TITLE PAGE

Role of Linagliptin in improving renal failure by improving CD34+ stem cell number, function and gene expression in renal function impaired type 2 diabetes patients

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PROTOCOL SYNOPSIS

Protocol Title:	Role of Linagliptin in improving renal failure by improving CD34+ stem cell number, function and gene expression in renal function impaired type 2 diabetes patients
Site Numbers & Names:	GWU Medical Faculty Associates
Research Hypothesis:	Both type 2 diabetes and CKD are associated with poor stem cell number and function. Poor viability and function of EPCs in CKD and diabetes affects the repair and regeneration of the endothelium and renal tubules. We hypothesize that use of Linagliptin (along with Insulin, metformin, or both) may help reduce cardiovascular risk by improving EPC survival and function above and beyond adequate glucose metabolism control.
Study Schema: Drugs / Doses / Length of Treatment)	We propose a 2-arm randomized, parallel group, longitudinal study of 12-week intervention duration. The 12-week time interval has been previously shown adequate to observe changes in biochemistry, EPCs and, importantly, pulse wave velocity (PWV) changes. Patients will be randomized to 2 groups: **Control, (n=20), Insulin/Metformin + Lifestyle + Placebo.** **Treatment, (n=20) Insulin/Metformin + Lifestyle + Linagliptin 5mg.**

Study Objectives: • **Primary:** • **Secondary:**	The primary objective is to ascertain if addition of Linagliptin improves CD34+ cell function and gene expression in early type 2 diabetes patients with CKD stage 1-3, which will be correlated to improvement in 24hr urinary protein estimation and creatinine clearance (obtained via micro-albumin/creatinine ratio from a spot urine sample). The secondary objective is to correlate the cellular outcome measures with other measures of endothelial function such as: 1. Arterial stiffness measures with pulse wave analysis and pulse wave velocity measurements 2. Serum biochemistry looking at surrogates of endothelial health, endothelial inflammation, appetite controlling hormone levels and fasting glucose, insulin and lipid profile. 3. Resting Metabolic Rate measurement **<u>Study Outcome Measures</u>** **Primary:** To investigate the effect of Linagliptin on Endothelial function in patients inadequately controlled (HbA1C ≥6.5 % to ≤10%) while being treated with stable Insulin, Metformin, or both. • **<u>Cellular markers.</u>** We will study pre and post Linagliptin treatment changes in number, function and gene expression of patients' peripheral blood-derived CD34+ cells. • 24hr urinary **protein estimation** and creatinine clearance. **Secondary:** • To investigate the effect of Linagliptin treatment on serum endothelial inflammatory markers including high sensitivity C-reactive protein (hs-CRP), IL-6, TNF-alpha, and fasting lipid profile including ApoA1 and ApoB. <u>Glycemic control</u> will be evaluated by measuring fasting blood glucose, insulin, and HbA1c levels and assessing insulin resistance using HOMA-IR • <u>Adiposity,</u> measured using the Tanita Body Composition Analyzer scale, measured as percentage body fat. • <u>Estimation of Creatinine clearance and Protenuria estimation.</u> • <u>Vessel health will be assessed by systolic and diastolic blood pressure and Arterial stiffness</u> assessed using vascular flow and wave measurement equipment, SphygmoCor CP system from ATCOR. • The sphygmocor system also allows us to estimate central and aortic blood pressure. • Resting Metabolic Rate (RMR, similar to Resting Energy expenditure measurement) at baseline, midway point (week 6) and post therapy.

