaired coronary microcirculation. Our novel findings may be useful in further investigation of the elevated risk of cardiovascular disease in asymptomatic patients with T2DM. We reveal that the tests primarily reflecting sympathetic autonomous control and activity had the strongest association with the CFR. Elevated cardiac sympathetic tone and damaged adrenergic receptors may play an important pathogenetic role in the development of myocardial injury and cardiac events in T2DM. It has been hypothesized that increased cardiac sympathetic tone may decrease myocardial vascularity, increase mitochondrial reactive oxygen species production, precipitate myocardial apoptosis, and promote myocardial remodelling (32), leading to impairment of the vascular performance of the heart and reduction of the coronary blood flow (29).

The agreement between cardiac autonomic function and CAC was investigated in a cross-sectional study of 160 patients with type 1 diabetes and 163 control subjects without diabetes (30). Reduced HRV (evaluated as total power) was associated with increased coronary calcification; however, the association lost significance after adjustment for systolic blood pressure (30). A cross-sectional study including patients with T2DM showed increased CAC was associated with lower HRV; however, this relationship became insignificance after adjustment for diabetes and other conventional risk factors (31).

We confirm the association between reduced HRV and increased CAC in our cohort and, likewise, that this association lost significance after adjustment for conventional risk factors, including systolic blood pressure, which could reflect shared risk factors.

The agreement between cardiac autonomic function measured by HRV and cardiac 123I-MIBG scintigraphy has been investigated in previous studies in T2DM. Murata et al. (4) observed a significant correlation, whereas Scholte et al. (32) observed disagreement between HRV and cardiac 123I-MIBG scintigraphy for the assessment of CAN. We demonstrate positive correlations between the late heart-to-mediastinum ratio and the time- and frequency-domain HRV indices. In unadjusted analyses, the late heart-to-mediastinum ratio correlated with the 30-to-15 ratio, an index of parasympathetic function, but not with the Valsalva test, which is a measure of parasympathetic activity. This was unexpected and might be a chance finding resulting from the limited sample size. After comprehensive adjustment, no significant agreement was found between the late heart-to-mediastinum ratio and any of the CARTs. These findings could be because of differences in measuring modalities or because the sample size limits its usability for complex statistical analyses. Outcomes of CARTs do not yield specific information about sympathetic function, whereas the late heart-to-mediastinum ratio is a measure reflecting the function of adrenergic receptors and sympathetic activity.

### Table 3: Unadjusted and stepwise adjusted associations between measures of cardiac autonomic function and CAC score

<table>
<thead>
<tr>
<th>Measure</th>
<th>Model 1 Unadjusted</th>
<th>Model 1 Adjusted for age and heart rate</th>
<th>Model 3 Adjusted for age, heart rate, * and other risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-to-I ratio (deep breathing)</td>
<td>1.58, 0.001</td>
<td>0.53, 0.39</td>
<td>-0.50, 0.35</td>
</tr>
<tr>
<td>Total power</td>
<td>0.97, 0.018</td>
<td>0.68, 0.051</td>
<td>0.08, 0.001</td>
</tr>
<tr>
<td>E-to-I ratio (response to standing)</td>
<td>1.14, 0.002</td>
<td>1.12, 0.001</td>
<td>1.27, 0.002</td>
</tr>
<tr>
<td>SDNN intervals</td>
<td>0.11, 0.05</td>
<td>0.83, 0.001</td>
<td>0.001, 0.001</td>
</tr>
<tr>
<td>RMSSD</td>
<td>1.77, 0.001</td>
<td>1.77, 0.001</td>
<td>1.77, 0.001</td>
</tr>
<tr>
<td>HF power</td>
<td>0.94, 0.043</td>
<td>0.61, 0.001</td>
<td>0.61, 0.001</td>
</tr>
<tr>
<td>LF power</td>
<td>0.90, 0.002</td>
<td>0.14, 0.001</td>
<td>0.14, 0.001</td>
</tr>
<tr>
<td>HF-to-LF ratio</td>
<td>0.07, 0.001</td>
<td>0.97, 0.001</td>
<td>0.97, 0.001</td>
</tr>
<tr>
<td>30-to-15 ratio (response to standing)</td>
<td>1.19, 0.005</td>
<td>1.11, 0.012</td>
<td>1.11, 0.012</td>
</tr>
<tr>
<td>Valsalva test</td>
<td>0.53, 0.23</td>
<td>0.93, 0.005</td>
<td>0.051, 0.032</td>
</tr>
</tbody>
</table>
| **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, HbA1c, UAER, and smoking.**
by cardiac $^{82}$Rb-PET/CT, in patients with T2DM. Limitations of the study include the small sample size increasing the likelihood of a type II error. Importantly, the sample size is rather limited for complex statistical analyses, and the results from the multivariate analyses have ideally to be confirmed in larger studies. Finally, the cross-sectional nature of this study makes it impossible to assess cause-and-effect associations and predictive value.

**Conclusions**

In patients with T2DM without clinical cardiovascular disease, we demonstrate a positive association between the late heart-to-mediastinum ratio, a measure reflecting the function of adrenergic receptors and sympathetic activity, and CFR, a measure of the coronary microcirculatory function. A reduced cardiac autonomic function and damage to adrenergic receptors may contribute to the development of cardiac microvascular dysfunction.

**Acknowledgments.** The authors thank all participants and acknowledge the work of study nurse L. Jelstrup and laboratory technicians A.G. Lundgaard, B.R. Jensen, T.R. Juhl, and J.A. Hermann (Steno Diabetes Center, Gentofte, Denmark).

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**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** B.J.v.S. conceived and designed the research, acquired the data, performed statistical analysis, and drafted the manuscript. C.S.H. acquired the data, performed statistical analysis, and critically revised the manuscript for key intellectual content. A.K., P.R., and P.H. conceived and designed the research, acquired the data, performed statistical analysis, and critically revised the manuscript for key intellectual content. B.J.v.S. 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.

**References**

Paper VII
Research: Treatment

Time course and mechanisms of the anti-hypertensive and renal effects of liraglutide treatment

B. J. von Scholten 1, M. Lajer 1, J. P. Goetze 2,3, F. Persson 1 and P. Rossing 1,3,4

1 Steno Diabetes Center, Gentofte, 2 Rigshospitalet, Copenhagen, 3 Aarhus University, Aarhus and 4 University of Copenhagen, Copenhagen, Denmark

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Abstract

Aims Glucagon-like peptide–1 receptor agonist studies have revealed clinically significant reductions in systolic blood pressure (SBP). The aim was to investigate the time course of the anti-hypertensive effect of liraglutide treatment and potential underlying mechanisms.

Methods We used an open-label, single-centre trial; 31 participants with Type 2 diabetes and hypertension completed the study. All participants were treated with liraglutide escalated to a maximum dose of 1.8 mg/day for 7 weeks, followed by a 21-day washout period. The primary outcome was a change in 24-h SBP.

Results Twenty-four-h SBP increased by 10 mmHg on day 3 (P = 0.008) and 7 mmHg on day 7 (P = 0.033, 0.6 mg/day). On day 29, (1.8 mg/day), 24-h SBP was 7 mmHg lower compared with baseline (P = 0.11). Following the treatment period (day 49) and after washout (day 70), 24-h BP was equivalent to baseline. In addition, extracellular volume (ECV) was reduced by 2.0 l [95% confidence interval (CI) = 1.0–3.1 l, P < 0.001] and midregional-pro-atrial natriuretic peptide (MR-proANP) was reduced by 20% (95% CI = 12–28%, P < 0.001). Also, urinary albumin excretion declined by 30% (95% CI = 12–44%, P = 0.003), GFR by 11 ml/min/1.73 m² (95% CI = 7.2–14.4 ml/min/1.73 m², P < 0.001) and fractional albumin excretion by 29% (95% CI = 3–48%, P = 0.032).

Conclusions Liraglutide treatment was associated with an initial increase in 24-h SBP, followed by a 7 mmHg reduction after escalation to 1.8 mg/day. This effect subsided after 4 weeks of maximum dose. Reductions in ECV and MR-proANP may explain the anti-hypertensive potential. Liraglutide treatment was associated with reversible reductions in albuminuria and GFR, which has to be confirmed in randomized trials.


Introduction

Glucagon-like peptide–1 (GLP-1) is an incretin hormone secreted by the intestinal L-cells in response to the ingestion of carbohydrates and lipids. GLP-1 binds to its receptor on pancreatic β-cells leading to glucose-dependent insulin secretion and thereby improvement of glycaemic control [1,2].

Liraglutide is a GLP-1 receptor agonist (GLP-1 RA) sharing 97% of the amino acid sequence identity of human GLP-1. In addition to blood glucose reduction in Type 2 diabetes, liraglutide and GLP-1 RA exenatide have been associated with weight loss, improved β-cell function and a reduction in office systolic blood pressure (SBP) [3].

The studies in the Phase 3 Liraglutide Effect and Action in Diabetes (LEAD) programme have revealed significant reductions in office SBP from 2.1 mmHg to 6.7 mmHg [4] and a meta-analysis of all six LEAD trials showed a reduction in office SBP of 2.5 mmHg from baseline and up to 26 weeks of treatment [5]. Analysis from LEAD-1, LEAD-2 and LEAD-4 revealed significant reductions in office SBP within the first 2 weeks of treatment when adding liraglutide 1.2 mg or 1.8 mg to oral anti-diabetic therapy. Because the greatest reduction in body weight occurs after ~ 8 weeks of treatment, reduction in SBP appears to occur before any significant weight loss [6].

Despite increasing interest in the possible anti-hypertensive and cardioprotective effect of liraglutide, the causative mechanism behind the observed reductions in SBP remains unclear. It has been speculated that the reduction in SBP might be due to increased natriuresis. Acute GLP-1 infusion...
What’s new?

- We are the first to have investigated the effect of treatment with the glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide on 24-h blood pressure and 24-h heart rate, in people with Type 2 diabetes and hypertension. Initially, both blood pressure and heart rate increased, but subsequently blood pressure returned to the baseline level.

- Reductions in extracellular volume and plasma midregional-pro-atrial natriuretic peptide may explain the anti-hypertensive potential of GLP-1 treatment.

- We are the first to have investigated the effect of liraglutide treatment on accurately measured GFR, 24-h urinary albumin excretion and fractional albumin excretion, and found treatment to be associated with significant and reversible reductions.

- Our novel findings will have an important impact on secondary renal endpoints.

May increase sodium excretion by acting on the Na+/H+ exchange in the kidney tubules [7], but the long-term effects are not known.

Novel mechanisms have been suggested linking stimulation of atrial natriuretic peptide (ANP) to GLP-1 RA increased natriuresis. Kim et al. [8] suggest that liraglutide activates GLP-1 receptors in atrial cardiomyocytes, which mediates ANP release and subsequently smooth muscle relaxation and natriuresis, both contributing to a reduction in BP.

The aim of this study was to investigate the time course of the anti-hypertensive effect of liraglutide treatment and the potential mechanisms behind this effect, including secondary renal endpoints.

Methods

Study design and participants

This open-label single-centre trial enrolled people with Type 2 diabetes (World Health Organisation criteria) with HbA1c > 48 mmol/mol (6.5%) on metformin. Hypoglycaemic treatment other than metformin was discontinued 2 weeks prior to baseline to avoid hypoglycaemia. Participants had hypertension defined as being on stable anti-hypertensive treatment or having elevated BP [SBP > 130 mmHg and/or diastolic BP (DBP) > 80 mmHg]. Participants were older than 18 years with an estimated GFR (eGFR) of > 60 ml/min/1.73 m². Exclusion criteria included insulin therapy, BP ≥ 170 mmHg systolic or 105 mmHg diastolic and a diagnosis of clinical heart failure.

The study was approved by the regional ethics committee and conducted according to the Declaration of Helsinki and Good Clinical Practice. The study was conducted at Steno Diabetes Center, Denmark.

The primary aim was to assess the time course of the anti-hypertensive effect of liraglutide treatment evaluated by 24-h BP. The second aim was to assess the effect of liraglutide treatment on GFR, extracellular volume (ECV), natriuresis and weight. Finally, explorative measurements of urinary albumin excretion rate (UAER), fractional albumin clearance, 24-h heart rate (HR), midregional-pro-atrial natriuretic peptide (MR-proANP), and arterial stiffness were included.

The study design is outlined in Fig. 1. After giving informed consent, participants were screened and underwent a 2-week washout with cessation of any hypoglycaemic treatment other than metformin. Participants attended a baseline examination visit and were instructed in subcutaneous injecting of the study drug. Participants were treated with liraglutide 0.6 mg/day for seven days, escalated to 1.2 mg/day for 14 days and to 1.8 mg/day for 28 days. The study treatment was followed by a 21-day washout period before the final visit.

Outcome measurements

Twenty-four-hour BP was measured as outlined in Fig. 1 using a BPro (HealthStats, Singapore), a watch-like device that captures radial pulse wave reflection and calculates brachial 24-h BP. BPro has been validated in people with diabetes and meets the European Society of Hypertension and Association for the Advancement of Medical Instrumentation standards [9,10].

BPro was calibrated with an oscillometric office BP device (model UA 787, A&D Medical) prior to BP measuring and was programmed to capture BP measurements every 15 min for 24 h. Mean SBP, DBP and HR were calculated using all readings over the 24-h. Discrimination between day- and night-time was based on the actual sleeping time reported by the participants. Only 24-h BP measurements with ≥ 20 readings during the daytime and ≥ 7 during the night-time were used for analysis. Nocturnal BP decrease was calculated as the difference in daytime and night-time SBP divided with daytime SBP [(daytime – night-time)/daytime] × 100%].

Renal function (GFR) and ECV were assessed during 4 h measurement of plasma 51Cr-EDTA by standard methods [11]. 51Cr-EDTA was performed at baseline, at the last day of treatment (day 49) and after 3 weeks of washout (day 70). eGFR was determined at day 1, 12, 21, 29, 49 and 70.

Twenty-four-hour urine collections were performed at baseline, during treatment (day 3, 4, 8, 9, 22, 23, 47 and 48) and during post-treatment washout (day 50, 51, 68, and 69) to measure urinary sodium, albumin (UAER) and creatinine. For technical issues, urinary sodium and urinary creatinine were not analysed in the 10 first study participants.
Arterial stiffness was measured as pulse-wave velocity (PWV) using a SphygmoCor device (AtCor Medical, Australia) at baseline, at the last day of treatment (day 49) and after 3 weeks of washout (day 70).

Fractional clearance of albumin (\(\theta_{\text{Alb}}\)) was determined as urinary albumin excretion/plasma albumin concentration × GFR in the 4-h urine collection during determination of GFR.

Laboratory procedures

HbA1c was measured by high-performance liquid chromatography calibrated against the IFCC standard. MR–proANP concentrations were measured on a Kryptor Compact plus apparatus (Brahms, Germany) according to assay details [12,13]. Inter-assay variations were < 6.5%. All other clinical laboratory variables were measured using standard clinical laboratory methods.

Statistical analysis

Demographic data are presented as mean (SD) or median (range) where skewed data are shown as geometric mean [interquartile range (IQR)] and analysed after log-transformation.

We limited our analyses to SBP because at middle and older age this is the predominant risk factor [14]. Comparisons were made by paired samples \(t\)-tests. Associations were evaluated in linear regression models. Values of \(P < 0.05\) were considered significant. Statistical analysis was performed using IBM SPSS 20.0 (IBM, Amonk, NY, USA).

Results

Patient recruitment and sample size

In total, 47 participants gave written informed consent, 12 failed screening, mainly due to HbA1c ≤ 48 mmol/mol (6.5%) or eGFR < 60 ml/min/1.73 m². Thirty-five participants were included and initiated treatment with liraglutide. Three participants dropped out (after less than 2 weeks of treatment) due to nausea/vomiting, and one was excluded due to lack of compliance. Therefore, 31 participants completed the study (Fig. 2).

Baseline characteristics

Data are presented in Tables 1 and 2. In total, 31 Caucasians with Type 2 diabetes completed the study, of these, eight (26%) were women, median age was 64 (range: 37–78) years, mean (SD) of HbA1c was 61 (14) mmol/mol (7.7 (1.3) %) and median diabetes duration was 6 (range: 1–18) years. All participants received anti-hypertensive treatment, of which 27 (87%) received renin-angiotensin-aldosterone system blocking treatment (Table 1). No change in anti-hypertensive medication was prescribed during the course of the study. Thirteen (42%) participants had micro- or macroalbuminuria (UAER ≥ 30 mg/day) at baseline.

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HbA1c and weight

HbA1c was reduced by 6 mmol/mol (0.6%) \( (P < 0.001) \) at the end of the treatment period (day 49) (Table 2). Baseline weight was 96.1 (14.2) kg and liraglutide treatment was associated with weight reduction throughout the treatment period, ranging from 0.6 kg at day 7 \( (P < 0.001) \) to the maximal reduction of 2.5 kg at day 49 \( (P < 0.001) \) (Table 2).

Twenty-four-hour ambulatory systolic blood pressure and heart rate

Mean (\( \bar{\mu} \)) of baseline 24-h BP was 131 (11) mmHg systolic and 83 (10) mmHg diastolic. For daytime BP, these values were 137 (12) mmHg and 86 (10) mmHg, respectively; and for night-time BP, the values were 125 (12) mmHg and 79 (10) mmHg, respectively. Twenty-four-hour SBP was increased by 10 mmHg on day 3 \( (P = 0.008) \) and by 7 mmHg on day 7 \( (P = 0.035) \) (0.6 mg/day). On day 29 (1.8 mg/day), 24-h SBP was 7 mmHg lower than baseline \( (P = 0.11) \). This change was associated with an increase in mean 24-h HR \( (P = 0.039) \) and borderline associated with a decrease in ECV \( (P = 0.064) \). At the end of the treatment period (day 49), mean 24-h SBP was 151 (32) mmHg, and at the end of washout period 24-h SBP was 130 (15) mmHg (Table 2, Fig. 3a).

No significant increase was observed in daytime SBP. By day 29, daytime SBP was reduced by 10 mmHg \( (P = 0.017) \) and by day 49 (end of treatment) daytime SBP was 2 mmHg lower compared with baseline \( (P = 0.38) \) (Table 2, Fig. 3b). Finally, an increase of 12 mmHg in night-time SBP was observed on day 3 \( (P = 0.004) \), while no significant reductions were observed during the treatment period. At the end of treatment, night-time SBP was increased by 3 mmHg \( (P = 0.44) \) (Table 2, Fig. 3c). Mean (\( \bar{\mu} \)) nocturnal BP decline was 10 (5)% at baseline, was reduced to 7 (5)% at the end of treatment \( (P = 0.017) \), and was normalized at the end of the study \( (P = 0.95) \).

Mean (\( \bar{\mu} \)) 24-h HR was 70.1 (10.7) bpm and was increased by 5 bpm on day 3 and day 7 \( (P < 0.001) \) (0.6 mg/day). On day 24 (1.8 mg/day), 24-h HR was 9 bpm higher than baseline \( (P < 0.001) \), and at the end of the treatment period (day 49) 24-h HR was 7 bpm higher \( (P = 0.001) \). At the end of washout period, 24-h HR was 71.8 (11.7) (p = 0.17) (Table 2). A similar time course was seen for both day- and night-time HR (data not shown).

Office systolic blood pressure

Mean baseline office SBP was 141 (17) mmHg and was reduced by 6–9 mmHg on day 12 \( (P = 0.043) \) and day 29 \( (P < 0.001) \) compared with baseline. At day 49, SBP was 4 mmHg lower than at baseline \( (P = 0.11) \), but returned towards baseline after post-treatment washout (Table 2, Fig. 3d).

Twenty-four-hour U-sodium excretion

Baseline urinary sodium was only available in 21 participants due to technical issues. Mean baseline urinary sodium was 189.9 (58.2) mmol/day, unchanged at day 3 \( (P = 0.90) \) and decreased during treatment to 142.8 (66.0) mmol/day at day 49 \( (P = 0.003) \). Urinary sodium returned towards baseline values at the end of the study \( (P = 0.57) \) (Table 2).

Extracellular volume

Baseline ECV was 14.9 (2.5) l and liraglutide treatment was associated with a 2.0 l reduction \([95\% \text{ confidence interval} (CI) = 1.0–3.1 \mathrm{l}, P < 0.001]\). During post-treatment washout, ECV returned towards the baseline value (Table 2).

Midregional-pro-atrial natriuretic peptide

At baseline, the geometric mean (IQR) of MR-proANP was 63.0 (41.8–85.4) pmol/l, and was reduced at the end of treatment (day 49) by 20% \( (95\% \text{ CI} = 12–28\%, P < 0.001) \). During washout, MR-proANP increased to 67.9 (42.1–81.4) pmol/l compared with baseline \( (P = 0.059) \) (Table 2).

Urinary albumin excretion rate

The geometric mean (IQR) of UAER was 32.7 (10.4–61.4) mg/day at baseline and liraglutide treatment was associated with reductions in albuminuria throughout the treatment period. UAER was decreased by 30% at the end of the treatment period. UAER was decreased by 30% at the end of the treatment period.

Table 1 Demographics of people with type 2 diabetes and hypertension

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Participants completing study ( n = 31 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64 (37–78)</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>6 (1–18)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>96.1 (14.2)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>31.9 (4.4)</td>
</tr>
<tr>
<td>Women, ( n ) (%)</td>
<td>8 (26)</td>
</tr>
<tr>
<td>Caucasian, ( n ) (%)</td>
<td>31 (100)</td>
</tr>
<tr>
<td>HbA1c, (mmol/mol)</td>
<td>61 (14)</td>
</tr>
<tr>
<td>HbA1c, (%)</td>
<td>7.7 (1.3)</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/L)</td>
<td>73.8 (17.4)</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td>99.5 (24.8)</td>
</tr>
<tr>
<td>renin-angiotensin-aldosterone system-blocking treatment, ( n ) (%)</td>
<td>27 (87%)</td>
</tr>
<tr>
<td>24-h systolic BP (mmHg)</td>
<td>131 (11)</td>
</tr>
<tr>
<td>Daytime systolic BP (mmHg)</td>
<td>157 (12)</td>
</tr>
<tr>
<td>Night-time systolic BP (mmHg)</td>
<td>125 (12)</td>
</tr>
<tr>
<td>24-h diastolic BP (mmHg)</td>
<td>83 (10)</td>
</tr>
<tr>
<td>Daytime diastolic BP (mmHg)</td>
<td>86 (10)</td>
</tr>
<tr>
<td>Night-time diastolic BP (mmHg)</td>
<td>79 (10)</td>
</tr>
</tbody>
</table>

Data are percentage (%) and mean (SD) or median (range). *(Geometric mean (IQR)).