Study Design:	Prospective, double-masked, randomized placebo-controlled trial
Accrual Goal: (Total number of subjects)	N=40
Accrual Rate: (Number of subjects expected per month)	**Recruit 40 patients in approximately 36 months.**
Estimated: **FPFV:** **LPFV:** **Follow Up:** **(dd-mm-yy)**	**FPFV: 09-15-2015.** **LPFV: 09-15-2018**
Co-relative Studies: (PK/PD, etc.)	N/A
Inclusion Criteria:	1. Adults aged 40-70 years. 2. Diagnosis of type 2 diabetes within the previous 15 years using criteria of the American Diabetes Association 3. Currently treated with a stable dose of Insulin, Metformin (1-2 grams/day), or a stable combination of the two as therapy. 4. HbA1C between 6.5 to 10% (both inclusive) 5. BMI 25 to 39.9 kg/m^2 (both inclusive) 6. CKD stages 1-3

Exclusion Criteria:	Patients with: 1. Implanted devices (e.g., pacemakers) that may interact with Tanita scale 2. Previous coronary or cerebrovascular event within 6 months of screening or active or clinically significant coronary and/or peripheral vascular disease 3. Low hematocrit (<28 UNITS). 4. Pre-existing liver disease and/or ALT and AST >2.5X's UNL 5. CKD stage 4 and 6. History of pancreatitis, or cancer (except basal cell carcinoma and cancer that is cured or not active or being treated in the past five years) 7. Statin use started or dose change in the last 3 months 8. Use of oral anti-diabetic medication other than Metformin, or Insulin. 9. Use of consistent long-term steroid medication in the last 3 months (oral, inhaled, injected). 10. Systolic BP> 140 mmHg and Diastolic BP> 90 mmHg 11. Active wounds or recent surgery within 3 months. 12. Inflammatory disease, or chronic current use of anti-inflammatory drugs within the last 3 months 13. triglycerides >450 mg/dL 14. untreated hyper/hypothyroidism 15. Auto antibody confirmed type 1 diabetes Additionally, patients who are active smokers, patients who are pregnant, nursing women, and post-menopausal women who are on hormone replacement therapy will be excluded. Patients on low dose oral contraceptives will be allowed to participate as these formulations contain lesser amount of estrogens.

Criteria for Evaluation: (Efficacy, safety, stopping rules, etc.)	Subjects will be screened at week -4, then evaluated for end points at weeks 0, 6 and 12. Follow up phone call will be done 30 days after last dose of study drug to assess for adverse effects. Study participation will be stopped for adverse effects at the discretion of the study investigator. Subjects may stop study participation at any time and for any reason.
Statistics:	The total sample size is requested, after accounting for attrition over the 12-week period, is 20 subjects per group or 40 subjects total. Sample size estimates were based on the effects of exercise on CD34+/KDR+ cells as described in the literature. The effect of a single session, as well as extended training, on healthy subjects or those with existing cardiovascular conditions appears to increase the CD34+/KDR+ cells. This is a pilot study as a result appropriate power calculation is not feasible. W however did conduct power analysis, which provided us with 73% power (see statistics section). A p-value of less than 0.05 will be considered statistically significant.

1 INTRODUCTION

Type 2 diabetes is a national epidemic (1, 2) with significant macro and microvascular complications (3). Insulin resistance in pre-diabetes and overt diabetes are associated with endothelial dysfunction (4).

A few studies indicate that stem cells particularly EPCs can act as a suitable bio-marker (5-7, 9) for monitoring cardiovascular morbidity. In this proposal we suggest that EPCs or CD34 positive cells (defined as CD34/VEGFR2+ cells) can act as a suitable cellular biomarker for estimating and following endothelial dysfunction in early type 2 diabetes patients with CKD. EPCs have been shown to be dysfunctional in both CKD patients and type 2 DM patients. (5, 7, 31, 35)

Linagliptin (TRADJENTA) tablets are indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus. No dose adjustment is recommended for patients with renal impairment. (29)

EPCs have been used as a regenerative tool in ischemic myocardium and diabetic wound healing (8, 10-13). Endothelial dysfunction with associated inflammation may be a consequence of excess intra-cellular super-oxide presence in a setting of diabetes which is a pro-oxidative stress condition ultimately leading to poor EPC function and senescence (14).

Though lifestyle modification has been proposed as a main stay for prevention and treatment of early type 2 diabetes, (2, 15-19) several new therapies for diabetes have been developed in recent years (2). Incretins and incretin mimetics appear to hold promise. Mechanism of positive effect of exercise and oral hypoglycemic agents can be very different. (44-50)

DPP-4 inhibitors have been shown to increase EPCs in patients with type 2 diabetes (20) reportedly via SDF-1 alpha up-regulation. Interestingly, up-regulation of SDF-1 alpha and vascular endothelial growth factor (VEGF), both chemotactic factors increase mobilization and recruitment of EPCs in the face of acute ischemic injury for repair and regeneration. (21-24).

Several studies have shown positive effect of incretins (Glucagon like peptide, GLP-1) and incretin receptor agonists (GLP-1 receptor agonists) on cardiovascular risk factors in type 2 diabetes patients (20, 25) and even in patients with chronic heart failure and left ventricular dysfunction who do not have diabetes (26, 27).

DPP-4 Inhibitors may have cardio-protective effects of their own, as they increase bio-availability of endogenous GLP-1. They improve blood flow and nitric oxide production in

endothelium (28, 29). These are unique properties not demonstrated by other oral diabetes medications (29). The mechanism underlying these effects may be mediated by increased nitric oxide bioavailability but is not completely known. However these beneficial effects appear to be independent of glycemia reduction.

It is however unknown whether Linagliptin will have any positive effect on human EPC function where two prominent cardiovascular risk factors co-exist such as CKD and type 2 diabetes.

Therefore we plan to investigate if Linagliptin can alter function and gene expression of CD34+ cells in a setting of CKD and type 2 diabetes. We choose to look at non geriatric adult population with early type 2 diabetes (≤15 years of duration) at an early phase of renal impairment (stages 1-3).

We specifically choose to exclude known CVD disease patients and early type 2 DM patients with early CKD as this cohort may be still retain capability to reverse or halt persistent endothelial damage seen in type 2 diabetes and CKD (3,30,35).

Our preliminary study results , which just finished, looked at effect of 150min/week of aerobic physical activity on EPCs in pre-diabetes population show that interventions even though non pharmaceutical can lead to quick (within 4 weeks) improvement in gene expression and function of CD34+ cells. These cellular changes are reproducible and consistent with other outcome measures such as serum biochemistry and vessel wall dilation measures such as flow mediated dilatation. (34)

Research Hypothesis:

We hypothesize that Linagliptin, a member of DPP-4 inhibitor group of drugs may be able to improve number and function of CD34+ endothelial progenitor cells by up-regulating chemotactic agent SDF1 alpha (DPP-4 degrades SDF-1) and its receptor CXCR4 (7, 20, 21) and thereby improving EPC migration and overall endothelial function. We also propose that this expected cardiovascular benefit is independent of HbA1C reduction. (30, 31)

In this proposed study we plan to recruit patients with type 2 diabetes of ≤15 years duration (in early phase of diagnosis), who are on a stable dose of insulin, Metformin, or both for at least 3 months either short or long acting but still remain inadequately controlled with HbA1C between 6.5% and 10% (both values inclusive), but not overtly out of control (> 10% of HbA1C).

The patient will adjust insulin levels so as to keep their HbA1C levels under 10.0%, for health measures. If the HbA1c rises above 10.0% then the PI will speak with the patient and provide a documented form indicating the insulin dose adjustment that should be

made. Initially life-style intervention with diet and regular aerobic activity (as per Standards of Diabetes Care: Diabetes Care Supplement: Jan 2014) along with insulin titration will be undertaken for initial 4 weeks (time between screening visit and 1st visit). Subjects will begin taking 5.0 mg of Linagliptin or placebo after initial 4 weeks. Subjects will be withdrawn from the study if the medication or placebo is not tolerated.

2 STUDY OBJECTIVES

2.1 Primary Objective

To investigate the effect of Linagliptin on Endothelial progenitor cells (CD34 positive cells) in patients whose HbA1C is between 6.5 to 10% (both inclusive) while being on treatment with a stable dose of insulin, Metformin, or both for the preceding 3 months before enrollment.