BP, blood pressure; UAER, urinary albumin excretion rate.
Table 2. Outcome measurements at baseline, at the end of treatment (day 49), and after post-treatment washout (day 70); n = 31

<table>
<thead>
<tr>
<th>Outcome measurement</th>
<th>Baseline</th>
<th>End of treatment</th>
<th>Change (95% CI)*</th>
<th>P**</th>
<th>End of study</th>
<th>Change (95% CI)**</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h SBP (mmHg)</td>
<td>131.3 (11.3)</td>
<td>131.4 (16.9)</td>
<td>0.5 (-4.0 to 5.0)</td>
<td>0.98</td>
<td>130.1 (12.2)</td>
<td>-0.8 (-5.7 to 4.2)</td>
<td>0.75</td>
</tr>
<tr>
<td>Daytime SBP (mmHg)</td>
<td>137.3 (12.4)</td>
<td>134.6 (16.1)</td>
<td>-1.6 (-5.9 to 2.6)</td>
<td>0.38</td>
<td>134.6 (15.6)</td>
<td>-1.4 (-7.3 to 4.4)</td>
<td>0.62</td>
</tr>
<tr>
<td>Night-time SBP (mmHg)</td>
<td>124.9 (11.7)</td>
<td>127.9 (17.1)</td>
<td>2.5 (-2.4 to 7.5)</td>
<td>0.44</td>
<td>123.4 (15.9)</td>
<td>-1.0 (-6.1 to 4.0)</td>
<td>0.67</td>
</tr>
<tr>
<td>24-h heart rate (bpm)</td>
<td>70.1 (10.7)</td>
<td>77.1 (8.5)</td>
<td>6.5 (3.2 to 9.9)</td>
<td>0.001</td>
<td>71.8 (11.7)</td>
<td>1.3 (-0.6 to 3.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>Office SBP (mmHg)</td>
<td>140.8 (16.6)</td>
<td>136.7 (17.8)</td>
<td>-4.1 (-9.3 to 1.0)</td>
<td>0.11</td>
<td>139.5 (18.2)</td>
<td>-1.8 (-6.2 to 2.7)</td>
<td>0.55</td>
</tr>
<tr>
<td>24-h sodium excretion (mmol/day)</td>
<td>189.9 (58.2)</td>
<td>142.8 (66.0)</td>
<td>-47.2 (-75.8 to -18.5)</td>
<td>0.003</td>
<td>180.7 (74.6)</td>
<td>-9.2 (-42.4 to 24.0)</td>
<td>0.57</td>
</tr>
<tr>
<td>24-h UAER (mg/day)</td>
<td>32.7 (10.4-61.4)</td>
<td>23.0 (8.5-43.4)</td>
<td>-30% (-44% to -12%)</td>
<td>0.002</td>
<td>28.1 (10.2-65.8)</td>
<td>-14% (-31% to 6%)</td>
<td>0.11</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td>99.5 (24.8)</td>
<td>88.7 (23.6)</td>
<td>-10.8 (-14.4 to -7.2)</td>
<td>&lt;0.001</td>
<td>97.5 (23.7)</td>
<td>-2.0 (-5.6 to 1.6)</td>
<td>0.26</td>
</tr>
<tr>
<td>Fractional albumin excretion (× 10⁻⁵)</td>
<td>63</td>
<td>4.4</td>
<td>-29% (-48% to -3%)</td>
<td>0.032</td>
<td>7.1</td>
<td>17% (-9% to 50%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Extracellular volume (L)</td>
<td>14.9 (2.6)</td>
<td>12.9 (2.2)</td>
<td>-1.9 (-3.1 to -1.0)</td>
<td>&lt;0.001</td>
<td>14.4 (2.8)</td>
<td>-0.5 (1.5 to 0.6)</td>
<td>0.42</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>89.7 (5.8)</td>
<td>85.0 (17.8)</td>
<td>-4.7 (-7.2 to 2.4)</td>
<td>&lt;0.001</td>
<td>88.2 (17.1)</td>
<td>-1.6 (4.3 to 1.3)</td>
<td>0.28</td>
</tr>
<tr>
<td>MR-proANP (pmol/L)</td>
<td>63.0 (41.8-85.4)</td>
<td>50.1 (37.1-72.2)</td>
<td>-20% (-28% to -12%)</td>
<td>&lt;0.001</td>
<td>67.9 (42.1-81.4)</td>
<td>8% (-1% to 17%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>96.1 (14.2)</td>
<td>93.5 (14.2)</td>
<td>-2.6 (-3.2 to -1.8)</td>
<td>&lt;0.001</td>
<td>94.5 (14.3)</td>
<td>-1.6 (-2.4 to -0.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pulse wave velocity (m/s)</td>
<td>11.3 (2.9)</td>
<td>11.3 (2.5)</td>
<td>0.2 (-0.6 to 1.0)</td>
<td>0.54</td>
<td>10.6 (2.6)</td>
<td>-0.6 (-1.4 to 0.3)</td>
<td>0.21</td>
</tr>
<tr>
<td>HbA₁c (mmol/mol)</td>
<td>61 (14)</td>
<td>53 (11)</td>
<td>-6 (-9 to -3)</td>
<td>&lt;0.001</td>
<td>58 (12)</td>
<td>-3 (-6 to 1)</td>
<td>0.17</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>7.7 (1.3)</td>
<td>7.1 (1.0)</td>
<td>-0.6 (-0.8 to -0.2)</td>
<td>&lt;0.001</td>
<td>7.3 (1.1)</td>
<td>-0.2 (0.3 to -0.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>138.0 (2.7)</td>
<td>139.0 (2.9)</td>
<td>1.0 (0.2 to 1.8)</td>
<td>0.014</td>
<td>138.1 (2.7)</td>
<td>0.1 (-0.8 to 0.9)</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Data represent mean (SD) or geometric means (IQR).
*From baseline to the end of treatment, **end of treatment vs. baseline, ***from baseline to the end of the study, P* value vs. baseline.
MR-proANP, midregional-pro-atrial natriuretic peptide; SBP, systolic blood pressure; UAER, urinary albumin excretion rate.
treatment period (95% CI = 12–44%, \( P = 0.003 \)), borderline associated with weight reduction (\( P = 0.066 \)). UAER returned towards the baseline value after post-treatment washout (Table 2, Fig. 4a). In participants with micro/macroalbuminuria (\( n = 13 \)), UAER was decreased by 49% at the end of treatment (\( P = 0.002 \)). Of nine participants with microalbuminuria, four (44%) regressed from micro- to normoalbuminuria, and two participants (50%) regressed from macro- to microalbuminuria. The change in UAER was not associated with a change in 24-h SBP or HbA1c.

To adjust for treatment-induced changes in GFR, which may influence the albuminuria reduction, fractional clearance was calculated. This was significantly reduced by 29% at the end of treatment (95% CI = 3–48%, \( P = 0.032 \)) (Table 2).

\[^{51}Cr	ext{-EDTA GFR}\]

Mean baseline GFR was 99.5 (24.4) ml/min/1.73 m\(^2\). At the end of treatment (day 49), GFR was reduced by 11 ml/min/1.73 m\(^2\) (95% CI = 7.2–14.4 ml/min/1.73 m\(^2\), \( P < 0.001 \)), and returned towards the baseline value after washout (97.5 ml/min/1.73 m\(^2\), \( P = 0.27 \)) (Table 2, Fig. 4b). The changes in eGFR showed a similar time course (Table 2, Fig. 4c).

Safety reporting

A total of 22 (62.9%) participants reported adverse events. The most common adverse event, reported by 14 (40%) of the participants, was nausea and three participants discontinued the study after the first 2 weeks due to nausea and vomiting. No serious adverse events occurred.

Discussion

In people with Type 2 diabetes, treatment with GLP-1 RA liraglutide initially led to an increase in 24-h SBP and HR. Reductions in ECV and MR-proANP might be important factors involved in the subsequent reduction in 24-h SBP, which was seen after 24 days, with a maximal reduction after 29 days of treatment. After 4 weeks of maximum dosage, the effect had subsided. Changes in BP were more pronounced during the daytime and for office BP compared with 24-h BP. We demonstrated significant and reversible BP-independent reductions in UAER, GFR and fractional clearance of albumin.

This is the first study to investigate the effect of liraglutide treatment on 24-h BP. We observed a rapid elevation in 24-h SBP by day 3, on liraglutide 0.6 mg/day, with a simultaneous increase in 24-h HR. This effect of GLP-1 administration...
has been described previously in murine models [15]. During further dose escalation, 24-h SBP decreased, until reduced by 7 mmHg by day 29 compared with baseline. During the remaining treatment period, 24-h SBP returned to baseline. A shared feature of the changes in 24-h and office SBP was that the largest reduction was observed at day 29 after the maximum dose of liraglutide had been injected for 1 week (Fig. 1). After 4 weeks of maximum dose (day 49), this effect had subsided, indicating a prompt anti-hypertensive effect when escalating to liraglutide 1.8 mg/day, followed by a steady-state condition after a longer period of maximum dosage. At the end of treatment, 24-h SBP was equivalent to

![Image of graphs showing changes in UAER, GFR, and eGFR](image_url)
baseline, with a tendency for increased night-time SBP and reduced daytime SBP of 2 mmHg.

The initial rise in 24 h SBP may be caused by the onset of the common side effects of liraglutide treatment (nausea, vomiting and headache), whereas the anti-hypertensive effect is present when escalating towards standard doses of liraglutide (1.2 and 1.8 mg/day).

The relatively low baseline 24 h SBP of 131 mmHg might explain our findings at day 49 (end of treatment), because a meta-analysis of the six LEAD trials showed that the anti-hypertensive effect of liraglutide was greatest among subjects with the highest baseline SBP (> 140 mmHg) [16].

Similar results were presented in a meta-analysis of GLP-1 RA exenatide in which patients with baseline SBP ≥ 150 mmHg showed the largest reduction [17]. In our study, only four patients had a baseline 24-h SBP ≥ 140 mmHg, and on day 29 (1.8 mg/day) we observed a borderline significant reduction of 22 mmHg, but no difference from baseline was observed at the end of treatment.

We speculate that liraglutide treatment is associated with a reduction in daytime BP, but with either no effect or a small increase in night-time BP. Subjects were instructed to inject the study medication in the morning, so the tendency for a difference in daytime and night-time SBP might be a result of the pharmacokinetics of liraglutide. Our observed decrease in office SBP during treatment was comparable with the findings in the LEAD trials [2,6].

We are the first to present data on the effect of liraglutide treatment on 24 h HR. Although a recent meta-analysis reports that liraglutide increased HR by 2.7 bpm vs placebo and 2.5 bpm vs active control [18], most of the included studies measured HR as a routine procedure as part of safety assessment. Measurement of 24-h HR improves accuracy, but our findings are in agreement with previous studies.

A recent study demonstrated the presence of GLP-1 receptors in the sinoatrial node in the heart [19], however, the mechanisms and clinical impact of elevated HR are being discussed. We did not find any association with liraglutide treatment and pulse wave velocity, but the long-term effect on arterial stiffness would be interesting to test.

ECV was reduced by 2 l, whereas natriuresis decreased during treatment. The subjects were not on a salt-restrictive diet, which ultimately weakens our data on 24-h urinary sodium. In addition, urinary sodium was only available for 21 participants, and we have not measured 24 h urinary sodium acutely. We measured urinary sodium at baseline (day 1) and after 2 days of liraglutide treatment. Although acute infusion studies have demonstrated an increase in natriuresis, we found unchanged sodium excretion after 3 days, but then a significant decline during treatment, with normalization during washout. We cannot exclude an acute initial increase in sodium excretion followed by normalization with the current design, which could contribute to the decline in ECV and MR-proANP and a compensatory increase in HR.

Potentially acting on this pathway, we found liraglutide treatment to be associated with a 20% reduction in MR-proANP and a simultaneous increase in plasma sodium. Whether the reduction in ECV leads to a reduction in circulating MR-proANP or vice versa cannot be determined by the present study, but a link between these observed changes may exist. ANP is known to be primarily produced in the cardiac atrium, however, upregulation of ANP mRNA expression has been observed in the kidneys [20]. Treatment with blockers of the renin-angiotensin-aldosterone system has shown a similar effect of lowering ANP in people with hypertension, whereas treatment with β-receptor blockers resulted in increased ANP, indicating an effect independent of SBP reduction [21,22]. The effect of the renin-angiotensin-aldosterone system on renal ANP production has been described previously [23], and it is likely that liraglutide acts on both cardiac and renal production of ANP. Acute experimental studies in mice have shown that liraglutide infusion increases ANP release, which induces smooth muscle relaxation and natriuresis, possibly explaining the anti-hypertensive effect [8], whereas we found a reduction in ANP, and a study in healthy men did not find any effect of GLP infusion on MR-proANP [24].

Previously, higher levels of MR-proANP have been related to cardiovascular morbidity, all-cause mortality and cardiovascular mortality in people with Type 2 diabetes [25]. By lowering MR-proANP, liraglutide treatment might have beneficial effects on these endpoints. Supporting this hypothesis, liraglutide treatment has been shown to reduce other cardiovascular risk biomarkers, including brain natriuretic peptide, plasminogen activator inhibitor-1 and highly sensitive C-reactive protein [3].

With regards to kidney function, liraglutide treatment was associated with a decrease in accurately measured GFR. However, after 3 weeks of washout, kidney function returned to the baseline level, thus indicating that the changes observed were benign and probably due to renal haemodynamic effects. Improvement in plasma glucose could contribute to the change in GFR, because hyperglycaemia (> 15 mmol/l) may increase GFR [26] and plasma glucose was not clamped during GFR measurements. However, adjustment for change in HbA1c or mean plasma glucose during GFR measurements did not explain the change in GFR (data not shown). Furthermore, analysis of the subgroup with plasma glucose below 15 mmol/l (n = 17) at the first examination did not change the findings for GFR over the time course. In relation to changes in GFR, it is possible that increases in distal sodium delivery in the tubular system through restoration of tubuloglomerular feedback might reduce GFR. This has recently been demonstrated with sodium glucose transporter 2 inhibition in Type 1 diabetic patients with hyperfiltration (GFR > 135 ml/min/1.73 m²), but not in those with normofiltration [27].
Although such an acute effect cannot be excluded, we only had two participants with hyperfiltration, and when GFR was measured the natriuresis was reduced compared with baseline.

Previous long-term studies have only evaluated eGFR without observing a similar decline in GFR, which might be misleading in the case of effects on renal handling of creatinine or changes in creatinine due to weight loss; however, our findings in measured GFR were paralleled by similar changes in eGFR.

We found a significant reduction in albuminuria and a high fraction of participants with regression of micro-/macroalbuminuria. This is potentially a beneficial effect of GLP-1 treatment, also recently described in another study with liraglutide [28], and studies with exenatide and the dipeptidyl peptidase-4 inhibitor saxagliptin have shown similar effects of lowering albuminuria [29–31]. As discussed, the time course in GFR might, to some extent, be related to changes in plasma glucose, which would also modify UAER, however, the significant change in fractional albumin excretion is adjusted for the changes in GFR. Furthermore, the change in UAER from baseline to the end of treatment was not explained by a change in HbA1c, or plasma glucose.

The effects of liraglutide on GFR, BP and albuminuria are reminiscent of known effects of a renin-angiotensin–aldosterone system blocking treatment, which in several studies has been shown to reduce BP, albuminuria and GFR in Type 2 diabetes [32,33]. Albuminuria is an important marker for renal and cardiovascular risk, and lowering albuminuria has been proved to possess a long-term renin- and cardioprotective effect [34,35]. To support our hypothesis of a possible GLP-1/renin-angiotensin–aldosterone system interplay, a study in healthy obese men discovered that GLP-1 affected the renin-angiotensin–aldosterone system by decreasing circulating levels of angiotensin II [36]. In addition, murine studies have found GLP-1 to inhibit angiotensin II and its pro-inflammatory effects on glomerular endothelial cells, indicating a possible protective effect of GLP-1 on the glomerulus [37].

We designed this study to be focused on the time course and mechanisms of the anti-hypertensive effect of liraglutide, so our study uses an open-label design. A double-blind randomized design with inclusion of a control group of placebo participants would have added to the impact of our study. A further limitation is the low number of participants and risk of Type 1 error. Ongoing studies with liraglutide in Type 2 diabetes designed to examine renal and cardiovascular safety will hopefully elucidate some of our observed changes (ClinicalTrials.gov: NCT01620489, NCT01179048), although further mechanistic studies are warranted.

In conclusion, this time course study demonstrates that liraglutide treatment is associated with an initial increase in 24-h SBP, whereas escalation to liraglutide 1.8 mg/day resulted in a 7 mmHg reduction. This effect subsided after 4 weeks of maximum dose. Reductions in ECV and MR-proANP may explain the anti-hypertensive potential. Liraglutide treatment was associated with a 30% reduction in albuminuria, a decline in GFR and increased 24-h HR. Results from ongoing placebo-controlled and randomized studies are highly anticipated in order to confirm these findings.

Acknowledgements

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Funding source

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Competing interests

Steno Diabetes Center, where BlyS, ML, FP and PR are employed, receives part of its core funding from unrestricted grants from Novo Nordisk Foundation and Novo Nordisk, and is owned by Novo Nordisk. ML owns shares in Novo Nordisk. JPG declares no conflicts of interest. FP reports having received research grant from Novartis. FP has received lecture fees from Novartis, Eli Lilly, Boehringer Ingelheim, Novo Nordisk, BMS, and AstraZeneca. PR reports having given lectures for Astra Zeneca, BMS and Boehringer Ingelheim, and has served as a consultant for AbbVie, Astra Zeneca, BMS, Eli Lilly, Boehringer Ingelheim, Astellas, Janssen, and Novo Nordisk, all fees given to Steno Diabetes Center, and has equity interest in Novo Nordisk.

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Paper VIII
Glucagon-like peptide 1 receptor agonist (GLP-1 RA): long-term effect on kidney function in patients with type 2 diabetes

Bernt Johan von Scholten a,⁎, Tine Willum Hansen b, Jens Peter Goetze b,c, Frederik Persson a, Peter Rossing a,c,d

a Steno Diabetes Center, Gentofte, Denmark
b Rigshospitalet, Copenhagen, Denmark
c Aarhus University, Denmark
d University of Copenhagen, Denmark

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MR-proANP

A B S T R A C T

Aim: In a short-term study including 31 patients with type 2 diabetes, glucagon-like peptide 1 receptor agonist (GLP-1 RA) treatment was associated with a significant reversible decline in GFR. Twenty-three patients re-initiated GLP-1 RA treatment after the primary study, and the aim was to investigate the long-term effect on kidney function.

Methods: We included 30 patients in a one-year extension study, all initially treated with liraglutide for seven weeks. During follow-up 23 were treated with liraglutide and seven untreated. Primary outcome was change in GFR (13Cr-EDTA plasma clearance).

Results: Patients were 61.5 (10.0) years and HbA1c 60.1 (13.8) mmol/mol. Baseline GFR was 100.6 (24.9) mL/min/1.73 m² and was reduced by 11 (95% CI: 6.6–15.7, p < 0.001) mL/min/1.73 m², independent of change in 24-h systolic blood pressure (SBP), weight, UAER or HbA1c (p > 0.33). Geometric mean (IQR) of UAER was 25.5 (9.9–50.9) mg/d and was reduced by 27 (95% CI: 5–44, p = 0.020). 24-h SBP was reduced by 8.2 (p = 0.048) mmHg. No changes occurred in untreated patients.

Conclusions: Long-term treatment with liraglutide was associated with a reduction in measured GFR similar to the effect during short-term treatment, suggesting a metabolic or haemodynamic reversible effect and not structural changes. Moreover, UAER and 24-h SBP were reduced.

Trial registration: ClinicalTrials.gov identifier: NCT01499108.

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1. Introduction

For more than five years, liraglutide has been available as an anti-diabetic treatment. The glucose-lowering effect is well proven, but recently it has been hypothesized that GLP-1 receptor agonism also may exert beneficial renal effects (Musiket, Smits, Morsink, & Diamant, 2014). So far, no randomized controlled trials with specific primary renal outcome have been performed, and liraglutide is not indicated in patients with impaired kidney function due to lack of clinical experience. Currently, there is an interest in expanding the label to include patients that have moderately impaired renal function.

The expression of GLP-1 receptors (GLP-1R) in the kidney has been described in both animals and humans, and GLP-1R MRNA was found in the proximal tubular cells in rats and pigs (Campos, Lee, & Drucker, 1994; Granados, Orichello, Pessio, et al., 2011; Korner, Stockli, Wasel, & Reshi, 2007; Pescezki, Muench, & Chellikani, 2012; Schlatter, Beglinger, Drewe, & Gutmann, 2007). Furthermore, studies in healthy humans, have shown GLP-1 infusion to increase sodium excretion by the kidneys (Gutzwiller, Frokiaer, et al., 2013), and in obese, insulin-resistant men, GLP-1 infusion was associated with an increased sodium excretion, a decreased urinary H+ secretion and a decreased glomerular filtration rate (GFR) (Gutzwiller, Tschopp, Bock, et al., 2004). In animal models, recent findings indicate that treatment with GLP-IRA decelerates the progression of diabetic nephropathy by inhibiting inflammatory actions and by improving endothelial...
GFR, measured with plasma clearance of $^{51}$Cr-EDTA, decreased (Rossing, 2015). In our seven-week study of liraglutide treatment, non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015), we have recently found that liraglutide treatment was associated with a significant reduction in albuminuria in type 2 diabetes in a non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015). In our seven-week study of liraglutide treatment, non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015), we have recently found that liraglutide treatment was associated with a significant reduction in albuminuria in type 2 diabetes in a non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015). In our seven-week study of liraglutide treatment, non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015), we have recently found that liraglutide treatment was associated with a significant reduction in albuminuria in type 2 diabetes in a non-randomised study (von Scholten, Lajer, Goetze, Persson, & Rossing, 2015).