Cellular markers: We will use patient's peripheral blood derived CD34+ cells looking at number, function and gene expression changes pre and post Linagliptin over 12 weeks of therapy.
Urinary Function Marker in CKD: We will estimate 24hr urinary protein estimation and creatinine clearance (This is measured via the micro-albumin/Creatinine ratio provided from a random spot urine sample).

2.2 Secondary Objectives

To investigate the effect of Linagliptin treatment on serum endothelium inflammatory markers including: C-reactive protein (hs-CRP), IL-6, TNF-alpha, and fasting lipid profile including ApoA1 and ApoB (5, 6, 30, 36).

Glycemic control will be evaluated by measuring fasting blood glucose, insulin levels and HbA1c. Fasting blood glucose, insulin and lipid profile will be used to assess insulin resistance (32, 37)

Adiposity will be measured using the Tanita Body Composition Analyzer scale, measured as percentage body fat (33). Serum will be used to estimate levels of SDF1-Alpha, VEGF, and GLP1 levels by ELISA (25, 28, 29, and 30).
We also intend to follow resting Metabolic Rate at baseline, midway, and post DPP4 inhibitor therapy to ascertain if Linagliptin has any effect on RMR (56).

Kidney health will be monitored by creatinine clearance and microalbumin/ creatinine levels, which will be obtained from the spot urine sample.

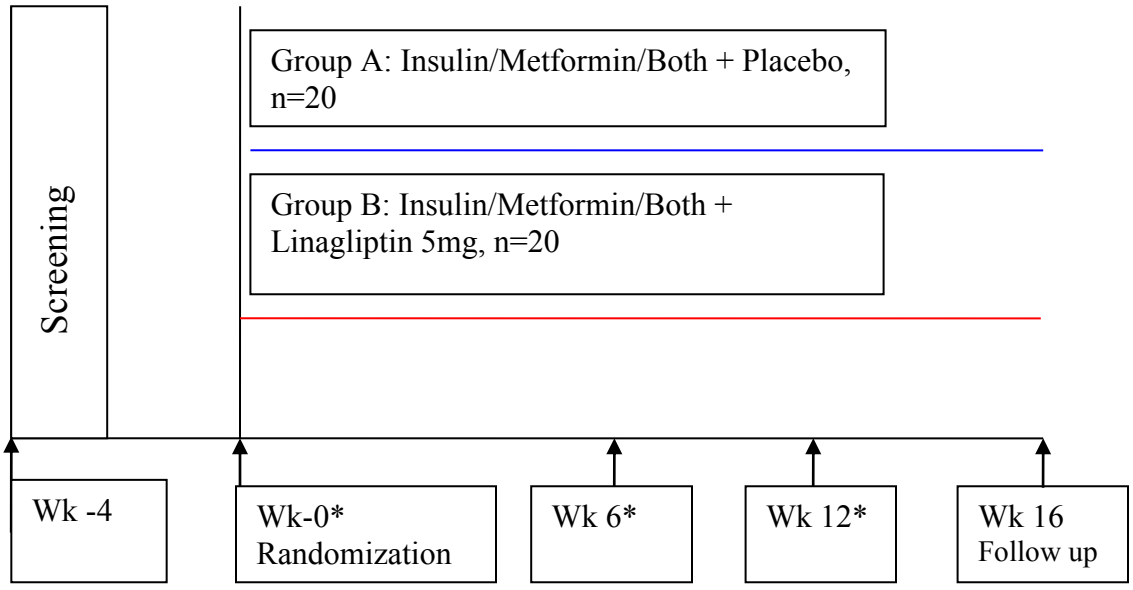
a. *Subjects will have the option of opting into a sub-study that is being where 10-20 mL of the excess fresh spot urine that has been collected will be used for exosome analysis. This is optional, and is not required of subjects. For details of urine-exosome study please see the appendix.*

The biochemical or bio-inflammatory markers are an important marker of endothelial inflammation and dysfunction along with glycemic markers and markers of arterial stiffness (36-43, 55, 57). Literature suggests that DPP4 inhibitors and GLP1modifies and modulates hemopoetic stem cells and vaso dilatation (37, 44, 45).
Vessel health will be assessed by degree of arterial stiffness, using arterial tonometry. The central and the aortic pressure is assessed by pulse wave analysis (PWA) and pulse wave velocity (PWV) . Arterial stiffness will be assessed using vascular flow and wave form analysis equipment, SphygmoCor CP system from ATCOR. (56)

3 INVESTIGATIONAL PLAN

3.1 Study Design and Duration

Study Schema, 40 Type 2 diabetic Subjects Aged 40-70 on stable dose of Insulin, Metformin, or both



+/- 3 day window for visits

*Assessed at week 0, 6 and 12: Biochemical and cellular markers of endothelial function and Pulse wave analysis and velocity (PWA and PWV), REE and Adiposity.

Week 16 - A telephone call to subjects will be made 30 days after last dose of study medication to determine if there have been any adverse events.

Available related studies and Expected Results: Studies measuring changes to EPCs in type 2 diabetes patients are very limited. To the best of our knowledge there are no studies examining the effect of Linagliptin in type 2 diabetes patients with CKD.

Use of CD34 positive cells as a cardiovascular risk surrogate has been reported in the past. Various studies (11, 34), have shown that various disease states, including those seen in diabetes, can deplete and damage EPC, thereby diminishing their regenerative potential. The inability to maintain or repair damaged endothelial tissue leads to cumulative vascular dysfunction and cardiovascular disease.

We hypothesize that monitoring **number, *function and gene expression of endothelial progenitors will allow us to quantify cardiovascular disease risk at the onset and regenerative potential post intervention at a cellular level. It will also help us to identify and correlate the best endothelial function bio-chemical inflammatory marker as an early indicator of cardiovascular disease progression in early type 2 diabetes with CKD and identify patients that are responders.***

Linagliptin along with other gliptins can increase SDF1 alpha levels and possibly CXCR4 receptor expression which will help EPC mobilization and gene expression of hemopoetic CD34 positive stem cells.

Linagliptin 5 mg or placebo will be added to patients who have type 2 diabetes diagnosed ≤15 years with HbA1C of 6.5 to 10 % (both inclusive) while being on Insulin, metformin, or both.

Insulin will be titrated upwards if HbA1C is above 10% with minimal dose adjustments so as to keep the adjusted HbA1C below overtly uncontrolled levels (above 10%).

Our study population will include equal numbers of adult male and female Type 2 diabetes (T2DM) patients aged 40-70 years, enrolled in both arms of the study. Patients will be treated with 5 mg of Linagliptin or placebo for 12 weeks and with adequate lifestyle modification for 16 weeks (as per American Diabetes Association guidelines). This time interval has been previously shown to be adequate to observe changes to Endothelial Progenitor Cells (EPCs), (5, 6).

3.2 Study Population

For entry into the study, the following criteria MUST be met.

3.2.1 Inclusion Criteria

1) Signed Written Informed Consent

- Before any study procedures are performed, subjects will have the details of the study described to them, and they will be given a written informed consent document to read. Then, if subjects consent to participate in the study, they will indicate that consent by signing and dating the informed consent document in the presence of study personnel.

2) Target Population

- Subjects with a diagnosis of Type 2 diabetes mellitus within the previous 15 years using criteria of the American Diabetes Association.

- Currently treated with Insulin (long or short acting or combination), Metformin (stable dose of 1-2 grams/day), or a stable dose of both.
- HbA1C between 6.5% and 10% (both inclusive)
- BMI 25-39.9 kg/m^2 both inclusive.
- History of Stage 1-3 CKD shown by a laboratory test taken within the past 2 years

CKD Staging:
Estimated Glomerular Filtration Rate Serum Cr should be used to estimate glomerular filtration rate (GFR). Estimated GFR (eGFR) is commonly reported by laboratories or can be estimated using formulae such as the Modification of Diet in Renal Disease (MDRD) study equation or the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. The latter is the preferred GFR estimating equation. GFR calculators are available at http:// www.nkdep.nih.gov.
Abnormal urinary albumin excretion and eGFR may be used to stage chronic kidney disease (CKD). The National Kidney Foundation classification (Table below) is based on **both kidney damage (UACR at or above 30 mg/g Cr) and eGFR.**
Normal UACR is defined as less than 30 mg/g Cr, and increased urinary albumin excretion is defined as at or above 30 mg/g Cr. Because of variability in urinary albumin excretion, ideally two (at least one) specimens of UACR should have been collected within a 3- to 6-month period before considering a patient to have albuminuria.