Long-term renal effects of GLP-1 RA treatment has only been assessed with estimated GFR (Davidson, Brett, Falahati, & Scott, 2011; Pawaskar, Tuttle, Li, Best, & Anderson, 2014), known to be imprecise. Potential effects on creatinine handling or the impact of body composition changes during GLP-1 RA treatment highlights the importance of a precise method to measure renal function.

The aim of this open-label extension study was to observe developments in GFR and UAER during long-term (one year) treatment with liraglutide on GFR, in patients with type 2 diabetes.

2. Materials and methods

Study design and participants has previously been described in details (von Scholten et al., 2015).

In brief, we enrolled patients with type 2 diabetes at Steno Diabetes Center (Gentofte, Denmark). All patients were older than 18 years, treated with metformin, HbA$_1c$ was $>48$ mmol/mol ($>6.5\%$) and estimated glomerular filtration rate (CKD-EPI-GFR) $>60$ mL/min/1.73 m$^2$. Exclusion criteria included ongoing insulin therapy, blood pressure $>170/105$ mmHg and diagnosis of clinical heart failure (NYHA class III-IV). All patients were treated with liraglutide for 49 days followed by a 21-day washout period before the last visit. A total of 31 patients completed the initial study.

2.1. Follow-up

At the last visit in the initial study, future antidiabetic treatment was discussed with each patient. A total of 23 patients chose to continue liraglutide treatment (1.2 or 1.8 mg/d), whereas eight patients ended the treatment. A one year follow-up visit was planned for all patients in order to determine the long-term effects of liraglutide treatment. Patients no longer on liraglutide treatment were included as untreated subjects. All patients except one untreated subject attended the follow-up visit. Written informed consent for participation in the study was obtained from all participants. The study was approved by the regional ethics committee and conducted according to the Declaration of Helsinki and Good Clinical Practice. This trial is registered at ClinicalTrials.gov, number NCT01499108.

2.2. Outcome measurements

GFR and extra cellular volume (ECV) were assessed during four hours measurement of plasma clearance $^{51}$Cr-EDTA (Brockner-Mortensen, 1972). Urinary sodium (U-sodium), albumin (UAER) and creatinine (U-creatinine) were measured in 24-h urine collections. Fractional clearance of albumin was determined as urinary albumin excretion/(plasma albumin concentration × GFR) in the four-hour urine collection during the GFR measurement.

Twenty-four-hour blood pressure and heart rate were measured by the BPro device (HealthStats, Singapore), a validated watch-like device that captures radial pulse wave reflection with tonometry and calculates brachial ambulatory blood pressure (Theilade, Joergensen, Persson, Lajer, & Rossing, 2012).

HbA$_1c$ was measured by high-performance liquid chromatography and MR-proANP concentrations on a Kryptor Compact plus apparatus (Brahms, Hennigsdorf, Germany) according to assay details (Hunter et al., 2011; Morgenstaler, Struck, Thorns, & Bergmann, 2004). All outcome measurements were determined both at baseline and end of study.

2.3. Statistical analysis

Clinical characteristics are presented as mean (SD) whereas variables with skewed distribution are shown as geometric mean (QR), and analysed after log-transformation. Comparisons between groups were performed by unpaired Student’s t-test and analyses of repeated measures by paired samples t-test. The effect on GFR and UAER of changes in other relevant variables was evaluated in adjusted linear regression models including 24-h SBP, weight and GFR-UAER (when appropriate). $p$-values $<0.05$ were considered significant. Statistical analysis was performed using IBM SPSS 20.0 (IBM Ammonk, NY, USA).

3. Results

3.1. Baseline characteristics (Table 1)

The 23 Caucasian patients treated with liraglutide during follow up, included 15 (65%) females, mean (SD) age was 59.8 (9.9) years, HbA$_1c$ 63.2 (14.2) mmol/mol (7.9 (1.3) %), and diabetes duration was median (range) 6.8 (1–16) years. The seven patients who did not start liraglutide again but attended the one-year follow-up visit were all male, age was 66.9 (8.5) years, had better glycemic control HbA$_1c$, 50.1 (5.3) mmol/mol (6.7 (0.5) %), tended to be less obese, and had diabetes duration 9 (2–18) years. All patients were treated with antihypertensive drugs, of which 21 patients (91%) in the liraglutide group and six (86%) of the untreated subjects received RAAS-blocking agents. The antihypertensive treatment was unchanged during the study for all patients. Nine (39%) patients in the liraglutide group and 3 (43%) of the untreated subjects had micro- or macroalbuminuria (geometric mean UAER ≥30 mg/d) at baseline.

After one year of follow-up no significant changes in any of the variables of interest occurred in the untreated group ($p ≥ 0.17$) (Table 2).

3.2. $^{51}$Cr-EDTA GFR

Baseline GFR was 100.6 (24.9) mL/min/1.73 m$^2$ and was reduced 11 (95% CI: 6.6 to 15.7) mL/min/1.73 m$^2$ ($p < 0.001$) after one year of

<table>
<thead>
<tr>
<th>Table 1 Baseline characteristics.</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Demographics</td>
<td>Liraglutide group</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.8 (9.9)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>97.9 (15.0)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32.9 (4.3)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>15 (65)</td>
</tr>
<tr>
<td>HbA$_1c$, (mmol/mol)</td>
<td>63.2 (142)</td>
</tr>
<tr>
<td>HbA$_1c$, (%)</td>
<td>7.9 (1.3)</td>
</tr>
<tr>
<td>Plasma creatinine (μmol/L)</td>
<td>71.7 (16.7)</td>
</tr>
<tr>
<td>$^{51}$Cr-EDTA GFR (mL/min/1.73 m$^2$)</td>
<td>100.6 (24.9)</td>
</tr>
<tr>
<td>RAAS-blocking treatment, n (%)</td>
<td>21 (91)</td>
</tr>
<tr>
<td>UAER (mg/d)$^{1}$</td>
<td>25.5 (9.8–50.9)</td>
</tr>
<tr>
<td>Systolic 24 h blood pressure (mmHg)</td>
<td>134 (14)</td>
</tr>
<tr>
<td>Diastolic 24 h blood pressure (mmHg)</td>
<td>83 (10)</td>
</tr>
</tbody>
</table>

Data are percentage [%] and mean ± SD or median (range). $p$-values are for unadjusted comparisons (t-test or chi$^2$ test) between the patients treated with liraglutide and the control subjects. $^{1}$Geometric mean (QR).
Data represent mean (SD) or geometric means (IQR).

### Table 2

Outcome measures for patients treated with liraglutide for one year and for untreated patients.

<table>
<thead>
<tr>
<th></th>
<th>Liraglutide group (n = 23)</th>
<th>Untreated group (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>End of study</td>
</tr>
<tr>
<td>131Cr-EDTA GFR (mL/min/1.73 m²)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>100.6 (24.9)</td>
<td>89.4 (25.7)</td>
</tr>
<tr>
<td>24 h urine albumin excretion (mg/d)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>25.5 (9.9 to 50.9)</td>
<td>18.6 (65.6 to 41.4)</td>
</tr>
<tr>
<td>Fractional albumin excretion (10^-3)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>5.4 (2.2 to 10.0)</td>
<td>3.2 (1.1 to 12.4)</td>
</tr>
<tr>
<td>MR-proANP (pmol/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>58.8 (40.0 to 83.6)</td>
<td>49.8 (35.2 to 65)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>97.9 (15.0)</td>
<td>94.4 (15.0)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>63.2 (14.2)</td>
<td>56.3 (12.6)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>79.5 (15.9)</td>
<td>76.5 (15.0)</td>
</tr>
<tr>
<td>Baseline End of study Change (95% CI)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>91.9 (26.2)</td>
<td>91.7 (28.9)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>91.6 (16.1)</td>
<td>83.7 (19.4)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>83.3 (11.1)</td>
<td>79.6 (12.7)</td>
</tr>
<tr>
<td>Serum calcium (mmol/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>2.53 (0.14)</td>
<td>2.53 (0.14)</td>
</tr>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>107.1 (81.7)</td>
<td>108.1 (81.2)</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>7.9 (1.3)</td>
<td>7.3 (1.1)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>133.8 (145)</td>
<td>125.7 (111.9)</td>
</tr>
<tr>
<td>Fractional albumin excretion (10^-3)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>83.3 (11.1)</td>
<td>79.6 (12.7)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>91.6 (16.1)</td>
<td>83.7 (19.4)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>187.5 (36.4)</td>
<td>153.1 (49.9)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>42.7 (3.3)</td>
<td>44.1 (3.1)</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>71.7 (16.7)</td>
<td>81.1 (21.4)</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>9.0 (0.9)</td>
<td>7.9 (0.4)</td>
</tr>
</tbody>
</table>

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### Fig. 1

GFR results. The effect of long-term (one year) liraglutide treatment on accurately measured kidney function (131Cr-EDTA GFR). Black line represents all patients treated with liraglutide for seven weeks (day 49) and with a subsequent 3-week washout period (n = 31). Blue line represents the patients who restarted liraglutide treatment after the initial study and continued treatment for one year (n = 23). Green line represents patients who remained untreated after the washout period (n = 7). Represents a significant change (p < 0.05) from baseline.

3.3. **UAER and fractional albumin excretion**

Baseline UAER was 25.5 (9.9–90.9) mg/d and was reduced with 27% (95% CI: 5 to 44%, p = 0.020) after one year of treatment (Table 2). The reduction in UAER was not associated with change in SBP, weight, GFR or HbA1c (p ≥ 0.17). To adjust for treatment-induced changes in GFR, which potentially influence the reduction in UAER we calculated fractional albumin excretion. This was reduced by 37% (95% CI: 13 to 54%; p = 0.007) at end of study (Table 2). GFR urine albumin excretion was reduced by 43% from baseline to end of study (95% CI: 21 to 58%, p = 0.002).

3.4. **MR-proANP and ECV**

At baseline, geometric mean (IQR) of MR-proANP was 58.8 (40.0–83.6) pmol/L, while reduced after one year of treatment by 15% (95% CI: 6 to 23%, p = 0.002, Table 2).

Baseline ECV was 14.8 (2.9) litres and was unchanged 14.4 (3.6) after one year of treatment (p = 0.75, Table 2).

3.5. **24-h systolic blood pressure and 24-h heart rate**

24-h systolic blood pressure was reduced by 8.2 mmHg (95% CI: 0.1 to 16.2, p = 0.048) (Table 2) after one year of treatment, while 24-h heart rate was changed by 2.5 bpm (95% CI: −0.5 to 5.4, p = 0.10, Table 2).

3.6. **HbA1c and weight**

HbA1c was reduced by 0.6% (95% CI: 0.1 to 1.2, p = 0.019, Table 2) (7 mmol/mol (95% CI: 1.2 to 12.6) and body weight by 3.5 kg (95% CI: 1.3 to 5.7, p = 0.003, Table 2).
4. Discussion

In patients with type 2 diabetes, one year of treatment with GLP-1 RA liraglutide was associated with significant reductions in accurately measured GFR, 24-h UAER and fractional albumin excretion. Moreover, a significant reduction in MR-proANP, HbA1c, weight and 24-h SBP was demonstrated.

The long-term renal effects of GLP-1 RA treatment has previously only been indirectly assessed using creatinine (estimated GFR). A meta-analysis of the six LEAD (Liraglutide Effect and Action in Diabetes) trials described no significant changes in creatinine from baseline in standard liraglutide doses (1.2 mg/d or 1.8 mg/d) after 26 weeks of treatment, suggesting that liraglutide treatment is not associated with changes in renal function (as measured by creatinine) (Davidson et al., 2011). However, the changes seen with GLP-1 RA treatment in body composition could have potential effects on creatinine levels and renal handling, which makes it important to measure renal function with a more precise method.

This is the first report on the long-term effect of GLP-1 RA treatment on accurately measured kidney function. Few studies have previously investigated the acute effect of GLP-1 on GFR in humans. One study found no effect of GLP-1 infusion on GFR (51Cr-EDTA) in healthy males (Skov et al., 2013), while Gutzwiller et al. observed GLP-1 infusion to decrease GFR (CrCl) by 6% in obese insulin-resistant males but found no significant effect in healthy subjects (Gutzwiller et al., 2004).

Our initial study with seven weeks of liraglutide treatment demonstrated a reduction in GFR (51Cr-EDTA) of 11 (95% CI = 7.2 to 14.4) mL/min/1.73 m², a change that was proven reversible, since values returned to baseline levels after three weeks of washout (von Scholten et al., 2015). Due to this reversible effect, we considered the reductions in GFR to be a renal haemodynamic phenomenon. However, we could not be certain that these reductions demonstrated in our initial study were not progressive when patients continued GLP-1 treatment for a longer period, since this had never been investigated previously. Our current findings indicate that one year of liraglutide treatment is associated with a similar reduction in GFR of 11 mL/min/1.73 m². Based on our initial study, this reduction is most likely reached already after few months of treatment, non-progressive and fully reversible when discontinuing the treatment (Fig. 1). However, the mechanism responsible for the reduction in GFR cannot be fully explained in this study. Of note, the patients in our study had a well-preserved kidney function, and the GFR level was still within normal limits at end of study.

The changes in GFR could be influenced by improvement in plasma glucose levels, as hyperglycaemia (>15 mmol/L) may increase GFR (Christiansen, Frandsen, & Parving, 1981), and plasma glucose was not clamped during GFR measurements. However, adjustment for change in HbA1c or mean plasma glucose during GFR measurements did not explain the change in GFR (data not shown). Furthermore, analysis of the subgroup with plasma glucose below 15 mmol/L at the first examination did not change the findings for GFR. Recently, a relatively large observational study showed that eGFR was unchanged during one year of liraglutide treatment (Zavattaro, Capoto, Sama, et al., 2015). However, most of the patients in that study were not treated with liraglutide in maximal doses. This might, in addition to the more inaccurate measure of GFR, explain some of the inconsistencies compared to our study.

Existing data on the antiproteinuric potential of GLP-1 are also sparse. We and others have previously reported liraglutide treatment to be associated with significant antiproteinuric effects (Imamura, Hirai, & Hirai, 2013; von Scholten et al., 2015), and studies with GLP-1 RA exenatide and the dipeptidyl peptidase-4 (DPP4) inhibitors saxagliptin and linagliptin have shown similar effects of lowering albuminuria (Bergenstal, Wysam, Macconnell, et al., 2010; Groop et al., 2013; Scirica, Bhatt, Braunwald, et al., 2013; Zhang, Zhang, Hu, & Lu, 2012). Our finding of a 27% reduction in UAER after one year of liraglutide treatment is interesting since albuminuria is considered a predictor of both cardiovascular and renal disease in patients with diabetes. However, as our study lacked a placebo-treated arm, it needs to be further examined in randomised trials with repeated measurements of albuminuria to account for the large day-to-day variability.

To exclude that the effect of liraglutide treatment on UAER was caused by the simultaneous decrease in GFR, we calculated fractional albumin excretion, which was also significantly decreased, thus suggesting that the reduction in UAER is not explained by the GFR reduction. It is tempting to hypothesise that the effects of liraglutide treatment on kidney parameters are due to the antihypertensive effect; however we did not find these changes to be associated. With regard to blood pressure, long-term treatment was associated with an 8 mmHg reduction in 24-h SBP. Our original hypothesis that the antihypertensive effect could be due to reductions in ECV and in MR-proANP might be factual, but this study indicates that the effects on ECV were transient, since the changes seen after seven weeks are not observed after one year of treatment, while MR-proANP was reduced by 15% as compared to a 20% reduction in our short-term study (von Scholten et al., 2015).

The effects of liraglutide treatment on GFR, UAER and blood pressure resemble the effects of RAAS blockade or increased RAAS-blocking in patients already on RAAS-blocking treatment, as in the current study where 91% were in single RAAS-blocking treatment. The GLP-1 receptor is expressed in renin-secreting cells of the juxtaglomerular arteries in the kidney (Pyke, Heller, Kirk, et al., 2014), and others (Skov et al., 2013; Mima et al., 2012) have demonstrated an effect of GLP-1 on Ang II, indicating a direct link, which we currently investigate further.

The heart rate-increasing effect of GLP-1 treatment has been well described and discussed, and a recent study demonstrated presence of GLP-1 receptors in the sinusoidal node in the heart (Pyke et al., 2014). We observed a 7 bpm increase in 24-h heart rate in our short-term study (von Scholten et al., 2015); however our follow-up data reveal no noticeable effect of liraglutide treatment on 24-h heart rate, suggesting the effect to be vane off, which has also previously described in a long-term liraglutide trial (Astrup, Carraro, Finer, et al., 2012).

One year of liraglutide treatment was associated with a weight reduction of 3.5 kg and with a decrease in HbA1c of 0.6% (7 mmol/mol) and could well be explaining factors for the changes in SBP as well as in renal parameters, although not directly associated. Moreover, treatment with liraglutide was associated with a significant reduction of 0.7 mmol/L in total cholesterol and an insignificant reduction in low-density lipoprotein and triglycerides (data not shown).

The current management of risk factors for diabetic nephropathy includes blood pressure control, glycaemic control, reduction of albuminuria, treatment of dyslipidaemia and weight loss. We demonstrate that one year of treatment with GLP-RA is associated with beneficial effects on all these risk factors. The current recommended preventive treatment of progression to diabetic nephropathy is RAAS-blockers, which are known to reduce both blood pressure and albuminuria in addition to loss of renal function and development of end stage renal disease. This report demonstrates that even in patients on stable RAAS-blocking treatment, the addition of liraglutide may lower both 24-h SBP and albuminuria. We suggest that GLP-RA treatment has the potential of both preventing and treating diabetic nephropathy. We speculate this effect to be driven in part by an effect on the RAAS although anti-inflammatory effects or effects on oxidative stress could be important as well.

4.1. Strengths and limitations

Our open-label extension study has several limitations. We lack a placebo-controlled group, and cannot conclude that our observations...
are not solely driven by the beneficial effects on glycemic control, SBP or weight, although we found no statistical association. Inclusion of an active comparator would have strengthened our study; however a single active comparator with known effect on all these parameters is not available. Moreover, number of participants is small, and the untreated group used for comparison is for obvious reasons not matched to the liraglutide-treated group. Strength of our study is the use of robust methods for the measurement of important renal and cardiovascular parameters. Our observed findings on the long-term effect of GLP-1 treatment are clear and novel, and they have, to the best of our knowledge, never been investigated before.

5. Conclusions

In patients with type 2 diabetes, long-term (one year) treatment with GLP-1 RA liraglutide was associated with a reduction in GFR, which was similar to that of short-term treatment suggesting the effect to be hemodynamic or metabolic, and not due to structural changes. Moreover, UAER and fractional albumin excretion were reduced. Besides well-known antihyperglycaemic and antihypertensive effects, GLP-1 treatment might possess the potential of renoprotection and prevention of diabetic nephropathy. Randomised longer trials are warranted to address this question.

Acknowledgements

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References

Paper IX
The effect of liraglutide on renal function: A randomized clinical trial

Bernt J. von Scholten MD | Frederik Persson MD, DMSc | Signe Rosenlund MD | Peter Hovind MD, DMSc | Jens Faber MD, DMSc | Tine W. Hansen MD, PhD | Peter Rossing MD, DMSc

1Department of Diabetes Complications Research, Steno Diabetes Center, Gentofte, Denmark
2Department of Clinical Physiology & Nuclear Medicine & PET, Rigshospitalet, Glostrup Hospital, Glostrup, Denmark
3Department of Endocrinology, Herlev University Hospital, Herlev, Denmark
4Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
5Health, Aarhus University, Aarhus, Denmark

Corresponding Author: Bernt Johan von Scholten MD, Steno Diabetes Center, Niels Steensens Vej 1, 2820, Gentofte, Denmark (bjos@steno.dk).

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The study was funded by an unrestricted grant from Novo Nordisk A/S, Bagsvaerd, Denmark, who had no influence over the trial’s conduct or analysis of the results.

Aims: Among patients with type 2 diabetes and albuminuria, cardiorenal morbidity and mortality are high despite multifactorial treatment. Short-term reduction in albuminuria is considered suggestive of long-term renoprotective effects. We evaluated the renal effects of the glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide on top of multifactorial care, including renin-angiotensin-system (RAS)-inhibition.

Materials and methods: Randomized, double-blind, placebo-controlled, cross-over trial including patients with type 2 diabetes and persistent albuminuria (urinary albumin-to-creatinine ratio >30 mg/g) and estimated glomerular filtration rate (eGFR) ≥30 mL/min/1.73 m². Patients received liraglutide (1.8 mg/d) and matched placebo for 12 weeks in a random order. The primary endpoint was change in 24-h urinary albumin excretion rate (UAER).

Results: A total of 32 patients were randomized and 27 completed the study. After placebo treatment, geometric mean (IQR) UAER was 199 (81-531) mg/24-h, mean (SD) measured GFR (mGFR) 75 (36) mL/min/1.73 m², 24-h blood pressure 145/80 (15/8) mm Hg and HbA1c 61 (11) mmol/mol. Liraglutide reduced HbA1c by 8 (95% CI: 5; 11) mmol/mol (P < .001) and weight by 1.8 (95% CI: 0.2; 3.4) kg (P = .032) compared to placebo. Furthermore, liraglutide reduced UAER by 32 (95% CI: 7; 50)% (P = .017) compared with placebo. The change in mGFR was −5 (95% CI: −11; 2) mL/min/1.73 m² (P = .15), and change in 24-h systolic blood pressure was −5 (95% CI: −10; 0) mm Hg (P = .07). In multivariate regression models, change in UAER was associated with change in 24-h systolic blood pressure (P = .025) but not with change in HbA1c, weight or mGFR (P ≥ .14), overall model R² = .39.

Conclusions: Our placebo-controlled randomized trial suggests that liraglutide has renoprotective effects on top of multifactorial treatment, including RAS-inhibition, in patients with type 2 diabetes and albuminuria.

KEYWORDS
diabetic nephropathy, GLP-1, liraglutide, randomized trial

1 INTRODUCTION

The GLP-1 analogue liraglutide is approved for management of hyperglycaemia in type 2 diabetes in doses up to 1.8 mg once daily, and has additional weight-loss effects. Furthermore, patients treated with liraglutide often experience moderate lowering of blood pressure, and in rodent models of diabetic nephropathy liraglutide was found to prevent kidney damage by diminishing renal oxidative stress. In non-randomized studies, we have previously shown that liraglutide treatment lowers albuminuria, and in the SCALE Diabetes Randomized Clinical Trial the maximal dose of 3.0 mg/d was...
associated with an 18% reduction in urinary albumin-to-creatinine ratio (UACR) compared to placebo in patients with type 2 diabetes. In a study exploring the safety and efficacy of liraglutide in 279 patients with type 2 diabetes and moderate renal impairment, albuminuria was 17% lower with liraglutide compared to placebo, although not statistically significant. However, none of the studies were designed to detect changes in albuminuria and levels of baseline albuminuria were low. Of note, the results of the LEADER trial recently demonstrated lower rates of renal outcomes, in particular, a lower risk of new-onset persistent macroalbuminuria, during liraglutide treatment.

Albuminuria is considered one of the best available risk markers of both renal and cardiovascular disease. Short-term treatment-induced reductions in albuminuria have been linked to long-term renal and cardiovascular protection. The wide implementation of renin-angiotensin-system (RAS)-blocking treatment in type 2 diabetes has made a pronounced contribution in slowing disease progression; however, patients with type 2 diabetes and albuminuria are still at high risk of both renal and cardiovascular complications. Hence, there is an unmet need for a novel treatment option that can reduce these complications, and such intervention is expected in short-term studies to lower albuminuria on top of RAS-inhibition.

In acute studies, GLP-1 treatment has been shown to interact with the RAS by decreasing angiotensin II (Ang II) levels in type 2 diabetic males. Further, GLP-1 infusion has been shown to reduce renin activity in healthy middle-aged men. Whether these effects of GLP-1 are only acute or sustained is unknown but might be an important mechanistic observation explaining the renoprotective effects of liraglutide.

The study hypothesis was that liraglutide treatment is associated with a reduction in albuminuria in patients with type 2 diabetes and persistent microalbuminuria who are already receiving stable RAS-blocking treatment, compared to placebo treatment. Further, it was hypothesized that the antiproteinuric and renoprotective effect is mediated through interaction with the RAS.

2 | METHODS

2.1 | Participants and study procedures

This randomized, placebo-controlled, double-blind, cross-over trial enrolled patients with type 2 diabetes (WHO criteria), HbA1c ≥8% mmol/mol (6.5%), persistent albuminuria (≥30 mg/g in at least 2 out of 3 consecutive morning spot urine samples) and who were receiving stable RAS-blocking treatment. Exclusion criteria included diagnosis of clinical heart failure and estimated glomerular filtration rate (eGFR) ≤30 mL/min/1.73 m². Patients were recruited from the Steno Diabetes Center, Gentofte, Denmark from April to October 2015 and the study was completed in April 2016.

The study design was illustrated in Figure 1. Patients were randomly assigned in a 1:1 ratio to 1 of the following 2 treatment groups: (1) liraglutide + standard therapy or (2) placebo + standard therapy. After 12 weeks of treatment, patients underwent a 4-week washout period prior to crossing over to the other treatment group for 12 weeks.

The study protocol was approved by the regional ethics committee and the Danish Medicine Agency and was conducted according to the Declaration of Helsinki and Good Clinical Practice. The study is registered at ClinicalTrials.gov with identifier NCT02545738 and was conducted at Steno Diabetes Center, Denmark. All patients gave written informed consent before any study procedure was initiated. The primary aim was to assess change in urinary albumin excretion rate (UAER) after 12 weeks of liraglutide treatment compared with placebo treatment. Secondly, the effect of liraglutide treatment on measured GFR (¹⁵⁰Cr-EDTA) and RAS hormones in plasma (renin, renin activity, angiotensin II and aldosterone) was assessed. Finally, explorative measurements included 24-hour systolic, 24-hour diastolic blood pressure, 24-hour heart rate and fractional albumin clearance. Participants attended a baseline examination visit and were instructed in subcutaneous injection of the study drug. Participants were treated with liraglutide/placebo 0.6 mg/d for 7 days, escalated to 1.2 mg/d for 7 days and lastly to 1.8 mg/d for the remaining 10 weeks or matching placebo.

2.2 | Procedures

Liraglutide injection pens and placebo/liraglutide (indistinguishable), as well as the random allocation sequence, were provided by Novo Nordisk A/S (Bagsvaerd, Denmark). The study medication was numbered sequentially and prior to each study day the correct drug number was assigned by a person not otherwise involved in the study. Participants, care providers and data managers were blinded.
2.3 | Outcome measurements

Two 24-hour urine collections were performed at baseline and two 24-hour urine collections were performed at the end of each treatment period to measure UAER using an enzyme immunoassay (Vitros, Raritan, New Jersey). Twenty-four-hour blood pressure was performed at baseline and at the end of each treatment period using a self-inflating cuff device (Takeda: A & D Medical, Tokyo, Japan). The blood pressure monitors were initiated to obtain readings at 15-minute intervals during daytime and 30-minute intervals at night. The device is validated according to the British Society of Hypertension and the Association for Advancement of Medical Instrumentation. Means of systolic and diastolic blood pressure and of heart rate were calculated using all readings during the 24-hour period. Only 24-hour blood pressure recordings with ≥20 readings during daytime and ≥7 at night were used for analysis. Two recordings were incomplete and were discarded from the analysis.

Renal function (mGFR) was assessed during 4-hour measurement of plasma $^{51}$Cr-EDTA by standard methods. $^{51}$Cr-EDTA was performed at the end of each treatment period. Fractional clearance of albumin (fAlb) was determined as urinary albumin excretion/plasma albumin concentration x GFR in the 4-hour urine collection period during determination of GFR.

2.4 | Laboratory procedures

Samples for plasma renin concentration, renin activity, Ang II and aldosterone levels were determined after 30 minutes of supine rest, and the plasma was frozen after centrifugation (−80°C). All RAS hormones were measured at baseline and at the end of each treatment period. Plasma renin concentration was measured by an in-house radioimmunoassay measured in the presence of 500 pmol/mL sheep angiotensinogen and renin activity was independent of endogenous substrate variations under these assay conditions. Plasma (25 μL) was incubated at 37°C for 3 hours with sheep angiotensinogen in a total reaction volume of 100 μL. The assay was calibrated against the international reference renin preparation 68/356 and results could therefore be expressed in terms of renin concentration (mIU/L). The enzymatic angiotensin I-generation step and the subsequent angiotensin I radioimmunoassay step were performed in the same tube. Under these assay conditions, renin activity is proportional to renin concentration. The intra-assay and inter-assay coefficients of variation were <5% and 8%-10%, respectively.

Plasma Ang II was measured by an in-house radioimmunoassay after ethanol extraction of plasma samples. Antibodies were raised in rabbits, and calibrators were purchased from NIBSC. Plasma aldosterone concentrations were determined by immunoassay using the Liaison autoanalyzer and kit from DiaSorin (Saluggia, Italy). Hba1c was measured by high-performance liquid chromatography calibrated against the IFCC standard. All routine clinical laboratory variables were measured using standard clinical laboratory methods.

2.5 | Statistical analysis

It was estimated that 24 patients completing the study could provide 80% power to demonstrate a significant difference between 2 treatments in antiproteinuric effect (measured as UAER) if the true difference was 15%. This was based on the assumption that intra-individual coefficient of variation for UAER was 13%.

Demographic data are presented as mean (SD) or median (range) where skewed data (UAER and all RAS-hormones) are shown as geometric mean (IQR) and are analysed after log-transformation. Each UAER was log-transformed and the geometric mean of the 2 UAERS is presented for each visit. The percent change in UAER was calculated using the geometric mean values at each time point. The advantage of a cross-over study is that each participant acts as his own control, which removes variability among participants. However, before comparing treatments, analyses were performed to test the assumption of no carryover effect, as described by Altman. We tested for period effect and treatment-period interaction using 2-sample t-tests comparing the mean difference and average, respectively, grouping participants according to order of treatment-period. No carryover effect was detected for any of our endpoints, allowing us to use paired samples t-tests for treatment comparison.

The change in all endpoints was analysed as end of placebo treatment vs. end of liraglutide treatment. Except for GFR (measured only after each treatment period), all endpoints were measured at all 4 study visits. In order to utilize all measures at all visits and to test the robustness of our results we additionally analysed and compared the changes within each treatment period. Associations between changes in the primary outcome UAER and changes in potential explanatory variables, Hba1c, weight, SBP, GFR and RAS-hormones were assessed by univariate and multivariate regression models. The proportion of the variability in UAER explained by the model is presented as the $R^2$. Two-sided P-values < .05 were considered significant. Statistical analysis was performed using IBM SPSS 23.0 (IBM Amonk New York).

3 | RESULTS

3.1 | Patient recruitment and sample size

In total, 40 patients gave written informed consent; 8 failed screening, mainly because of UACR < 30 mg/g. Thirty-two patients were included and initiated treatment with liraglutide/placebo. Five patients dropped out because of gastrointestinal side effects (nausea, vomiting, diarrhea), all during liraglutide treatment.

Therefore, 27 participants completed the study (Figure 2).

3.2 | Baseline characteristics

Data are presented in Table 1. Of the completing participants, 22 (81%) were men, mean (SD) age was 65 (7) years, diabetes duration was 15 (7) years, Hba1c was 61 (12) mmol/mol (7.7 (1.1)%) and 21 (78%) were treated with insulin (Table 1). Seventeen participants (63%) had microalbuminuria (UAER ≥ 30 and ≤ 300 mg/d) and 10 (37%) had macroalbuminuria (UAER ≥ 300 mg/d) at baseline. Retinopathy was present in 19 participants (70%) at baseline.
3.3 | HbA1c and weight

Mean HbA1c was 53 (11) mmol/mol at the end of liraglutide treatment compared with 61 (11) mmol/mol at the end of placebo treatment ($P < .001$). Mean body weight was 98 (19) kg at the end of liraglutide treatment compared with 100 (18) kg at the end of placebo treatment ($P = .032$).

### TABLE 1  Baseline characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients randomized (n = 32)</th>
<th>Patients completing (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 (7)</td>
<td>65 (7)</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>84</td>
<td>81</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>16 (7)</td>
<td>15 (7)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>98.5 (18.3)</td>
<td>99.7 (18.8)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>31.5 (4.8)</td>
<td>31.9 (5.0)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>61.8 (10.7)</td>
<td>61.3 (11.6)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.8 (1.0)</td>
<td>7.8 (1.1)</td>
</tr>
<tr>
<td>UAER (mg/d)</td>
<td>233 (84-587)</td>
<td>197 (85-528)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>138 (18)</td>
<td>135 (18)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>75 (10)</td>
<td>75 (11)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>90.3 (30.0)</td>
<td>91.7 (31.5)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²) (CKD-EPI)</td>
<td>76.6 (21.8)</td>
<td>75.7 (22.7)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>1.9 (0.6)</td>
<td>1.9 (0.6)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>30 (14)</td>
<td>30 (15)</td>
</tr>
<tr>
<td>RAS-treatment (%)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Macroalbuminuria (%)</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>75</td>
<td>70</td>
</tr>
</tbody>
</table>

Abbreviations: ALT; alanine aminotransferase; eGFR, estimated glomerular filtration rate; RAS, renin-angiotensin-system; UAER, urinary albumin excretion rate.

Data are percentage (%), mean (SD) or geometric mean (IQR).

3.4 | Primary endpoint: Urinary albumin excretion rate

Geometric mean UAER was 32 (95% CI: 7; 50) lower after liraglutide treatment (UAER [IQR] 135 [59-292] mg/24-h) compared with after placebo treatment (UAER [IQR] 199 [81-531] mg/24-h) ($P = .017$). When analysing the effect within each treatment period, the UAER was reduced by 26 (95% CI: 5; 43) from start to end of liraglutide treatment, and increased by 9 (95% CI: -6 to 22) from start to end of placebo treatment (Table 2; Figure 3). Comparing the changes within these 2 treatment periods, the UAER was significantly reduced by 35 (95% CI: 9; 53) during liraglutide treatment compared with placebo treatment ($P = .015$).

Seven patients (26%) had a reduction in UAER > 50%, and 12 patients (44%) had a reduction >30% after liraglutide treatment compared with placebo treatment.

In univariate regression models, change in UAER was associated with change in 24-hour systolic blood pressure ($P = .022$, $R^2 = .18$) and with change in HbA1c ($P = .032$, $R^2 = .16$), but not with change in GFR ($P = .63$) or weight ($P = .70$). In multivariate regression models, change in UAER was associated with change in 24-hour systolic blood pressure ($P = .025$), but not with HbA1c, GFR or weight ($P ≥ .14$), overall model $R^2 = .39$.

Of the 10 patients with macroalbuminuria, 3 regressed to microalbuminuria, 1 regressed to normoalbuminuria. Four patients regressed from micro- to normo-albuminuria.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Before liraglutide</th>
<th>After liraglutide</th>
<th>Change for the liraglutide group (95% CI)</th>
<th>Before placebo</th>
<th>After placebo</th>
<th>Change for placebo group (95% CI)</th>
<th>P value for comparison between therapies (end vs. end)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UAER, mg/24-h</strong></td>
<td>183 (75-534)</td>
<td>135 (59-292)</td>
<td>-26 (-43; -5%), P = .022</td>
<td>181 (84-353)</td>
<td>199 (81-531)</td>
<td>9 (-6; 22%), P = .21</td>
<td>.017</td>
</tr>
<tr>
<td><strong>GFR (mL/min/1.73 m²)</strong></td>
<td>70 (30)</td>
<td>75 (36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.15</td>
</tr>
<tr>
<td><strong>eGFR (mL/min/1.73 m²)</strong></td>
<td>75 (23)</td>
<td>74 (24)</td>
<td>-1 (-4; 1), P = .33</td>
<td>73 (23)</td>
<td>72 (23)</td>
<td>-1 (-4; 2), P = .57</td>
<td>.41</td>
</tr>
<tr>
<td><strong>24-h systolic blood pressure, mm Hg</strong></td>
<td>79 (14)</td>
<td>140 (11)</td>
<td>-4 (-9; 1), P = .13</td>
<td>142 (12)</td>
<td>145 (15)</td>
<td>3 (-2; 9), P = .22</td>
<td>.07</td>
</tr>
<tr>
<td><strong>24-h diastolic blood pressure, mm Hg</strong></td>
<td>72 (12)</td>
<td>77 (13)</td>
<td>-1 (-6; 2), P = .45</td>
<td>76 (6)</td>
<td>80 (6)</td>
<td>4 (0; 7), P = .029</td>
<td>.25</td>
</tr>
<tr>
<td><strong>HbA1c, mmol/mol</strong></td>
<td>9.7 (18.7)</td>
<td>98.0 (18.6)</td>
<td>-1.7 (-3.0; -0.4), P = .012</td>
<td>99.5 (17.8)</td>
<td>100.2 (17.9)</td>
<td>0.7 (-0.2; 1.5), P = .11</td>
<td>.032</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>99.7 (34.5-322.0)</td>
<td>52.4 (140-204.1)</td>
<td>-37 (-59; -5%), P = .030</td>
<td>81.9 (30.0-241.0)</td>
<td>60.1 (15.9-242.3)</td>
<td>-27 (-56; 18%), P = .22</td>
<td>.57</td>
</tr>
<tr>
<td><strong>RAS hormones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Renin concentration, mU/L</td>
<td>8.37 (3.0-50.5)</td>
<td>5.5 (1.1-29.4)</td>
<td>-43 (-64; -9%), P = .022</td>
<td>9.0 (4.0-39.0)</td>
<td>6.4 (1.6-33.9)</td>
<td>-28 (-57; 17%), P = .20</td>
<td>.53</td>
</tr>
<tr>
<td>p-Aldosterone, nm/L</td>
<td>21.4 (316.8-29.2)</td>
<td>213.6 (58.0-319.9)</td>
<td>-1 (-19; 18), P = .96</td>
<td>225.0 (182.0-299.0)</td>
<td>206.4 (157.8-293.5)</td>
<td>-6 (-20; 13), P = .45</td>
<td>.63</td>
</tr>
</tbody>
</table>

**Abbreviations:** GFR, glomerular filtration rate; RAS, renin-angiotensin-system; UAER, urinary albumin excretion rate.

Data represent mean (SD) or geometric means (IQR).
3.5 | GFR, fractional albumin excretion and ambulatory blood pressure

Mean of measured GFR ($^{51}$Cr-EDTA) was 70 (30) mL/min/1.73 m$^2$ at the end of liraglutide treatment compared with 75 (36) mL/min/1.73 m$^2$ at the end of placebo treatment ($P = .15$, Table 2). Estimated GFR was 74 (24) mL/min/1.73 m$^2$ at the end of liraglutide treatment compared with 72 (23) mL/min/1.73 m$^2$ at the end of placebo treatment ($P = .4$) (Table 2). Fractional clearance of albumin was 23 (95% CI: 5; 38)% lower at the end of liraglutide treatment compared with placebo treatment ($P = .019$). Mean 24-hour systolic blood pressure was 140 (15) mm Hg at the end of liraglutide treatment compared with 145 (15) mm Hg at the end of placebo treatment ($P = .07$) and mean 24-h diastolic blood pressure was 78 (6) mm Hg at the end of liraglutide treatment compared with 80 (8) mm Hg ($P = .25$) (Table 2).

When analysing the effect within each treatment period, 24-hour systolic blood pressure was reduced by 4 (95% CI: -9; 1) mmHg ($P = .13$) from start to end of liraglutide treatment, and increased by 3 (95% CI: -2 to 9) mmHg ($P = .22$) from start to end of placebo treatment. Comparing the changes within these 2 treatment periods, 24-hour systolic blood pressure was significantly reduced by 7 (95% CI: 2; 14) mmHg during liraglutide treatment compared with placebo treatment ($P = .014$). Liraglutide treatment was associated with a mean increase in 24-hour heart rate of 3 (95% CI: 0; 6) bpm compared with placebo treatment ($P = .035$) (Table 2).

3.6 | RAS hormones

No significant differences were observed when comparing levels of RAS hormones at the end of liraglutide treatment with levels at the end of placebo treatment ($P = .53$, Table 2). When analysing the effect within each treatment period, mean plasma renin concentration was reduced by 37% (95% CI: 5; 59%) ($P = .003$), renin activity was reduced by 35 (95% CI: -2; 59%) ($P = .060$), Ang II was reduced by 43 (95% CI: 9; 64%) ($P = .022$) and aldosterone remained unchanged ($P = .95$). Comparing changes within the 2 treatment periods, no significant differences were observed during liraglutide compared with placebo treatment ($P = .51$), as renin and Ang II also decreased during placebo treatment, although to a lesser extent (Table 2).

No significant association was observed between changes in RAS hormones and change in UAER ($P ≥ .38$) during liraglutide treatment. Changes in plasma renin concentration, renin activity and Ang II were associated with change in GFR ($R^2 = .15$, $P = .044$). Change in plasma renin activity was associated with change in 24-hour systolic blood pressure ($R^2 = .21$; $P = .031$).

3.7 | Safety

A total of 12 (44%) patients reported adverse events. The most common adverse event, reported by 9 (33%), was nausea. Five patients discontinued the study during liraglutide treatment because of nausea, vomiting or diarrhoea. No patients discontinued the study during placebo treatment. A total of 4 serious adverse events were reported throughout the study. One serious adverse event was reported during placebo therapy (ankle fracture). Three serious adverse events were reported during liraglutide treatment (atrial fibrillation, $n = 1$; pneumonia, $n = 1$; acute myocardial infarction, $n = 1$). None of the serious adverse events were considered to be drug-related and none led to study discontinuation. No change in antihypertensive medication was prescribed during the course of the study.

4 | DISCUSSION

In this first randomized placebo-controlled trial to investigate the effect of liraglutide treatment on renal function and RAS hormones in type 2 diabetic patients with persistent albuminuria, we have demonstrated liraglutide treatment, on top of stable RAS-inhibition, to be associated with a statistically and clinically significant reduction in albuminuria. We speculate that the effect is partly driven by the antihypertensive and partly by the glucose-lowering effect. Further, the impact of liraglutide on the RAS might contribute to the beneficial effect on albuminuria, since reductions in Ang II of 43% and in renin concentrations of 37% were seen with liraglutide compared with reductions of 28% and 27%, respectively, with placebo treatment.

Diabetes is the most common cause of end-stage renal disease in the developed world. In outcome trials of patients with diabetic nephropathy, retrospective analyses demonstrate a robust relationship between magnitude of short term albuminuria reduction and long-term slowing of chronic kidney disease (CKD) progression as well as reduced cardiovascular event rates,$^{3,15}$ although most studies were performed with antihypertensive agents.

By demonstrating that liraglutide treatment reduces albuminuria by 32%, we suggest liraglutide as a novel treatment option with the capability to lower albuminuria on top of existing RAS-inhibition in patients with persistent albuminuria. In addition, 8 patients regressed in albuminuric status and 7 patients had a reduction in UAER > 50%, while no patients progressed in albuminuric status. Findings of a recent meta-analysis concluded that short-term albuminuria reduction is associated with long-term renal protection across different interventions and populations. Further, the authors suggest that for new interventions, a 30% reduction in albuminuria on top of guideline-
recommended care seems necessary to confer a detectable renoprotective treatment effect. As many of the studies included in this meta-analysis used UACR rather than UAER, we can report that the magnitude of change in UACR in our study was similar to the change in UAER (55% CI: 9-53%). However, whether the >30% reduction in albuminuria associated with liraglutide treatment will translate into renoprotection will have to be demonstrated in a long-term outcome study in a relevant study population.

Recently, several novel therapeutic approaches have been shown to possess albuminuria lowering effects and promising renoprotective effects. The endothelin antagonist atrasantan, the nonsteroidal mineralocorticoid receptor antagonist finerenone and CCR2 inhibition with CCX140-B have all been demonstrated to lower albuminuria in patients with type 2 diabetes and nephropathy. Of ongoing trials, only the phase 3 randomized controlled trials with atrasantan, finerenone or canagliflozin may result in a new therapeutic indication, while most other trials are phase 2 trials that will provide proof-of-concept. These novel treatment opportunities are interesting and might prove to be important in the treatment of diabetic nephropathy. However, the risk of side effects and adverse events is still a matter of concern. Since the glucose-lowering drugs used in the studies investigating atrasantan, finerenone and CCR2 inhibition were mainly metformin, sulfonylureas and insulin, it is unclear whether newer anti-nephropathy treatments would be needed if a GLP-1 receptor agonist was being used instead of these drugs. Liraglutide has been available for the treatment of type 2 diabetes for 7 years and, along with lowering HbA1c, the treatment has shown weight-lowering and antihypertensive potential. The SGLT-2 inhibitors have also demonstrated antihypertensive, antialbuminuric and potentially renoprotective effects in recent studies beyond the glucose-lowering effect which is the primary indication. The current management of risk factors for diabetic nephropathy includes blood pressure control, glycemic control, reduction of albuminuria and weight loss, and while SGLT-2 inhibitors and GLP-1 receptor agonists are primarily prescribed for their glucose-lowering effects, these agents may possess pleiotropic effects on renal risk factors.