Stage -- Description -- GFR (mL/min/1.73 m2)
Stage 1 Kidney damage* with normal or increased GFR >=90
2 Kidney damage* with mildly decreased eGFR 60–89
3 Moderately decreased eGFR 30–59
4 Severely decreased eGFR 15–29
5 Kidney failure ,15 or dialysis
*Kidney damage is defined as abnormalities on pathological, urine, blood, or imaging tests.[1]

3) Age and Reproductive Status

Men and women, 40 to 70 years of age.

- Enrolled women must not be pregnant, should not be breast-feeding and should not be taking hormone replacement therapy (HRT) during randomization.

The following women are WOCBP:

- Women using the following methods to prevent pregnancy: Oral contraceptives, other hormonal contraceptives (vaginal products, skin patches, or implanted or injectable products), or mechanical products such as intrauterine devices or barrier methods (diaphragm, condoms, spermicides).
- Women who are practicing abstinence.

- Women who have a partner who is sterile (e.g., due to vasectomy).WOCBP must be using an acceptable method of contraception to avoid pregnancy throughout the study and for up to 4 weeks after the last dose of study drug in such a manner that the risk of pregnancy is minimized.
- WOCBP must have a negative serum or urine pregnancy test result (minimum sensitivity 25 IU/L or equivalent units of HCG) within 0 to 72 hours before the first dose of study drug.
- Women must not be breast-feeding.

Post-menopause is defined as:

- Women who have had amenorrhea for $\geq$ 12 consecutive months (without another cause) and/or who have a documented serum follicle-stimulating hormone (FSH) level > 35 mIU/mL.
- Women who have irregular menstrual periods and a documented serum FSH level > 35 mIU/mL.
- Women who are taking hormone replacement therapy (HRT).

3.2.2 Exclusion Criteria

- Type 1 diabetes mellitus
- History of diabetic ketoacidosis or hyperosmolar non-ketotic coma
- Low hematocrit (< 28 Units)
- History of pancreatitis, or cancer (except basal cell carcinoma and cancer that is cured or not active or being treated in the past five years)
- Previous coronary event or history of cerebrovascular event within 6 months of screening or active or clinically significant coronary and/or peripheral vascular disease
- CKD Stages 4 and 5 (estimated CrCl <30 mL/min)
- Beginning Statin medications or of change of Statin dose in the last 3 months,
- Use of oral or injectable anti-diabetic medication other than Insulin and Metformin. We did not want to bring in too many variables in the control arm and wanted to avoid a combination of insulin and other OHAs as the results might be difficult to interpret.
- Patients will be off other OHA (other than Metformin) before randomization and inclusion in the study.
- Use of consistent long-term steroid medication (oral, inhaled, injected) within the last 3 months
- Untreated Systolic BP> 140 mmHg and Diastolic BP> 90mmHg
- Active wounds or recent surgery within 3 months
- Inflammatory disease, or current chronic use of anti-inflammatory drugs in the last 3 months.

- Untreated hyper/hypothyroidism
- Contraindications for moderate exercise
- Implanted devices (e.g., pacemakers) that may interact with Tanita scale

Physical and Laboratory Test Findings

- Pre-existing liver disease and/or ALT and AST >2.5X's UNL,
- serum creatinine levels ≥2.0 with estimated CrCl < 30 mL/min)
- Triglycerides >450 mg/dL

Allergies and Adverse Drug Reactions

- Subjects with a history of any serious hypersensitivity reaction to Linagliptin or another DPP-4 inhibitor.

Sex and Reproductive Status

- Women in reproductive age group will be included in the study but encouraged to use contraceptive method to avoid pregnancy within 16 weeks of study duration.
- Women who are pregnant or breast-feeding will be excluded.