What are the mechanisms explaining the effect of liraglutide treatment on albuminuria? Reductions in systolic blood pressure, HbA1c, GFR and body weight may all contribute to lowering albuminuria; and in this study we found changes in 24-hour systolic blood pressure and changes in HbA1c to be associated with changes in albuminuria. Since reductions in 24-hour systolic blood pressure were significantly associated with the change in UAER in both univariate and multivariate regression models, we suggest that the reduction in systolic blood pressure associated with liraglutide contributes to improved renal function. The fact that the fractional albumin excretion, which is adjusted for the changes in GFR, was significantly reduced supports the notion that the reduction in UAER occurred independently of the reduction in GFR.

Also, based on experimental and clinical studies investigating the renal effects of liraglutide treatment, we hypothesize that the effects might be influenced by a direct impact on the RAS. GLP-1 receptor expression has been observed in renin-secreting cells of the juxtaglomerular apparatus and recent evidence supports a predominant presence of GLP-1 receptors in the renal vasculature, including the afferent arteriole. Acute GLP-1 administration has been demonstrated to reduce renin activity in healthy subjects and Ang II in type 2 diabetic patients. In this study in albuminuric patients, we found significant reductions in renin concentration and Ang II during 12 weeks of liraglutide treatment; however, these were not statistically different when compared with placebo treatment. As recently highlighted by Drucker, investigation of components of the RAS in subjects treated with GLP-1 receptor agonists has not identified consistent changes in plasma levels of renin, Ang II or aldosterone. Of note, inclusion criteria, duration of treatment and the RAS hormones selected for analyses have differed, when the effect of GLP-1 receptor agonists on the RAS has been investigated. The exploratory investigation of an effect of liraglutide treatment compared to placebo on RAS hormones lacked the statistical power to demonstrate significance, in part because the reductions are smaller than those seen with RAS-blocking treatment and, potentially, because all patients were on stable RAS blockade before and throughout the study. Further, we measured systemic changes in the RAS, whereas the effect of liraglutide might better be reflected in changes in the local (tissue) activation of the RAS. While there was no significant association between changes in RAS-hormones and change in UAER, changes in plasma renin concentration, renin activity and Ang II were all associated with change in GFR and change in renin activity was associated with change in 24-hour systolic blood pressure. Whether the changes in GFR and 24-hour systolic blood pressure caused the changes in the RAS-hormones, or if it is the other way around, cannot be determined in the present study; however, these findings point to a potential link between the renal effects of liraglutide and impact on the RAS.

In our previous open-label study in 31 type 2 diabetic patients, 7 weeks of liraglutide treatment was associated with a short-term and reversible decrease in mGFR. We speculated that the changes were benign and probably the consequence of renal haemodynamic effects; this was partly confirmed when we determined the effect on mGFR of reinitiating liraglutide treatment for 1 year. While mGFR was 5 mL/min/1.73 m² lower with liraglutide treatment than with placebo treatment in the present study, the difference did not reach statistical significance, and in a recent study including 19 overweight type 2 diabetic patients with a baseline mGFR of 79 mL/min/1.73 m², no changes in mGFR were observed after 12 weeks of liraglutide treatment compared with placebo. The discrepancy could be explained by the fact that we included patients with lower mGFR for the present study (75 mL/min/1.73 m² vs. 100 mL/min/1.73 m²), as a larger reduction may be expected with a higher start level of mGFR. This is demonstrated by the fact that, in the 2 patients with hyperfiltration (mGFR > 135 mL/min/1.73 m²) included in this study, mGFR was reduced by >30% during liraglutide treatment (data not shown). However, based on the observed variability in mGFR, it is also plausible that our sample size was insufficient to make definitive statements about whether or not a change in mGFR occurred. Data on eGFR from the LEAD studies (type 2 diabetes without renal impairment) as well as from the LIRA-RENAL study inadequately controlled type 2 diabetes and moderate renal impairment reflected no significant changes in eGFR after 26 weeks of liraglutide treatment. However, a short-term decrease followed by stabilization in
the eGFR over time – a mechanism similar to that of RAS-inhibition as well as SGLT-2 inhibition – was demonstrated in the LIRA-RENAL study.6

The beneficial effects of acute reductions in mGFR and eGFR have been demonstrated previously in intervention studies with RAS-inhibitors,29,30 and recently the EMPA-REG OUTCOME trial with the SGLT2 inhibitor empagliflozin demonstrated that an acute fall in eGFR was followed by a slower rate of chronic GFR decline.31 Empagliflozin has been shown to reduce the proximal tubular sodium reabsorption, thereby increasing distal sodium delivery to the macula densa, which has been shown to activate the tubulo-glomerular feedback, leading to afferent vasomodulation and a decrease in hyperfiltration.31 Short-term liraglutide treatment in type 2 diabetic patients has been shown previously to reduce proximal tubular sodium reabsorption,11 and it has been suggested that the antihypertensive and renal effects of GLP-1 may be mediated through increased natriuresis. However, we could not confirm this in our previous non-randomized study5 and we did not perform an acute measure of urinary sodium excretion in the present study.

The LEADER trial (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results)32 demonstrated, in 9340 patients with type 2 diabetes followed for a median of 3.8 years, that liraglutide treatment is superior to placebo in reducing cardiovascular events with a hazard ratio of .87; 95% confidence interval (CI): .78; .97; \( P = .03 \). Interestingly, there was also a marked lower incidence of new onset nephropathy in the liraglutide treated group, with a hazard ratio of 0.78 (95% CI: .67; .92; \( P = .003 \)) compared to placebo. Further analyses of the LEADER trial data are expected; however, large-scale outcome studies examining the long-term effects of GLP-1 treatment on CKD progression in diabetic nephropathy are still warranted.

4.1 | Strengths and limitations

This is the first randomized trial designed to investigate the effect of liraglutide treatment on albuminuria in type 2 diabetic patients with elevated albuminuria. We included patients with persistent albuminuria despite being on RAS blocking treatment, and we applied robust methods for evaluation of important renal parameters. Limitations include that we did not include an active comparator with glucose-lowering effects. Also, the primary outcome of the study was the change in UAER during 12 weeks of treatment. Despite previous studies linking reductions in albuminuria to renal and cardiovascular protection, the reduction in albuminuria demonstrated in this study may not necessarily be linked to a reduction in progression to end-stage renal disease, cardiovascular disease or all-cause mortality.

4.2 | Conclusion

In this randomized clinical trial of type 2 diabetic patients with persistent albuminuria, liraglutide treatment, on top of RAS-inhibition, was associated with a clinically relevant reduction in UAER. This effect may be partly explained by a reduction in 24-hour systolic blood pressure, HbA1c, plasma levels of renin concentration and Ang II, and liraglutide may represent a new additional treatment option to prevent progression of diabetic kidney disease.

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Clinical trial registration: ClinicalTrials.gov, NCT02545738.

Conflict of interest

Steno Diabetes Center, where B. J. vS., F. P., S. R., T. W. H. and P. R. are employed, receives part of its core funding from unrestricted grants from Novo Nordisk Foundation and Novo Nordisk, and is owned by Novo Nordisk. B. J. vS. declares having given lectures for Novo Nordisk and BMS, all fees having been given to Steno Diabetes Center, and that he has equity interest in Novo Nordisk. D. P. claims having given lectures from Novartis, Eli Lilly, Boehringer Ingelheim, Novo Nordisk, BMS and AstraZeneca. S. R. has equity interest in Novo Nordisk. P. H. declares no conflicts of interest. J. F. declares having given lectures for Novo Nordisk, Astrazeneca, Eli Lilly, Boehringer Ingelheim; and that he has served as a consultant for Astra Nordisk and Novartis. T. W. H. has equity interest in Novo Nordisk. P. R. declares having given lectures for Astra Zeneca, BMS and Boehringer Ingelheim; that he has served as a consultant for AstraZeneca, BMS, Eli Lilly, Boehringer Ingelheim, Astellas, Janssen and Novo Nordisk, all fees having been given to Steno Diabetes Center; and that he has equity interest in Novo Nordisk.

Author contributions

B. J. vS. conceived and designed the research, analysed and interpreted the data, performed statistical analysis and wrote the manuscript. F. P. conceived and designed the research, analysed and interpreted the data and made a critical revision of the manuscript for key intellectual content. S. R. conceived and designed the research, analysed and interpreted the data and made a critical revision of the manuscript for key intellectual content. B. J. vS. and F. P. conceived and designed the research, performed statistical analysis and wrote the manuscript. P. R. conceived and designed the research, analysed and interpreted the data, performed statistical analysis and wrote the manuscript. T. W. H. analysed and interpreted the data and made a critical revision of the manuscript for key intellectual content. J. F. conceived and designed the research, performed statistical analysis and wrote the manuscript. P. H. conceived and designed the research, performed statistical analysis and wrote the manuscript. J. F. conceived and designed the research, performed statistical analysis and wrote the manuscript.
REFERENCES


Paper X
Effects of liraglutide on cardiovascular risk biomarkers in patients with type 2 diabetes and albuminuria: A sub-analysis of a randomized, placebo-controlled, double-blind, crossover trial

Bernt Johan von Scholten MD1 | Frederik Persson MD, DMSc1 | Signe Rosenlund MD1 | Jesper Eugen-Olsen PhD2 | Tomasz Pielak BSc3 | Jens Faber MD, DMSc4 | Tine W. Hansen MD, PhD1 | Peter Rossing MD, DMSc1,5,6

1Department of Diabetes Complications Research, Steno Diabetes Center Copenhagen, Gentofte, Denmark
2Department of Clinical Biochemistry, Clinical Research Centre, Copenhagen University Hospital Hvidovre, Hvidovre, Denmark
3Nutopi Sp. z o.o., Poznan, Poland
4Department of Endocrinology, Herlev Hospital, Herlev, Denmark
5Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
6Faculty of Health, Aarhus University, Aarhus, Denmark

Correspondence
Bernt Johan von Scholten MD, Steno Diabetes Center, Niels Steensens Vej 1, 2820 Gentofte, Denmark.
Email: bvon0013@regionh.dk

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We assessed the effects of liraglutide treatment on five cardiovascular risk biomarkers, reflecting different pathophysiology: tumour necrosis factor (TNF)-α; soluble urokinase plasminogen activator receptor (suPAR); mid-regional pro-adrenomedullin (MR-proADM); mid-regional pro-atrial natriuretic peptide (MR-proANP); and copeptin, in people with type 2 diabetes with albuminuria. In a randomized, double-blind, placebo-controlled, crossover trial we enrolled people with type 2 diabetes and persistent albuminuria (urinary albumin-to-creatinine ratio [UACR] >30 mg/g) and estimated glomerular filtration rate (eGFR) ≥30 mL/min/1.73 m². Participants received liraglutide (1.8 mg/d) and matched placebo for 12 weeks, in random order. The primary endpoint was change in albuminuria; this was a prespecified sub-study. A total of 32 participants were randomized, of whom 27 completed the study. TNF-α level was 12% (95% confidence interval [CI] 3; 20) lower after liraglutide treatment compared with placebo (P = .012); MR-proADM level was 4% (95% CI 0; 8) lower after liraglutide treatment compared with placebo (P = .038), and MR-proANP level was 13% (95% CI 4; 21) lower after liraglutide treatment compared with placebo (P = .006). In the present study, we showed anti-inflammatory effects of liraglutide treatment, reflected in reductions in levels of TNF-α and MR-proADM, while the reduction in MR-proANP levels may represent a clinically relevant benefit with regard to heart failure.

KEYWORDS clinical trial, GLP-1 analogue, liraglutide

1 | INTRODUCTION

People with type 2 diabetes, and especially those with concomitant albuminuria, are at high risk of cardiovascular disease.1 Endothelial dysfunction is an early marker of atherosclerosis, and inflammatory processes play an important role in atherosclerotic cardiovascular disease.2 Liraglutide has pleiotropic effects3 and, in addition to lowering glucose, liraglutide also lowers weight, blood pressure, albuminuria and lipid levels,4 which may all contribute to its cardioprotective properties. Anti-inflammatory and vasodilatory effects of liraglutide have also been suggested as important mechanisms.5

We evaluated the effect of liraglutide on five biomarkers that are associated with cardiovascular disease in patients with diabetes: tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine; soluble urokinase plasminogen-activator receptor (suPAR), a marker of endothelial dysfunction and inflammation; mid-regional pro-adrenomedullin (MR-proADM), a marker of systemic inflammation;
mid-regional pro-atrial natriuretic peptide (MR-proANP), a natriuretic peptide⁹; and copeptin, a surrogate marker of arginine vasopressin.¹⁰

The hypothesis was that liraglutide treatment is associated with a reduction in inflammatory and cardiovascular risk biomarkers in high-risk patients.

2 | METHODS

A randomized, placebo-controlled, double-blind, crossover trial was conducted, enrolling patients with type 2 diabetes, a glycated haemoglobin (HbA1c) concentration of ≥48 mmol/mol (6.5%) and albuminuria. The primary study aimed to assess liraglutide’s effect on albuminuria and has been published.⁴ The present study was a pre-specified sub-study. Participants were randomly assigned (1:1) to liraglutide + standard therapy or placebo + standard therapy. After 12 weeks of treatment, participants underwent a 4-week washout period before crossing over to the other treatment group. The study was approved by the relevant authorities and conducted according to the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (NCT02545738).

A full description of methods used for the biomarker analyses can be found in Appendix S1.

2.1 | Statistical analysis

Power calculation was performed for the primary outcome, and a detailed statistical specification of the crossover design has been described previously.⁴ In brief, no carryover effect was detected for any of the biomarkers allowing paired-samples t-tests for treatment comparison. Change in endpoints was analysed as end of placebo vs end of liraglutide treatment. All biomarkers were analysed after log10-transformation. Except for norepinephrine, all endpoints were measured at all study visits. In order to use all measures, we additionally analysed and compared changes within each treatment period. Associations between changes in outcome variables and changes in potential explanatory variables were assessed using univariate and multivariate models. Two-sided P values < .05 were taken to indicate statistical significance.

3 | RESULTS

A total of 32 participants were included and treatment with liraglutide/placebo was initiated. Five participants withdrew from the study as a result of gastrointestinal side effects; all during liraglutide treatment; therefore, 27 participants completed the study.⁴

In brief, of the completing participants, 22 (81%) were men, the mean (standard deviation [s.d.]) age was 65 (7) years, mean (s.d.) diabetes duration was 15 (7) years, the mean HbA1c concentration was 7.7 (1.1) % (61 [12] mmol/mol), and 21 participants (78%) were treated with insulin.

The effects of treatment on HbA1c level, weight, 24-hour blood pressure, urinary albumin excretion rate (UAER) and 24-hour heart rate have been published.⁴ LDL cholesterol level was 0.21 mmol/L (95% confidence interval [CI] 0.03;0.40) lower after liraglutide treatment compared with placebo (P = .028), total cholesterol was

![Figure 1](image-url)

**FIGURE 1** Changes in TNF-α and MR-proANP. Individual effects of 12 weeks of liraglutide treatment on: A, plasma levels of TNF-α and B, plasma levels of MR-proANP. Distribution of both variables was skewed and log10-transformation was applied.
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**Table 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (IQR) Before placebo</th>
<th>Median (IQR) After placebo</th>
<th>% change for the placebo treatment periods (end vs end)</th>
<th>P value for comparison between treatment periods (end vs end)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, pg/mL</td>
<td>1.72 (1.30-2.04)</td>
<td>1.48 (1.17-1.89)</td>
<td>-22; 95% CI: -42; 11; P = .042</td>
<td>1.66 (1.36-2.19)</td>
</tr>
<tr>
<td>suPAR in plasma, ng/mL</td>
<td>3.9 (3.1-4.9)</td>
<td>4.0 (3.1-5.5)</td>
<td>-24; 95% CI: -46; 10; P = .19</td>
<td>4.0 (3.2-6.1)</td>
</tr>
<tr>
<td>MR-proADM, nmol/L</td>
<td>0.83 (0.64-1.08)</td>
<td>0.80 (0.64-0.95)</td>
<td>-4; 95% CI: -10; 2; P = .056</td>
<td>0.81 (0.64-1.12)</td>
</tr>
<tr>
<td>MR-proANP, pmol/L</td>
<td>98 (57-197.2)</td>
<td>76 (58-140.4)</td>
<td>-20; 95% CI: -41; 7; P = .053</td>
<td>91 (58-192.8)</td>
</tr>
<tr>
<td>Norepinephrine, ng/mL</td>
<td>7.6 (5.1-22.7)</td>
<td>7.6 (5.1-22.2)</td>
<td>-0; 95% CI: 0; 0; P = .78</td>
<td>2.8 (2.1-3.2)</td>
</tr>
</tbody>
</table>

Abbreviation: IQR, interquartile range.

3.1 | TNF-α

TNF-α level was 12% (95% CI 3: 20) lower after liraglutide treatment compared with placebo (P = .012; Figure 1). When analysing the effect within each treatment period, TNF-α was reduced by 12% (95% CI 1: 22; P = .042) from start to end of liraglutide treatment, and increased by 9% (95% CI -6; 21; P = .16) from start to end of placebo treatment (Table 1). Comparing the changes within the two treatment periods, TNF-α level was reduced by 19% (95% CI 4: 33), a significant reduction, during liraglutide treatment compared with placebo (P = .024). In univariate and multivariate models, no associations between change in TNF-α level and change in HbA1c, weight, 24-hour systolic blood pressure, UAER, 24-hour heart rate, LDL cholesterol or total cholesterol levels were observed (P ≥ .064).

3.2 | suPAR

No differences were observed in plasma or urinary suPAR at the end of liraglutide treatment compared with at the end of placebo treatment (P = .61 and P = .49, respectively; Table 1). When analysing the effect within each treatment period on plasma suPAR, no differences were observed during liraglutide or placebo treatment (P = .78 and P = .78, respectively). Similarly, no differences were observed in urinary suPAR level during liraglutide or placebo treatment (P = .19 and P = .58, respectively).

3.3 | MR-proADM

The MR-proADM level was 4% (95% CI 0: 8) lower after liraglutide treatment compared with placebo (P = .038). When analysing the effect within each treatment period, MR-proADM was reduced by 4% (95% CI -1; 7; P = .056) from the start to the end of liraglutide treatment, and unchanged (0% reduction [95% CI: -3; 4]; P = .89) from the start to the end of placebo treatment (Table 1). Comparing the changes within the two treatment periods, the MR-proADM level was reduced by 4% (95% CI 0; 8) during liraglutide treatment compared with placebo (P = .074).

In univariate models, change in MR-proADM was associated with change in weight (P = .022, R² = .18), but not with HbA1c, 24-hour systolic blood pressure, UAER, 24-hour heart rate or LDL cholesterol and total cholesterol level. In multivariate models, no associations were observed (P ≥ .11).

3.4 | MR-proANP

The MR-proANP level was 13% (95% CI 4: 21) lower after liraglutide treatment compared with placebo (P = .006). When analysing the effect within each treatment period, MR-proANP was reduced by 11% (95% CI 1: 20; P = .035) from the start to end of liraglutide treatment, and increased by 1% (95% CI -6; 8; P = .79) from the start to end of placebo treatment (Table 1). Comparing the changes within the two treatment periods, MR-proANP was reduced by 14% (95% CI 0.020:0.58) lower (P = .034), while there was no significant effect on triglyceride (P = .64) or HDL cholesterol levels (P = .12).
In univariate models, reduction in MR-proANP level was associated with an increased 24-hour heart rate (P < .001; $R^2 = 0.53$), but not with change in HbA1c, 24-hour systolic blood pressure, UAER or weight (P ≥ .38). In multivariate models, change in MR-proANP was associated with change in 24-hour heart rate (P = .001), but not with other variables (P ≥ .32).

3.5 | Copeptin and norepinephrine

No significant differences were observed when comparing copeptin or norepinephrine levels at the end of liraglutide treatment with those at the end of placebo treatment (P = .75 and P = .58, respectively; Table 1).

4 | DISCUSSION

In the present randomized, placebo-controlled, double-blind, cross-over trial, we investigated the effect of liraglutide treatment on cardiovascular risk biomarkers in a high-risk population with type 2 diabetes, and showed that liraglutide reduced TNF-α, MR-proADM and MR-proANP levels, independently of glucose-lowering, weight-lowering, antihypertensive or antiproteinuric effects.

The Liraglutide Effect and Action in Diabetes: Evaluation of cardiovascular outcome (LEADER) study showed that liraglutide treatment was superior to placebo in reducing cardiovascular events in 9340 patients with type 2 diabetes followed for 3.8 years; however, the delayed separation of the Kaplan-Meier curves in the LEADER study (>12 months for cardiovascular death and >18 months for all-cause mortality and hospitalization for heart failure) suggests that the reduction in cardiovascular risk may not be entirely attributable to the favourable differences in cardiometabolic factors such as HbA1c, systolic blood pressure, weight or albuminuria.

Studies have suggested that glucagon-like peptide-1 (GLP-1) analogues have a protective effect on the development of atherosclerosis, potentially mediated via the GLP-1 receptors expressed on endothelial cells, smooth muscle cells, and in monocytes/macrophages. Treatment with GLP-1 analogues can improve endothelial function, and liraglutide reduced intima-media thickness after 8 months of treatment in patients with type 2 diabetes.

We expand the literature on anti-inflammatory actions of liraglutide by showing beneficial effects on markers of endothelial dysfunction and inflammation, as reflected in reduced circulating TNF-α and MR-proADM levels. In a cohort of 200 people with type 2 diabetes, we recently showed that markers of inflammation and endothelial dysfunction, and in particular TNF-α, were associated with cardiovascular disease after 6 years of follow-up. MR-proADM has been shown to be associated with cardiovascular disease in a cohort of 1243 people with type 2 diabetes. The key finding of the LEADER study was a 13% reduction in the primary outcome, including a 22% reduction in cardiovascular death. The authors of that study suggested that liraglutide modifies progression of atherosclerotic disease, which we suggest is attributable to anti-inflammatory actions, together with glucose-lowering, antihypertensive, antiproteinuric, weight and lipid-lowering effects.

SuPAR has gained attention as a marker of inflammation and as a risk marker of cardiovascular disease in type 1 diabetes. We are the first to evaluate the impact of liraglutide on suPAR levels in people with type 2 diabetes and found no effect of treatment.

N-terminal pro-brain natriuretic peptide and MR-proANP are risk markers of cardiovascular disease in patients with type 2 diabetes. Also, ANP has been reported to mediate the antihypertensive effects of GLP-1 analogues in mice, where it was shown that GLP-1 receptor activation induced vasodilatation and natriuresis. By contrast, we previously found liraglutide to reduce MR-proANP after 7 weeks of treatment. After 1 year of liraglutide treatment, we observed reductions in 24-hour systolic blood pressure and in MR-proANP levels, indicating that it is unlikely that the antihypertensive effects of GLP-1 are driven by increased ANP level. In the present study, we confirm that liraglutide reduces MR-proANP levels, which might reflect reduced atrial and ventricular distention, and perhaps a prognostic benefit in heart failure, although this was not identified as significant in the LEADER study. We observed a powerful association between reduction in MR-proANP level and increase in 24-hour heart rate. The chronotropic effect of liraglutide has been suggested to be mediated through activation of the GLP-1 receptors on cells in the cardiovascular system, which may directly influence the expression of the cardiac hormone, potentially through changes in cardiac output. With regard to the changes in 24-hour heart rate, we measured norepinephrine to assess impact on the sympathetic nervous system as a potential explanation for the known heart rate increase observed during liraglutide treatment; however, no changes in norepinephrine level were observed. Copeptin is involved in multiple pathways and is considered a risk marker of cardiovascular disease in type 2 diabetes, but levels were unchanged during liraglutide treatment.

Strengths of the present study include the randomized crossover design and inclusion of a well-characterized high-risk population with type 2 diabetes. Limitations include the lack of an active comparator with glucose-lowering effects. Also, it cannot be determined in the present study whether the observed beneficial effects on the biomarkers can be translated into actual cardioprotection, but the findings may contribute to a better understanding of the LEADER study results. In addition, the potential confounding effect of weight changes may be limited, given the lack of association between changes in the biomarkers and changes in weight.

In conclusion, in the present randomized study in people with type 2 diabetes with albuminuria, liraglutide treatment was associated with reductions in circulating levels of cardiovascular risk biomarkers.

ACKNOWLEDGMENTS

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Conflict of interest

B. J. v. S. reports having given lectures for Novo Nordisk and BMS (all fees given to the Steno Diabetes Center), and has equity interest in Novo Nordisk. F. P. reports having received a research grant from Novartis and has received lecture fees from Novartis, Eli Lilly, Boehringer Ingelheim, Novo Nordisk, BMS, and AstraZeneca. S. R. has equity interest in Novo Nordisk. J. F. has given lectures for Astra Zeneca, BMS, Novo Nordisk, and has served as a consultant for Novo Nordisk and Novartis. T. W. H. has equity interest in Novo Nordisk. P. R. reports having given lectures for Astra Zeneca, BMS, Novo Nordisk, and Boehringer Ingelheim, and has served as a consultant for AbbVie, Astra Zeneca, BMS, Eli Lilly, Boehringer Ingelheim, Astellas, Janssen, and Novo Nordisk (all fees given to the Steno Diabetes Center), and has equity interest in Novo Nordisk.

Author contributions

B. J. v. S. conceived and designed the research, analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. S. R. conceived and designed the research, analysed and interpreted the data, handled funding and supervision, and made critical revision of the manuscript for key intellectual content. T. W. H. analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. J. F. analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. T. P. analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. J. E. O. analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. S. R. conceived and designed the research, analysed and interpreted the data, performed statistical analysis and wrote the manuscript. F. P. conceived and designed the research, analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. B. J. v. S. reports having given lectures for Astra Zeneca, BMS, Novo Nordisk, and Boehringer Ingelheim, and has served as a consultant for AbbVie, Astra Zeneca, BMS, Eli Lilly, Boehringer Ingelheim, Astellas, Janssen, and Novo Nordisk (all fees given to the Steno Diabetes Center), and has equity interest in Novo Nordisk.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

REFERENCES


Paper XI
The influence of pharmaceutically induced weight changes on estimates of renal function: A patient-level pooled analysis of seven randomised controlled trials of glucose lowering medication

Bernt Johan von Scholten a,⁎, David Dynnes Ørsted b, Anne Louise Svendsen b, Frederik Persson a, Peter Rossing a,c,d

a Steno Diabetes Center, Gentofte, Denmark
b Novo Nordisk, Denmark
c Aarhus University, Denmark
d University of Copenhagen, Denmark

Abstract

Background: Estimation of kidney function (eGFR) is essential in monitoring of patients with kidney disease. Estimates of kidney function based on serum creatinine are derived from cross-sectional studies. If body weight (BW) changes, this might affect creatinine and eGFR. The Cockcroft–Gault (CG) equation includes creatinine and BW, whereas the Modification of Diet in Renal Disease (MDRD) and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations only include creatinine.

Methods: Data were pooled from the six LEAD (Liraglutide Effect and Action in Diabetes) trials and the LIRA-DPP4 trial. The trials were conducted in patients with type 2 diabetes and of 26 weeks duration. We investigated changes in eGFR for patients treated with liraglutide, and for patients treated with glucose-lowering medications with less weight-reducing effects (insulin glargine, glimepiride, exenatide and rosiglitazone).

Results: We included 5100 patients (liraglutide n = 3173, comparator n = 1927). Mean (SD) CKD-EPI eGFR was 81.2 (20.6) ml/min/1.73 m² for liraglutide and 81.6 (20.3) ml/min/1.73 m² for comparator. For liraglutide, BW changed −1.9 (95% CI (−2.0; −1.8)) kg, for comparator BW changed 0.2 (95% CI (0.03; 0.3)) kg. Using regression modelling, a 10% BW decrease yielded no change in creatinine, MDRD eGFR or CKD-EPI eGFR for both liraglutide and comparator, but was associated with a 10.2% (−11.3%; −9.1%) decrease in CG eGFR for liraglutide, and a 10.6% (−12.0%; −9.1%) decrease for comparator.

Conclusions: A liraglutide-induced weight reduction of 1.9 kg was not associated with change in creatinine. Accordingly, there was no change in weight-independent estimates of GFR, whereas weight-dependent estimates were changed. The MDRD and CKD-EPI equations can be used in patients experiencing pharmaceutically induced weight reductions.

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1. Introduction

Estimation of glomerular filtration rate (eGFR) is essential in the diagnosis and monitoring of patients at risk of developing or with established kidney disease, and for correct dosage of drugs eliminated from the circulation by the kidneys. Estimation of GFR based on serum creatinine is most commonly used, since it has proven to be a reliable and inexpensive technique. The different eGFR equations based on serum creatinine are derived from cross-sectional studies. Skeletal muscle mass is the main determinant of creatinine generation/production with creatinine being the final catabolite of muscular energetic metabolism (Wyss & Kaddurah-Daouk, 2000). Hence, if body weight (BW) or body composition and in particular muscle mass change over time, and serum creatinine is also affected, this could influence estimates of renal function, without actual changes in true GFR. Whether these factors indeed influence eGFR depends on the applied equations, as for example the Cockcroft–Gault (CG) equation (Cockcroft & Gault, 1976) includes both creatinine and BW, whereas
the 4-variable Modification of Diet in Renal Disease (MDRD) (Levey et al., 1999) and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (Levey, Stevens, Schmid, et al., 2009) equations only include creatinine and as a result would not be influenced by changes in BW alone.

The glucagon like peptide 1 (GLP-1) analogue liraglutide is a glucose-lowering agent, approved for management of type 2 diabetes in doses up to 1.8 mg once-daily. Furthermore, liraglutide treatment has been associated with sustained weight reductions in patients with type 2 diabetes of up to 3.4 kg (Bode, 2012).

Our pre-specified aim of the present study was to investigate changes in eGFR based on CC, MDRD and CKD-EPI in patients with type 2 diabetes treated with liraglutide, and in patients treated with other glucose-lowering medications with less or no weight-reducing effects (insulin glargine, glimepiride, exenatide, rosiglitazone), with the assumption that true renal function is not affected by these agents. Our secondary aim was to determine the weight reduction associated with liraglutide treatment would decrease muscle mass and creatinine leading to different changes in eGFR depending on the applied equation.

2. Methods

Data for this patient-level pooled analysis were pooled from the LEAD (Liraglutide Effect and Action in Diabetes) clinical trials (Buse, Rosenstock, Sesti, et al., 2005; Garber, Henry, Ratner, et al., 2005; Marré, Shaw, Brandle, et al., 2009; Nauck, Frid, Hermansen, et al., 2009; Russell-Jones, Vaag, Schmitz, et al., 2009; Zinman, Gerich, Buse, et al., 2009) and from the LIRA-DPP4 trial (Pratley, Nauck, Bailey, et al., 2012), all investigating liraglutide for the treatment of type 2 diabetes. The LEAD programme consisted of six phase 3, multicentre, parallel-group, placebo and active-controlled trials and the LIRA-DPP4 trial was a multicentre, active-controlled, parallel-group trial. This allows comparison of estimates of renal function in patients on weight stable or weight reducing glucose lowering agents not assumed to affect renal function.

A total of 5100 patients with type 2 diabetes were included in this patient-level pooled analysis. 192 patients did not have serum creatinine measurements at baseline. Consistent with standards for drug development at the time, patients with elevated serum creatinine levels were excluded from the individual trials as follows: monotherapy study (Garber et al., 2005) (≥1.7 mg/dL) and combination therapy studies (Buse et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009) (males ≥1.4–1.5 mg/dL; females ≥1.3 mg/dL), whilst a CC eGFR below 50 mL/min was used as exclusion criterion in the LIRA-DPP4 trial (Pratley et al., 2012).

Three dosages of liraglutide, 0.6 mg daily, 1.2 mg daily and 1.8 mg daily, were compared with active comparator or placebo for efficacy and safety assessments. The active comparators were insulin glargine (Russell-Jones et al., 2009), glimepiride (sulfonylurea) (Garber et al., 2009), exenatide (GLP-1 receptor agonist) (Buse et al., 2009), rosiglitazone (glitazone) (Marre et al., 2009) and sitagliptin (DPP-4 inhibitor) (Pratley et al., 2012). In the LEAD studies including a placebo arm, placebo-treated patients also received background oral antidiabetic drug therapy. Clysacemic control, mean body weight, and systolic blood pressure were analysed to determine efficacy of liraglutide. Serum creatinine levels were compared between treatment arms for safety and tolerability assessment.

2.1. Estimation of GFR

For the CG eGFR the following equation was used:

\[ \text{CrCl} = \left(140 - \text{age}\right) \times \left(\text{weight in kg}\right) \times 0.85 \text{ if female} \times 0.72 \text{ if male} \]


For the MDRD eGFR the following equation was used:

\[ \text{eGFR} = 175 \times \left(\text{SCr}\right)^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \]

(Levey et al., 1999).

For the CKD-EPI the following equations were used (Levey et al., 2009):

\[
\begin{align*}
\text{Scr} & \text{ Serum Creatinine, } S_c \text{ (mg/dL)} \\
\text{Male} \geq 5.9 & \text{ GFR} = 141 \times (S_c)^{-1.094} \times (1.210)^\text{age} \times (0.85 \text{ if female}) \\
\text{Female} \geq 5.7 & \text{ GFR} = 141 \times (S_c)^{-1.094} \times (1.210)^\text{age} \times (0.85 \text{ if female}) \\
\text{Male} \leq 5.9 & \text{ GFR} = 141 \times (S_c)^{-1.073} \times (1.210)^\text{age} \times (0.85 \text{ if female}) \\
\text{Female} \leq 5.7 & \text{ GFR} = 141 \times (S_c)^{-1.073} \times (1.210)^\text{age} \times (0.85 \text{ if female})
\end{align*}
\]

2.2. Statistical analyses

All analyses used the full analysis population defined as patients exposed to at least one dose of trial product. Missing data were imputed using last observation carried forward. Comparisons of mean at baseline with mean at end of trial were done using paired t-test. The association between change in body weight on the endpoints creatinine, CG eGFR, MDRD eGFR, and CKD-EPI eGFR was investigated using separate lognormal linear regression models for liraglutide and comparator. Each trial was modelled separately and then the pooled analysis was analysed using both a fixed effect and a random effect approach. In the fixed effects model the effect of body weight is assumed to be the same for all trials, whereas in the random effect model the effect is assumed to follow a distribution. Specifically, the log transformed relative change in the endpoint was modelled using country and previous treatment as fixed factors and the log transformed endpoint at baseline, the log transformed body weight at baseline, and the log transformed relative change from baseline in body weight as covariates. For the pooled model the fixed effects approach also included trial whereas the random effects approach also included the random effect of log transformed relative change in body weight by trial. All analyses were programmed and executed by the study statistician and were independently validated.

3. Results

This patient-level pooled analysis included 3173 patients treated with liraglutide (1.2 or 1.8 mg/d) and 1927 patients treated with comparators. Baseline characteristics for the two groups are shown in Table 1. Patients were 52.7% male in the liraglutide treated group and 54.7% in the comparator group. Baseline characteristics for the two groups are shown in Table 1. Patients were 52.7% male in the liraglutide treated group and 54.7% in the comparator group. Baseline characteristics for the two groups are shown in Table 1. Patients were 52.7% male in the liraglutide treated group and 54.7% in the comparator group. Baseline characteristics for the two groups are shown in Table 1. Patients were 52.7% male in the liraglutide treated group and 54.7% in the comparator group.

During 26 weeks of treatment, for liraglutide BW changed −1.9 kg (95% CI (−2.0 to −1.8), p < 0.0001) and for comparator BW changed 0.2 kg (95% CI (0.03–0.3)), p = 0.017).

3.1. Serum creatinine

Creatinine was unchanged in the liraglutide group (0.003 mg/dL (95% CI −0.005 to 0.01, p = 0.44)) and increased by 0.01 mg/dL (95%
CI (0.01-0.02; p = 0.0003) in the comparator group after 26 weeks of treatment. A 10% decrease in BW yielded no change in creatinine for both liraglutide and comparator (−0.8% (−2.0% to 0.5%); p = 0.22) for liraglutide and 0.9% (−0.8%; 2.6%; p = 0.32) for comparator (Fig. 1A).

3.2. CG eGFR

CG eGFR changed by 3.5 ml/min (95% CI (−4.1 to −2.9), p < 0.0001) in the liraglutide group and by −1.0 ml/min (95% CI (−1.9 to −0.2), p = 0.01) in the comparator group. A 10% decrease in BW was associated with a −10.2% (−11.3%; 9.1%) (p < 0.0001) change in CG eGFR for liraglutide and a −10.6% (−12.0%; −9.1%) (p < 0.0001) change for comparator (Fig. 1B).

3.3. MDRD eGFR

MDRD eGFR was changed by −0.7 ml/min (95% CI (−1.2 to −0.2), p = 0.006) ml/min/1.73 m² in the liraglutide group and by −1.0 ml/min/1.73 m² (95% CI (−1.7 to −0.4), p = 0.002) in the comparator group. A 10% decrease in BW corresponded to no change in MDRD eGFR for both liraglutide and comparator (−0.3% (−1.7%; 1.1%); p = 0.08) for liraglutide and −0.7% (−2.6%; 1.2%); p = 0.44) for comparator (Fig. 1C).

3.4. CKD-EPI eGFR

CKD-EPI eGFR changed by −0.5 ml/min/1.73 m² (95% CI (−1.0 to −0.1, p = 0.01) in the liraglutide group and by −0.8 ml/min/1.73 m² (95% CI (−1.4 to −0.3), p = 0.002) in the comparator group. A 10% decrease in BW corresponded to no change in CKD-EPI eGFR for both liraglutide and comparator (−0.3% (−1.8%; 1.1%) for liraglutide (p = 0.65) and −0.5% (−2.4%; 1.5%) for comparator (p = 0.61)) (Fig. 1D).

4. Discussion

In a patient-level pooled analysis including seven randomised clinical trials with 5100 patients with type 2 diabetes and preserved/normal kidney function, we found that a mean weight reduction of 1.9 kg in patients treated with the GLP-1 receptor analogue liraglutide was not associated with changes in serum creatinine. Accordingly, there was no change in weight independent estimates of GFR (MDRD, CKD-EPI), whereas weight dependent estimates (CG) were influenced. Thus, the novel eGFR equations (MDRD and CKD-EPI) can be applied in patients experiencing a clinically significant pharmacologically induced weight reduction.

In clinical practice measuring plasma creatinine and estimating renal function is fundamental for diagnosis and monitoring of patients with kidney disease. In addition, it is frequently used for correct dosage of drugs, primarily for safety reasons. Moreover, it is essential in selection of novel/alternative treatment options not yet approved for renal impaired patients. Many equations based on serum creatinine have been suggested for eGFR and especially MDRD and CKD-EPI have been proved to be reliable, compared to accurately measured GFR. Measuring GFR by insulin-clearance (Berger, Farber, & Earle, 1948), chromium-EDTA clearance (Brochner-Mortensen, 1972) or inohed clearance (Kruizien, Back, Nilsson-Ehle, & Nilsson-Ehle, 1984) is considered the “gold standard” of GFR. However, it is time consuming (typically a four hour examination) and expensive, and hence not feasible as a routine measurement in clinical practice. Health professionals therefore must rely on creatinine-based kidney function equations for clinical decisions, although plasma creatinine is not kidney specific and is often influenced and changed due to non-kidney related diseases and disorders. In patients with type 2 diabetes weight reductions are often desired and due to the introduction of treatment options with weight reducing effects, more patients are experiencing pharmacologically induced weight reductions. Depending on the degree of change in muscle mass associated with this, plasma creatinine and eGFR may be affected.

Studies investigating the effect of liraglutide on weight by DXA assessments have demonstrated liraglutide treatment to be associat-ed with significant reductions in both total body fat mass and total lean body tissue mass when compared to glimepiride treatment. The authors concluded that in general, liraglutide treatment reduced fat mass more than lean tissue mass (Jendle, Nauck, Matthews, et al., 2009). Since muscle mass is part of lean body tissue mass, the data suggest that liraglutide treatment is associated with changes in muscle mass. However, in a subgroup analysis, change in limb tissue mass, a surrogate for skeletal muscle mass, was not statistically different between treatment arms (Jendle et al., 2009).

Our data suggest that the changes in muscle mass associated with the mean weight reductions are limited, or at least not sufficient to yield changes in serum creatinine. In a different clinical setting, in patients undergoing gastric by-pass surgery and subsequently experiencing large weight reductions, muscle mass may be affected to a higher degree. In a study of 37 patients, a mean weight reduction of 37 kg 6 months post-operatively was associated with a statistically significant reduction in creatinine from 0.83 mg/dL to 0.72 mg/dL, and as a result MDRD eGFR was increased and CG eGFR was decreased (Getty, Hamdallah, Shamseddine, et al., 2012). A prospective cohort study found that accurately measured GFR, determined using renal inohedamate clearance, decreased in a cohort of women during the first year after bariatric surgery. Importantly, serum creatinine and creatinine-based eGFR did not detect this change in kidney function because of a large decrease in creatinine generation. Preoperatively, the CKD-EPI equation significantly underestimated GFR; postoperatively, it overestimated GFR when patients lost weight and muscle mass (Lieskje et al., 2014). These data illustrate our original hypothesis that the change in muscle mass in relation to weight reduction is important for the interpretation of estimations of GFR and that serum creatinine based eGFR should be carefully evaluated in patients that have experienced a large weight reduction. Our data on the other hand highlights that in type 2 diabetes patients with smaller, however clinically significant, weight reductions, the MDRD and CKD-EPI equations are unaffected and can be used. The magnitude of a weight loss associated with sufficient impact on muscle mass reduction to affect plasma creatinine and eGFR cannot be determined by the present patient-level pooled analysis. Additional studies aiming for larger pharmacologically induced weight reductions are warranted in order to answer this.

Table 1

Baseline characteristics of 5100 patients with type 2 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>Liraglutide group</th>
<th>Comparator group</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 3175)</td>
<td>(n = 1927)</td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>1671 (52.7)</td>
<td>1054 (54.7)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.7 (10.0)</td>
<td>56.0 (10.0)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>6.4 (0.1-40.3)</td>
<td>6.5 (2.4-43.5)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.5 (18.9)</td>
<td>89.6 (18.8)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.3 (12)</td>
<td>7.8 (13)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>56 (15)</td>
<td>62 (14)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>129.0 (15.1)</td>
<td>131.6 (15.7)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.85 (0.21)</td>
<td>0.85 (0.21)</td>
</tr>
<tr>
<td>MDRD eGFR (ml/min/1.73 m²)</td>
<td>87.2 (31.2)</td>
<td>87.9 (21.5)</td>
</tr>
<tr>
<td>CKD-EPI eGFR (ml/min/1.73 m²)</td>
<td>81.2 (20.6)</td>
<td>81.6 (20.3)</td>
</tr>
<tr>
<td>CG eGFR (ml/min)</td>
<td>117.3 (38.7)</td>
<td>120.8 (41.6)</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD), median (range) or number of patients (%).
question. In contrast, estimates of renal function based on CG which includes weight and creatinine in the equation predicted a decline in renal function as weight was reduced and creatinine unchanged, in patients treated with liraglutide.

4.1. Strengths and limitations

Our study was limited to studies performed by Novo Nordisk, in a clinical development programme, providing access to individual

A) Creatinine

B) CG eGFR

Fig. 1. A) Creatinine. Percent change in creatinine for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side. B) CG eGFR. Percent change in CG eGFR for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side. C) MDRD eGFR. Percent change in MDRD eGFR for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side. D) CKD-EPI eGFR. Percent change in CKD-EPI eGFR for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side.
patient level data. It is possible that including smaller liraglutide studies and other studies using GLP-1 agonists (i.e., exenatide) could provide results of broader applicability, but the current analysis included patient level data on 5100 individuals, which would not have been possible in a study level analysis. Thus the results of our study must be interpreted in the context of the included population in the LEAD-trials and the LIRA-DPP4 trial. Another limitation is that GFR is not measured with a precise method such as plasma clearance of an exogenous marker, and thus the true GFR and its change are not known. Therefore, this study cannot assess if the study medication has any effect on GFR. Another limitation of our study is the exclusion of patients with elevated serum creatinine, which was consistent with standards for drug development at the time. Measurement of serum creatinine itself is only a surrogate of glomerular filtration rate because of the influences of age, sex, race, and muscle mass on creatinine levels. As the equations are originally developed with

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**C) MDRD eGFR**

<table>
<thead>
<tr>
<th>Comparator</th>
<th>Liraglutide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random</td>
<td></td>
</tr>
<tr>
<td>Pool</td>
<td></td>
</tr>
<tr>
<td>Sitagliptin</td>
<td></td>
</tr>
<tr>
<td>Exenatide</td>
<td></td>
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<tr>
<td>Insulin glargine</td>
<td></td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td></td>
</tr>
<tr>
<td>Glimepiride</td>
<td></td>
</tr>
<tr>
<td>Metformin + glimepiride</td>
<td></td>
</tr>
<tr>
<td>Glimepiride + rosiglitazone</td>
<td></td>
</tr>
</tbody>
</table>

Percent change in MDRD eGFR for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side.

**D) CKD-EPI eGFR**

<table>
<thead>
<tr>
<th>Comparator</th>
<th>Liraglutide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random</td>
<td></td>
</tr>
<tr>
<td>Pool</td>
<td></td>
</tr>
<tr>
<td>Sitagliptin</td>
<td></td>
</tr>
<tr>
<td>Exenatide</td>
<td></td>
</tr>
<tr>
<td>Insulin glargine</td>
<td></td>
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<tr>
<td>Rosiglitazone</td>
<td></td>
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<tr>
<td>Glimepiride</td>
<td></td>
</tr>
<tr>
<td>Metformin + glimepiride</td>
<td></td>
</tr>
<tr>
<td>Glimepiride + rosiglitazone</td>
<td></td>
</tr>
</tbody>
</table>

Percent change in CKD-EPI eGFR for a 10% body weight decrease in a random effects and pooled model including all 7 trials and for each trial separately. The comparator(s) for liraglutide used in each trial is listed on the left hand side.

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Fig. 1. (continued)
creatinine measurements performed with different methods, it is difficult to compare estimated GFR based on the different methods, but changes over time in creatinine, as applied in this study, would not be dependent on the method. Therefore the changes in estimates can be compared.

Strengths of our study include the pooled-analysis of individual data from a large patient population included in randomised clinical trials conducted according to GCP, including central lab assessment of creatinine levels.

5. Conclusions

In a pooled analysis of 5100 patients with type 2 diabetes and normal renal function, weight reduction of 1.9 kg was not associated with changes in serum creatinine. Accordingly, there was no change in weight independent estimates of GFR (MDRD and CKD-EPI), whereas weight dependent estimates (CG) were changed. The novel eGFR equations (MDRD and CKD-EPI) can be used in patients experiencing clinically significant pharmacologically induced weight reduction, whilst CG eGFR should be carefully interpreted.

Contributor statements

B.v.S. conceived and designed the research, and drafted the manuscript. D.D.O. acquired the data, and made critical revision of the manuscript for key intellectual content. A.L.S. acquired the data, and made critical revision of the manuscript. D.D.Ø. acquired the data, and made critical revision of the manuscript. P.R. conceived and designed the research, and drafted the manuscript. The results presented in this paper have not been published previously in whole or part, except in abstract form.

References


Paper XII
Effect of large weight reductions on measured and estimated kidney function

Bernt Johan von Scholten 1*, Frederik Persson 1, Maria S. Svane 2, Tine W. Hansen 1, Sten Madsbad 2 and Peter Rossing 1,3,4

Abstract

Background: When patients experience large weight loss, muscle mass may be affected followed by changes in plasma creatinine (pCr). The MDRD and CKD-EPI equations for estimated GFR (eGFR) include pCr. We hypothesised that a large weight loss reduces muscle mass and pCr causing increase in eGFR (creatinine-based equations), whereas measured GFR (mGFR) and cystatin C-based eGFR would be unaffected if adjusted for body surface area.

Methods: Prospective, intervention study including 19 patients. All attended a baseline visit before gastric bypass surgery followed by a visit six months post-surgery. mGFR was assessed during four hours plasma 51 Cr-EDTA clearance. GFR was estimated by four equations (MDRD, CKD-EPI-pCr, CKD-EPI-cysC and CKD-EPI-pCr-cysC). DXA-scans were performed at baseline and six months post-surgery to measure changes in lean limb mass, as a surrogate for muscle mass.

Results: Patients were (mean ± SD) 40.0 ± 9.3 years, 14 (74%) were female and 5 (26%) had type 2 diabetes, baseline weight was 128 ± 19 kg, body mass index 41 ± 6 kg/m² and absolute mGFR 122 ± 24 ml/min. Six months post-surgery weight loss was 27 (95% CI: 23; 30) kg, mGFR decreased by 9 (−17; −2) from 122 ± 24 to 113 ± 21 ml/min (p = 0.024), but corrected for current body surface area (BSA) mGFR was unchanged by 2 (−5; 9) ml/min/1.73 m² (p = 0.52). CKD-EPI-pCr increased by 12 (6; 17) and MDRD by 13 (8; 18) (p < 0.001 for both), while CKD-EPI-cysC was unchanged by 2 (−15; 4) ml/min/1.73 m² (p = 0.51). Lean limb mass was reduced by 3.5 (−4.4; −2.6; p < 0.001) kg and change in lean limb mass correlated with change in plasma creatinine (R² = 0.28, p = 0.032).

Conclusions: Major weight reductions are associated with a reduction in absolute mGFR, which may reflect resolution of glomerular hyperfiltration, while mGFR adjusted for body surface area was unchanged. Estimates of GFR based on creatinine overestimate renal function likely due to changes in muscle mass, whereas cystatin C based estimates are unaffected.

Trial registration: ClinicalTrials.gov, NCT02138565. Date of registration: March 24, 2014.

Keywords: Glomerular filtration rate, Bariatric surgery, Creatinine, Muscle mass, Cystatin C, DXA scan
Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [3] equations include plasma creatinine and would not be affected by body weight changes alone. Cystatin C is a filtration marker that is less influenced by changes in muscle mass and may be a more suitable marker of renal function in subjects experiencing fast and large weight reductions [4]. In this prospective intervention study, we investigated the effect of a large weight loss (after Roux-en-Y gastric bypass surgery (RYGB)) on measured GFR (mGFR) \( \left( { }^{51} \text{Cr}-\text{EDTA plasma clearance} \right) \) and on estimated GFR (using both plasma creatinine and cystatin C). Dual energy X-ray absorptiometry (DXA)-scans were performed before and after RYGB to estimate changes in skeletal muscle mass.

We hypothesised that a large weight loss reduces muscle mass (lean limb mass) and plasma creatinine leading to increases in eGFR (creatinine-based equations), whereas mGFR and cystatin C-based eGFR would be unaffected when adjusted for the change in body surface area (BSA).

**Methods**

**Participants and study design**

This prospective, open-label intervention study included 23 obese patients all scheduled for RYGB at Hvidovre University Hospital, Denmark. Three patients never had the surgery performed and one patient declined to participate in the post-surgery visit. Therefore, a total of 19 patients completed the study. Type 2 diabetes was diagnosed according to the WHO criteria. Patients were recruited from March 2014 and the study was completed in June 2016.

The study design is illustrated in Fig. 1. Patients attended the baseline visit within two weeks prior to the scheduled RYGB and the follow-up visit was performed six months (± two weeks) after RYGB.

The study protocol was approved by the regional ethics committee and was conducted according to the Declaration of Helsinki. All patients gave written informed consent before any study procedure was initiated. The study is registered at ClinicalTrials.gov with identifier NCT02138565. The two study-related visits were conducted at Steno Diabetes Center, Gentofte, Denmark, and the RYGB was performed at Hvidovre University Hospital, Hvidovre, Denmark as described previously [5].

The primary aim was to assess the effect of a large weight reduction on measured GFR \( \left( { }^{51} \text{Cr}-\text{EDTA plasma clearance} \right) \) and on estimated GFR (applying different equations based on plasma creatinine or cystatin C). Secondly, we assessed the effect on body composition (determined by DXA-scan) in order to relate these changes with changes in renal outcome measures.

**Outcome measurements**

Renal function (mGFR) and extracellular volume were assessed during four hours measurement of plasma \( { }^{51} \text{Cr}-\text{EDTA clearance} \) by standard methods [6]. \( { }^{51} \text{Cr}-\text{EDTA} \) was performed within two weeks prior to RYGB and six months (± two weeks) after surgery.

For the present study, mGFR was represented by two approaches: 1) Absolute mGFR, where mGFR was presented as the raw mGFR (ml/min) and 2) mGFR corrected for concurrent body surface area (BSA): BSA-corrected mGFR (ml/min/1.73 m\(^2\)).

For the estimation of BSA we used the Du Bois & Du Bois formula [7].

1DMS-traceable plasma creatinine was analysed using the enzymatic Creatinine Plus method (Vitros 5600, Ortho Clinical Diagnostics, Illkirch Cedex, France). Standardized plasma cystatin C was analyzed on the Cobas 8000® (Roche Diagnostics, Indianapolis, IN).


![Fig. 1 Study design](image-url)
DXA measurements of body composition were performed in all patients at baseline and six months after surgery using a Hologic Discovery A, series 82800-A (Hologic, Bedford, MA, USA).

The following parameters were obtained: Lean body mass (in kg), lean limb mass (in kg), fat mass (in kg) and fat mass (in percent). Lean limb mass is considered to be the best surrogate measure of skeletal muscle mass and was calculated as the total non-bone and non-fat lean mass of the extremities: Lean mass of left arm + lean mass of right arm + lean mass left leg + lean mass of right leg [9].

Urinary albumin-to-creatinine ratio (UACR) was calculated as the geometric mean of three consecutive morning spot urine samples performed at baseline and six months after surgery.

Twenty-four-hour blood pressure was performed at baseline and six months after surgery using BPro (HealthStats, Singapore), a watch-like device that captures radial pulse wave reflection and calculates brachial 24–h BP. BPro has been validated in people with diabetes and meets the European Society of Hypertension and Association for the Advancement of Medical Instrumentation standards [10, 11]. Mean of systolic and diastolic blood pressure and heart rate was calculated using all readings during the 24 h. Only 24-h blood pressure recordings with ≥20 readings during daytime and ≥7 during night-time were used for analysis. One recording was incomplete and was discarded for the analysis.

The urinary albumin concentration of the morning spot samples was analysed using a turbidimetric immunoassay (Vitros 5600, Ortho Clinical Diagnostics, Illkirch Cedex, France).

Statistical analysis
Outcome measures are presented as mean (SD) and skewed data (UACR) are shown as geometric mean (IQR), and analysed after log-transformation.

The change in outcome measures was analysed from levels at baseline to six months after surgery and compared using the paired samples t-tests.

Associations between changes in outcome measures were assessed by linear regression models. The proportion of the variability explained by the models is presented as the $R^2$. Due to the exploratory nature of the study, no power calculation was performed, however based on a previous related study, we anticipated that a total of 20 subjects would be sufficient [12].

Two-sided $p$-values < 0.05 were considered statistical significant. Statistical analysis was performed using IBM SPSS 23.0 (IBM Amonk NY, USA).

Results
Baseline demographics
Patients were (mean ± SD) 40 ± 9 years, 14 (74%) were female and 5 (26%) had type 2 diabetes. Baseline weight was 128 ± 19 kg, body mass index 41 ± 6 kg/m², absolute mGFR 122 ± 24 ml/min and CKD-EPI-pCr eGFR 93 ± 18 ml/min/1.73 m². Six patients received antihypertensive treatment at baseline and no changes were prescribed during the course of the study.

Renal outcome measures
Six months after RYGB, absolute GFR was reduced by mean 9 (95% confidence interval: 2; 17; $p = 0.021$) ml/min, while BSA-corrected GFR was unchanged by 2 (−5; 9; $p = 0.52$) ml/min/1.73 m² (Table 1).

Plasma creatinine was reduced by 9 (5; 14; $p < 0.001$) μmol/l, and plasma cystatin C was unchanged by 0.02 (−0.04; 0.07; $p = 0.61$) six months after RYGB. MDRD eGFR increased by 13 (8; 18; $p < 0.001$) ml/min/1.73 m², CKD-EPI-pCr eGFR increased by 12 (6; 17; $p < 0.001$) ml/min/1.73 m², and CKD-EPI-pCr-cysC eGFR was unchanged by 5 (−0.5; 10; $p = 0.074$) ml/min/1.73 m². CKD-EPI-cysC eGFR was unchanged by 2 (−8; 4; $p = 0.51$) ml/min/1.73 m². Plasma urea was reduced by 0.7 (−1.3; −0.02; $p = 0.043$) mmol/l and UACR was reduced by 23 (−35; −9; $p = 0.005$) %, while extracellular volume was unchanged ($p = 0.99$) (Table 1).

Weight loss and body composition outcome measures
Six months after RYGB weight was reduced by mean 27 (23; 30; $p < 0.001$) kg or 21 (18; 24; $p < 0.001$) % and body mass index was reduced by 8 (−10; −7; $p < 0.001$) kg/m². Lean body mass was reduced by 3.5 kg (−4.4; −2.6; $p < 0.001$) kg, lean limb mass was reduced by 6.5 (−7.9; −5.0; $p < 0.001$) kg, and fat mass was reduced by 20 (−23; −18; $p < 0.001$) kg (Table 2).

Linear correlations
At baseline, BSA-corrected mGFR correlated significantly with plasma creatinine and with all estimates of GFR ($R^2 ≥ 0.25$, $p ≤ 0.029$), except for MDRD ($p = 0.093$). After RYGB, BSA-corrected mGFR correlated with plasma creatinine and all estimates of GFR ($R^2 ≥ 0.34$, $p ≤ 0.011$). Change in BSA-corrected mGFR correlated with change in plasma creatinine and MDRD eGFR ($R^2 = 0.24$, $p ≤ 0.048$) and not with changes in other renal measures.

Lean limb mass correlated significantly with plasma creatinine at baseline ($R^2 = 0.28$, $p = 0.025$) and after RYGB ($R^2 = 0.37$, $p = 0.010$). Change in lean limb mass correlated with change in plasma creatinine ($R^2 = 0.28$, $p = 0.032$) and with change in UACR ($R^2 = 0.28$, $p = 0.034$).

Lean body mass correlated significantly with plasma creatinine at baseline ($R^2 = 0.32$, $p = 0.012$) and after RYGB ($R^2 = 0.42$, $p = 0.004$). Change in lean body mass
correlated with change in UACR ($R^2 = 0.38$, $p = 0.011$), and not with change in plasma creatinine or other renal measures ($p \geq 0.38$).

Discussion

In this prospective intervention study investigating the effects of a fast and large (mean 27 kg) weight loss, obtained by Roux-en-Y gastric bypass surgery, we found a reduction in absolute mGFR, while BSA-corrected mGFR was unchanged. Plasma creatinine was reduced causing increases in creatinine-based eGFR (MDRD and CKD-EPI), while cystatin C-based eGFR was unchanged (all adjusted for BSA). Lean limb mass, a surrogate measure of skeletal muscle mass, was reduced by mean 3.5 kg and might explain the reduction in plasma creatinine, since we found a significant correlation between these changes.

Monitoring GFR is important for diagnosis and monitoring of patients with kidney disease. Furthermore, it is often used for dosage of drugs, mainly for safety reasons. Numerous equations based on plasma creatinine have been suggested for estimation of GFR and when compared to accurately measured GFR, particularly MDRD and CKD-EPI have been proven to be reliable. Of note, creatinine-based eGFR equations have not been validated in morbidly obese adults or in patients with change in body composition after RYGB [13]. Cystatin C is less affected by muscle mass and diet than is creatinine, while reports have found an association between cystatin C concentrations and body weight and fat mass [14–17]. In our study, cystatin C levels tended to be associated with body weight, but were not associated with fat mass, fat percent or body mass index (data not shown). Nonetheless, it has been anticipated that cystatin C would provide a more accurate estimate of GFR than creatinine [18]. Measuring GFR by inulin-clearance [19], chromium-EDTA clearance [6] or iohexol clearance [20] is considered the “gold standard” of GFR. However, it is expensive and time consuming (usually a four hour examination), and therefore not realistic as a routine measurement in clinical practice or in large-scale studies. Whether mGFR should be presented absolute or

Table 1 Renal outcome measures

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>6 months post-surgery</th>
<th>Change from baseline (95% CI)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGFR ($^{51}$Cr-EDTA), ml/min (absolute)</td>
<td>122 (24)</td>
<td>113 (21)</td>
<td>$-9$ ($-17$; $-2$)</td>
<td>0.027</td>
</tr>
<tr>
<td>mGFR ($^{51}$Cr-EDTA), ml/min/1.73 m$^2$ (corrected for body surface area)</td>
<td>88 (17)</td>
<td>90 (16)</td>
<td>$2$ ($-5$; $9$)</td>
<td>0.52</td>
</tr>
<tr>
<td>Body surface area, m$^2$</td>
<td>2.38 (0.22)</td>
<td>2.14 (0.23)</td>
<td>$0.24$ ($0.20$; $0.28$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/l</td>
<td>76 (18)</td>
<td>66 (12)</td>
<td>$9$ ($-14$; $-5$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Plasma cystatin C, mg/l</td>
<td>0.94 (0.19)</td>
<td>0.96 (0.19)</td>
<td>$0.02$ ($-0.04$; $0.07$)</td>
<td>0.61</td>
</tr>
<tr>
<td>CKD-EPI-sCr eGFR, ml/min/1.73 m$^2$</td>
<td>93 (18)</td>
<td>105 (15)</td>
<td>$12$ ($6$; $17$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>MDRD eGFR, ml/min/1.73 m$^2$</td>
<td>84 (21)</td>
<td>97 (22)</td>
<td>$13$ ($8$; $18$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>CKD-EPI-cysC eGFR, ml/min/1.73 m$^2$</td>
<td>89 (19)</td>
<td>87 (23)</td>
<td>$-2$ ($-8$; $4$)</td>
<td>0.51</td>
</tr>
<tr>
<td>24-h systolic blood pressure, mmHg</td>
<td>122 (14)</td>
<td>124 (13)</td>
<td>$2$ ($-5$; $10$)</td>
<td>0.56</td>
</tr>
<tr>
<td>24-h diastolic blood pressure, mmHg</td>
<td>82 (10)</td>
<td>79 (11)</td>
<td>$-3$ ($-9$; $4$)</td>
<td>0.41</td>
</tr>
<tr>
<td>Urinary albumin-to-creatinine ratio, mg/g</td>
<td>6.3 (2.7–8.1)</td>
<td>4.8 (2.1–5.2)</td>
<td>$-23$ ($-35$; $-9$ %)</td>
<td>0.005</td>
</tr>
<tr>
<td>Extracellular volume, l</td>
<td>204 (5.9)</td>
<td>204 (6.3)</td>
<td>$0$ ($-2.5$; $2.6$)</td>
<td>0.99</td>
</tr>
<tr>
<td>Plasma urea, mmol/l</td>
<td>5.0 (1.5)</td>
<td>4.3 (1.1)</td>
<td>$-0.7$ ($-1.3$; $-0.02$)</td>
<td>0.047</td>
</tr>
<tr>
<td>Plasma calcium, mmol/l</td>
<td>1.28 (0.04)</td>
<td>1.28 (0.03)</td>
<td>$0$ ($-0.02$; $0.02$)</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Values represent mean (SD) or geometric mean (IQR).

GFR: glomerular filtration rate.

Table 2 DXA outcome measures

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>6 months post-surgery</th>
<th>Change from baseline (95% CI)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean body mass, kg</td>
<td>66.2 (12.2)</td>
<td>59.7 (13.2)</td>
<td>$-6.5$ ($-7.9$; $-5.0$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Lean body mass + bone mineral content, kg</td>
<td>69.1 (12.4)</td>
<td>62.6 (13.3)</td>
<td>$-6.5$ ($-7.9$; $-5.0$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Lean limb mass, kg</td>
<td>30.6 (6.5)</td>
<td>27.1 (6.6)</td>
<td>$-3.5$ ($-4.4$; $-2.6$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>59.0 (12.0)</td>
<td>38.5 (9.9)</td>
<td>$-20.4$ ($-23.1$; $-17.7$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Fat mass, %</td>
<td>46.1 (5.8)</td>
<td>38.1 (6.2)</td>
<td>$-7.9$ ($-9.1$; $-6.7$)</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>128 (18)</td>
<td>101 (18)</td>
<td>$-27$ ($-30$; $-23$)</td>
<td>$&lt;$0.001</td>
</tr>
</tbody>
</table>

Values represent mean (SD).
demonstrated that a pharmaceutically induced weight loss was overestimated due to the reduction in body weight and extracellular volume was reduced. However GFR indexed with extracellular mass [12]. Of note, these studies did not control for renal function and body composition after RYGB. In order to resolve whether bariatric surgery is inducing function in obese subjects and after weight changes are debated but still indeterminate. The purpose of the present study was to obtain a better understanding of how a large weight loss influence measured and estimated kidney function. Absolute mGFR decreased significantly, while creatinine-based equations 

Strengths of this study include the prospective design and the use of robust methods to determine changes in renal function and body composition after RYGB. Limitations include the small sample size and lack of a non-surgical control group with a comparable weight

BSA-corrected (expressed as per 1.73 m$^2$) in the setting of obesity is still unclear and may depend on the situation [13]. However, studies have indeed questioned the use of BSA-correction and concluded that data obtained for GFR indexed by BSA should either be avoided or interpreted with caution, especially in obese subjects [21, 22]. Due to the limitations of BSA-correction, the extracellular volume has been proposed as a better parameter for body size adjustment of GFR than BSA [23]. In our study, the extracellular volume was essentially unchanged six months after RYGB, hence mGFR adjusted for extracellular volume was reduced. However GFR indexed with extracellular mass was unchanged. Of note, these studies did not control for renal function and body composition after RYGB. In order to resolve whether bariatric surgery is inducing function in obese subjects and after weight changes are debated but still indeterminate. The purpose of the present study was to obtain a better understanding of how a large weight loss influence measured and estimated kidney function. Absolute mGFR decreased significantly, while creatinine-based equations 

Other studies have demonstrated similar results. In a study of 37 patients, a weight loss of 37 kg six months after surgery was associated with a significant reduction in mean creatinine, and accordingly an increase in muscle mass. We found absolute mGFR and creatinine-based eGFR was unchanged, while creatinine-based eGFR was increased after a weight reduction of mean 27 kg. By applying robust methods for determination of body composition, we were able to demonstrate that changes in muscle mass correlated with changes in plasma creatinine. This suggests that for monitoring changes in renal function over time in patients experiencing a large weight loss, cystatin C-based estimates of GFR may be more useful. Other studies have demonstrated similar results. In a study of 37 patients, a weight loss of 37 kg six months after surgery was associated with a significant reduction in mean creatinine, and accordingly an increase in MDRD, while Cockcroft Gault eGFR (including both creatinine and body weight) was decreased [25]. A small prospective study demonstrated that BSA-corrected mGFR, determined using clearance of iothalamate, was reduced in a cohort of 11 women during the first year after bariatric surgery. Notably, serum creatinine and creatinine-based eGFR did not identify this change in renal function, which was explained by a large reduction in creatinine production. Preoperatively, the CKD-EPI equation underestimated mGFR; postoperatively, mGFR was overestimated due to the reduction in body weight and muscle mass [12]. Of note, these studies did not measure actual changes in body composition. In a recent pooled analysis including more than 5000 patients, we assessed whether a pharmacologically induced weight loss was associated with changes in plasma creatinine. We demonstrated that a “stable” weight reduction of mean 1.9 kg was not associated with a change in plasma creatinine and concluded, that in patients experiencing a smaller weight reduction, creatinine-based equations (MDRD and CKD-EPI) are unaffected and can be applied [26]. The extent and rate of a weight reduction associated with enough impact on skeletal muscle mass reduction to affect levels of creatinine and eGFR is currently unknown and cannot be determined by the present study. Depending on the magnitude of the weight reductions, a non-creatinine-based equation (e.g. cystatin C) should be considered for these studies, in order to obtain reliable estimates of kidney function. Our present study expands on previous studies investigating the effects of bariatric surgery on mGFR. In studies examining mGFR in patients with normal or supranormal kidney function, absolute mGFR decreased significantly, while the BSA-corrected mGFR was unchanged one year after surgery [13, 27]. In the present study, we can confirm these findings and in a sub-analysis of subjects with hyperfiltration (baseline mGFR > 130 ml/min, n = 5) mGFR was significantly reduced by 24 ml/min (data not shown). This illustrates that the GFR-lowering effect of bariatric surgery is more pronounced in subjects with supranormal baseline levels of mGFR. It has been suggested that the decrease in the absolute mGFR is a resolution of glomerular hyperfiltration which may result in decreased intraglomerular pressure and kidney injury [13, 27, 28].

In a recent study, including 985 patients treated with bariatric surgery and 985 matched controls, it was concluded that patients undergoing bariatric surgery had a 58% lower risk of an eGFR decline ≥30% and a 57% lower risk of doubling of serum creatinine or developing end-stage renal disease compared with the controls. Of note, end-stage renal disease occurred in only eight surgery and ten non-surgery patients, indicating that the vast majority of the kidney outcomes were based on levels of creatinine [29]. While the study was well-designed and provided valuable information with important clinical implications, a major limitation is the use of a creatinine-based eGFR for determination of kidney outcomes, as also highlighted by the authors themselves. In our study, plasma creatinine was reduced in all patients except two, likely explained by a reduction in muscle mass. We found absolute mGFR and creatinine-based eGFR to change in opposite directions after RYGB. In order to resolve whether bariatric surgery is reducing the risk of adverse kidney outcomes, cystatin C-based eGFR may be a more suitable measure.
reduction. As a result, we are unable to rule out that our findings are not specifically caused by the RYGB-related changes in the renal outcome measures.

Conclusions
Major weight reductions are associated with a reduction in absolute mGFR, which may reflect resolution of glomerular hyperfiltration, while mGFR adjusted for body surface area was unchanged. Estimates of GFR based on plasma creatinin- one overestimate renal function likely due to changes in muscle mass, whereas cystatin C based estimates are unaffected. Our results have important implications for both clinicians and researchers and provide a better understanding of the physiology of glomerular filtration rate and emphasize the limitations of using plasma creatinine in the setting of obesity and following weight changes.

Abbreviations
BSA: Body surface area; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; Cystatin C: DNA: Dual energy X-ray absorptiometry; mGFR: Estimated glomerular filtration rate; GFR: Glomerular filtration rate; IQR: Interquartile range; MDRD: Modification of Diet in Renal Disease; mGFR: Measured glomerular filtration rate; RYGB: Roux-en-Y gastric bypass surgery; SD: Standard deviation; UACR: Urinary albumin-to-creatinine ratio

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
BJvS conceived and designed the research, analysed and interpreted the data, performed statistical analysis and wrote the manuscript. FP conceived and designed the research, analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. MS conceived and designed the research and made critical revision of the manuscript for key intellectual content. PR conceived and designed the research, analysed and interpreted the data, and made critical revision of the manuscript for key intellectual content. TWH made critical revision of the manuscript for key intellectual content. MS conceived and designed the research and made critical revision of the manuscript for key intellectual content. BJvS 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. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study protocol was approved by The Research Ethics Committee, Capital Region of Denmark with the protocol number: H-1-2013-074, and the study was conducted according to the Declaration of Helsinki. All patients gave written informed consent before any study procedure was initiated.

Author details
1. Steno Diabetes Center Copenhagen, Niels Steensens Vej 1, 2820-Gentofte, Denmark. 2. Hvidovre University Hospital, Hvidovre, Denmark. 3. University of Copenhagen, Copenhagen, Denmark. 4. Aarhus University, Aarhus, Denmark.

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Paper XIII
Effect of weight reductions on estimated kidney function: Post-hoc analysis of two randomized trials

Bernt Johan von Scholten a,b,⁎, Melanie J. Davies b, Frederik Persson a, Tine W. Hansen a, Sten Madshbad c, Lars Endahl d, Cecilie H. Jepsen d, Peter Rossing a,c,e

a Steno Diabetes Center Copenhagen, Region H, Copenhagen, Denmark
b Diabetes Research Centre, University of Leicester, Leicester, UK
c Midnord University Hospital, Hvidovre, Denmark
d University of Copenhagen, Copenhagen, Denmark
e Aarhus University, Aarhus, Denmark

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Serum creatinine

Aims: Weight loss-induced serum creatinine reduction may increase creatinine-based estimated glomerular filtration rate (eGFR) producing incorrect estimates of kidney function. We investigated whether weight changes in the SCALE program with liraglutide 3.0 mg were associated with changes in serum creatinine.

Methods: Post hoc analysis of two 56-week, randomized, double-blind trials: SCALE Obesity and Prediabetes (n = 3731, without type 2 diabetes [T2D], randomized [2:1] to liraglutide 3.0 mg [n = 2487] or placebo [n = 1244]); SCALE Diabetes (n = 846 with T2D, randomized [2:1:1] to liraglutide 3.0 mg [n = 423], 1.8 mg [n = 21], or placebo [n = 212]). NCT01272219/NCT01272232.

Results: In SCALE Obesity and Prediabetes, mean (± SD) weight loss (baseline to week 56) with liraglutide was 8.0 ± 6.7% (2.6 ± 6.9% with placebo); baseline creatinine with liraglutide was 76 ± 15 μmol/L and 74 ± 15 μmol/L after 56 weeks (similar across treatment groups). In SCALE Diabetes, weight loss with liraglutide was 5.9 ± 5.5% (2.0 ± 4.3% with placebo); baseline creatinine was 79 ± 19 μmol/L (77 ± 16 μmol/L, placebo) and 79 ± 20 μmol/L after 56 weeks (76 ± 15 μmol/L, placebo). No association between changes in weight and changes in serum creatinine was observed (P ≥ 0.05, both trials, all tests).

Conclusions: Moderate gradual body weight reductions observed in the SCALE program were not associated with changes in serum creatinine.

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1. Introduction

Accurate assessment of glomerular filtration rate (GFR) is important, both to evaluate the effect of weight loss or gain on kidney function, as well as to assess progression in nephropathy, decide drug dosing and patient counseling. However, methods for monitoring kidney function in the presence of moderate weight loss over time have not been validated.

Measuring kidney function using plasma clearance of an exogenous marker such as inulin is considered the 'gold standard' of

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GFR. However, this procedure is time-consuming and expensive, and hence not feasible as a routine measurement in clinical practice or in large randomized clinical trials. Clinicians therefore must rely on estimation of GFR, and equations based on serum creatinine are most commonly used (e.g. the 4-variable Modification of Diet in Renal Disease [MDRD] and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI), as they are considered to be reliable and inexpensive. Skeletal muscle mass is the main determinant of creatinine generation/production with creatinine being the final catabolite of muscular energetic metabolism. Hence, if body weight – and muscle mass in particular – changes over time, this would influence estimates of kidney function, if serum creatinine is also affected, without actual changes in measured GFR.

In a recent pooled analysis including 5100 patients, we demonstrated that a pharmacologically induced weight loss of 1.9 kg on average was not associated with changes in serum creatinine. However, in participants experiencing a fast and large weight loss following bariatric surgery, it has been well described that levels of serum creatinine decrease, resulting in increases in creatinine-based estimated GFR (eGFR); presumably explained by reductions in muscle mass. Furthermore, these studies demonstrated a pronounced discrepancy between changes in eGFR and changes in measured GFR. The magnitude and rate of a weight loss associated with sufficient impact on muscle mass reduction to affect serum creatinine and thereby eGFR are currently unknown.

For the present study, we hypothesized that a weight loss of a magnitude observed in the Satiety and Clinical Adiposity – Liraglutide Evidence trials in individuals with and without diabetes (SCALE) program would lead to reductions in serum creatinine causing increases in eGFR (creatinine-based equations).

2. Materials and methods

2.1. Study design and participants

Post-hoc analysis of two 56-week, randomized, double-blind trials was performed: the SCALE Obesity and Prediabetes trial and the SCALE Diabetes trial.

The SCALE Obesity and Prediabetes trial included 3731 participants without type 2 diabetes (T2D) but with body mass index (BMI) ≥ 30 kg/m² or a BMI ≥ 27 kg/m² if they had treated or untreated dyslipidemia or hypertension. Participants were randomly assigned in a 2:1 ratio to receive once-daily subcutaneous injections of liraglutide at a dose of 3.0 mg (n = 2487) or placebo (n = 1244); both groups received counseling on lifestyle modification. Patients were stratified according to prediabetes status (American Diabetes Association 2010 criteria) at screening and according to BMI ≥ 30 vs. < 30 kg/m².

The SCALE Diabetes trial included 846 adult participants with T2D and a BMI ≥ 27.0 kg/m², taking zero to three oral glucose-lowering agents with stable body weight, and HbA1c level ≥ 7.0% to 10.0%. Participants were randomly assigned (2:1:1) to receive once-daily subcutaneous liraglutide (3.0 mg; n = 423), liraglutide (1.8 mg; n = 211) or placebo (n = 212); all received counseling on lifestyle modification.

For the present post-hoc analysis, only participants treated with liraglutide 3.0 mg or placebo were included; hence, in the SCALE Diabetes trial, a total of 211 subjects randomized to liraglutide 1.8 mg were excluded from the present investigation.

Clinical trial registration: ClinicalTrials.gov number: NCT01272219 and ClinicalTrials.gov number: NCT01272232.

2.2. Outcome measures

Changes from baseline in body weight and serum creatinine were assessed at week 28 and week 56.

2.3. Laboratory procedures

Serum creatinine was measured at a central laboratory using a rate-blanked and compensated-modified Jaffe method on the Roche BMD instrument (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Estimated GFR was calculated by the CKD-EPI equation from creatinine.

2.4. Statistical analysis

Descriptive statistics are presented as averages and standard deviations are calculated using last observation carried forward at week 56 and through available cases at week 28. Scatterplots between changes in body weight and changes in serum creatinine were visually

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SCALE Obesity and Prediabetes trial</th>
<th></th>
<th>SCALE Diabetes trial</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3293</td>
<td>1244</td>
<td>412</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Patients, no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prediabetes, no.</td>
<td>3731</td>
<td>2487</td>
<td>423</td>
<td>211</td>
</tr>
<tr>
<td>Predictors, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>45.2 (12.1)</td>
<td>45.0 (12.0)</td>
<td>55.5 (10.8)</td>
<td>54.7 (9.8)</td>
</tr>
<tr>
<td>Female, %</td>
<td>78.7</td>
<td>78.1</td>
<td>48.0</td>
<td>54.2</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>38.3 (6.4)</td>
<td>38.3 (6.3)</td>
<td>37.1 (6.3)</td>
<td>37.4 (7.1)</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>76 (15)</td>
<td>76 (15)</td>
<td>79 (19)</td>
<td>77 (15)</td>
</tr>
<tr>
<td>3036</td>
<td>1244</td>
<td>412</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Changes from baseline to week 28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>−8.6 (5.6)</td>
<td>−3.1 (5.6)</td>
<td>−6.3 (5.0)</td>
<td>−2.9 (4.0)</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>−2</td>
<td>−1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>1.6</td>
<td>1.2</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Changes from baseline to week 56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>−8.4 (7.3)</td>
<td>−2.8 (6.5)</td>
<td>−6.3 (6.0)</td>
<td>−2.2 (4.8)</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>−2</td>
<td>−2</td>
<td>0</td>
<td>−1</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>2.1</td>
<td>1.8</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values are mean (SD) or %. eGFR, estimated glomerular filtration rate

* In total, 423 patients were randomized to the liraglutide 3.0 mg group; however, serum creatinine measurements were only available for 412 and therefore only these patients were included in this current analysis.
inspected with the aid of non-parametric smoothed curves based on moving averages (figures not shown). As the association between changes in body weight and changes in serum creatinine seems linear in both treatment groups in both trials, the data were analyzed using ANCOVA models, with change in serum creatinine as the dependent variable and weight change by treatment group as the independent variable adjusted for age and sex. The scatterplots with the linear regression lines by treatment group averaged over the age and sex distribution within each treatment group are presented together with the P-value for test of slope = 0 (i.e. test of the hypothesis of no association between change in serum creatinine and change in body weight).

3. Results

Table 1 shows baseline characteristics and outcome measures after 28 and 56 weeks for the analyzed participants in the two trials according to treatment group.

After 28 weeks in the SCALE Obesity and Prediabetes trial, participants in the liraglutide group had a mean (±SD) weight loss of 8.2% ± 5.1% (8.6 ± 5.6 kg), whereas participants in the placebo group had a mean weight loss of 2.9% ± 5.0% (3.1 ± 5.6 kg). After 56 weeks, participants in the liraglutide group had a mean weight loss of 8.0% ± 6.7% (8.4 ± 7.3 kg), whereas participants in the placebo group had a mean loss of 2.6% ± 5.7% (2.8 ± 6.5 kg).

After 28 weeks in the SCALE Diabetes trial, participants in the liraglutide group had a mean weight loss of 6.0% ± 4.6% (6.3 ± 5.0 kg), whereas participants in the placebo group had a mean loss of 2.7% ± 3.8% (2.9 ± 4.0 kg). After 56 weeks, participants in the liraglutide group had a mean weight loss of 6.0% (6.3 kg), whereas participants in the placebo group had a mean loss of 2.0% (2.2 kg).

In the SCALE Obesity and Prediabetes trial, serum creatinine was 76 ± 15 μmol/L at baseline and 74 ± 15 and 74 ± 15 μmol/L at week 28 and 56, respectively, with no apparent differences between the treatment groups. In the SCALE Diabetes trial, serum creatinine with liraglutide 3.0 mg was 79 ± 19 μmol/L at baseline and 79 ± 19 and 79 ± 20 μmol/L at week 28 and 56, and with placebo 77 ± 15 μmol/L at baseline and 77 ± 16 and 76 ± 15 μmol/L at week 28 and 56, respectively, with no difference between groups.

No significant interaction with treatment group (liraglutide/placebo) in the association between change in body weight and serum creatinine was revealed (P ≥ 0.05 for all four tests).

3.1. Associations between changes in body weight and changes in serum creatinine

In the SCALE Obesity and Prediabetes trial, no association was observed after 28 weeks (P > 0.05) or 56 weeks (P > 0.05, Fig. 1) of treatment. In the SCALE Diabetes trial, no association was observed after 28 weeks (P > 0.05) or 56 weeks of treatment (P > 0.05, Fig. 2).

3.2. Additional analysis

Analyses restricted to participants with weight loss >15% (n = 333 for liraglutide 3.0 mg and n = 134 for placebo at week 28, n = 329 and n = 42, respectively, at week 56) in the SCALE Obesity and Prediabetes trial demonstrated no association between changes in body weight and changes in serum creatinine after 28 weeks (P > 0.05) or 56 weeks (P > 0.05, Fig. 3) of treatment. Participants with weight loss >15% (n = 9 for liraglutide 3.0 mg and n = 1 for placebo at week 28; n = 22 and n = 6, respectively, at week 56) in the SCALE Diabetes trial demonstrated no association between changes in body weight and changes in serum creatinine after 28 weeks (P > 0.05) or 56 weeks (P > 0.05, Fig. 4) of treatment.

4. Discussion

In this post-hoc analysis of data from the SCALE program including 4366 participants with obesity, prediabetes or diabetes treated with liraglutide 3.0 mg/day (n = 2910) or placebo (n = 1456), moderate body-weight reductions of mean 6%-8% were not associated with changes in serum creatinine. Hence, in weight-management trials with a moderate weight loss, creatinine-based eGFR equations are applicable to monitor treatment effects and safety.

GFR measurement by inulin-clearance,11 chromium-EDTA clearance12 or iohexol clearance13 is considered the ‘gold standard’. However, these procedures are time-consuming (typically a 4-h examination) and expensive, and hence not feasible as a routine measurement in clinical practice or in large clinical trials. Estimating GFR is therefore central for diagnosis and monitoring of participants with kidney disease and it is frequently used for correct dosage of drugs. Further, estimations of GFR are often included in clinical trials for safety reasons and for monitoring the potential effect of treatment on kidney function. Several equations based on serum creatinine have been suggested for estimating GFR; the CKD-EPI equation has been proven to be especially dependable when compared with measured
GFR. However, creatinine-based eGFR equations have not been validated in persons with morbid obesity or large change in body composition. Cystatin C is less affected by muscle mass and diet than creatinine, and it has been anticipated that cystatin C would provide a more accurate estimate of GFR than creatinine, in particular in populations where weight reductions are intended.

The purpose of the present analysis was to elucidate whether a weight reduction observed in a large weight-management trial would lead to reductions in levels of serum creatinine and thereby increases in creatinine-based eGFR, which might not reflect actual changes in kidney function.

In a recent prospective interventional study, a mean weight reduction of 27 kg (21%) 6 months after Roux-en-Y gastric bypass surgery was associated with reductions in serum creatinine and consequently increases in creatinine-based eGFR. Measured GFR (51Cr-EDTA plasma clearance) adjusted for concurrent body surface area and the cystatin C-based eGFR were unchanged. By applying robust methods for determination of body composition (dual-energy X-ray absorptiometry [DXA] scans), we were able to demonstrate that changes in muscle mass correlated with changes in serum creatinine, and concluded that creatinine-based eGFR did not reflect the true eGFR in this patient group.

In the present analysis, no association between moderate weight reductions of 6%–8% (6.4–8.4 kg) and serum creatinine was observed, either in people without diabetes, participants with prediabetes or participants with T2D. Findings were similar in analyses restricted to high weight-loss responders (reduction >15%), both after 28 and 56 weeks. This suggests that even in the participants with the most pronounced weight reductions in the SCALE program, creatinine-based eGFR equations are reliable, superseding the need for non-creatinine-based eGFR equations in weight management programs.

However, what is the explanation for the discrepancy between the present results and findings in the setting of bariatric surgery? One explanation could be that while body weight is gradually decreasing in the SCALE program over the time course of 1 year, bariatric surgery is associated with a much more rapid and larger weight reduction. After bariatric surgery, the body quickly develops a catabolic state, perceived as being in starvation, and in general the body prefers to use muscle as energy before consuming fat after bariatric surgery.
contributing to loss of muscle mass.17 Additionally, a greater caloric deficit after bariatric surgery may lead to the greater loss in muscle mass. It might therefore be that large and rapid weight reductions following bariatric surgery are associated with greater reductions in muscle mass when compared to the more moderate weight reductions over a longer time window obtained in the SCALE program. 

Accordingly, in a subgroup of participants (n = 15) from the iraglutide 3.0 mg phase 2 study, body composition was measured at randomization and week 20 by DXA and single-slice abdominal computerized axial tomography, and lean tissue mass was reduced by 2.0%.18 When we assessed changes in body composition, using DXA scans, in 19 participants before and 26 weeks after gastric bypass surgery, a 10% reduction in lean tissue mass was observed.8 

Taken together, our data suggest that a pronounced but gradual body-weight reduction observed in a large weight-management program does not affect levels of serum creatinine, or that the creatinine release and clearance may be decreased to a similar extent.

Further, our findings are in accordance with our previous study, where smaller weight reductions (mean of 1.9 kg), obtained with smaller dosages of iraglutide (0.6–1.8 mg), did not affect levels of serum creatinine. Accordingly, there was no change in weight-independent estimates of GFR (MDRD, CKD-EPI), whereas weight-dependent estimates (Cockcroft–Gault) were influenced.13

4.1. Strengths and limitations

Strengths of our study include the analyses of individual data from a large population enrolled in two randomized clinical trials conducted according to good clinical practice, including central laboratory assessment of serum creatinine levels. Limitations include that all participants were recommended a reduced-calorie diet and increased physical activity, which may have weight-independent effects on creatinine. Further, cystatin C-based measurements of eGFR were not available, and GFR was not measured with a precise method such as plasma clearance of an exogenous marker, and thus the true GFR and its change are not known. Therefore, this post-hoc analysis of phase 3 data cannot assess if the weight changes have any effect on GFR. However, in our previous weight-loss study using bariatric surgery, we demonstrated that measured GFR was stable when measured with 125I-EDTA plasma clearance.9 Lastly, we did not perform DXA scans in all participants and therefore lack information on exact changes in body composition.

5. Conclusion

Moderate gradual body weight reductions observed in the SCALE program were not associated with changes in serum creatinine.

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References


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