Prohibited Treatments and/or Therapies

Treatment with a strong cytochrome P450 3A4 (CYP3A4) or P-gp inducer (i.e. rifampin)

Other Exclusion Criteria

- Prisoners or subjects who are involuntarily incarcerated.
- Subjects who are compulsorily detained for treatment of either a psychiatric or physical (e.g., infectious disease) illness.

Additionally, patients who are active smokers, patients who are pregnant, nursing women, and women who are on hormone replacement therapy will be excluded. Patients on low dose oral contraceptives will be allowed to participate as these formulations contain very low amounts of estrogens.

Eligibility criteria for this study have been carefully considered to ensure the safety of the study subjects and to ensure that the results of the study can be used. It is imperative that subjects fully meet all eligibility criteria.

3.2.3 Discontinuation of Subjects from Treatment

Subjects MUST discontinue investigational product (and non-investigational product at the discretion of the investigator) for any of the following reasons:

- Withdrawal of informed consent (subject's decision to withdraw for any reason).
- Any clinical adverse event, laboratory abnormality, or inter-current illness which, in the opinion of the investigator, indicates that continued participation in the study is not in the best interest of the subject.

- Patients in CKD stages 1-3 who progress to stages 4 and beyond, post screening, will be excluded also.
- Concomitant treatment with a strong systemic cytochrome P450 3A4 (CYP 3A4) or P-gp inducer such as rifampicin. Use of alternative treatments to Linagliptin is recommended.
- Pregnancy
  - Pregnant patients will be advised to contact the investigator or study staff immediately if they suspect they might be pregnant (e.g., missed or late menstrual period) at any time during study participation.
- Loss of ability to freely provide consent through imprisonment or involuntary incarceration for treatment of either a psychiatric or physical (e.g., infectious disease) illness.
- If pancreatitis is suspected, promptly discontinue treatment with Linagliptin

All subjects who discontinue should comply with protocol-specified follow-up procedures outlined before. The only exception to this requirement is when a subject withdraws consent for all study procedures or loses the ability to consent freely (i.e., is imprisoned or involuntarily incarcerated for the treatment of either a psychiatric or physical illness). If a subject withdraws before completing the study, the reason for withdrawal must be documented appropriately.

4 TREATMENTS

4.1 Study Treatment: Linagliptin

Definition of Investigational Product: A pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical study, including products already with a marketing authorization but used or assembled (formulated or packaged) in a way different from the authorized form, or used for an unauthorized indication, or when used to gain further information about the authorized form. In this protocol, the investigational product is Linagliptin.

Definition of Non-Investigational Product: Other medications used in the study as support or escape medication for preventative, diagnostic, or therapeutic reasons as components of a given standard of care. In this protocol, the non-investigational product is Insulin, Metformin, or both. Patients will continue on Insulin, Metformin, or both as per their study entry dose added to Linagliptin or placebo. As mentioned before, patients on oral hypoglycemic agents other than Metformin will not be invited to join the study as OHA plus DPP4 inhibitor could have a different effect on vasculature or hemopoetic CD34+ stem cells compared to insulin and DPP4 inhibitor.

This study is being conducted under FDA mandated guideline for use in type 2 diabetes. Though in this particular study Linagliptin is referred to as the investigational drug, it is not a novel drug and we should not need a separate IND for execution of this study.

4.2 Method of Assigning Subjects to a Treatment

Subjects, as per selection criteria below will be randomized to treatments using a permuted block design, developed by the Epidemiology & Biostatistics Research Core. This approach ensures groups will be approximately balanced at any time during the study and at study completion.

4.3 Selection and Timing of Dose for Each Subject

The recommended dose of Linagliptin is 5 mg PO will be given once daily. Linagliptin can be taken with or without food.

4.3.1 Dose Modifications

No dose modification is necessary in CKD stage 1-3

4.4 Blinding/ Unblinding

Blinding is critical to the integrity of this clinical study. However, in the event of a medical emergency or pregnancy in a subject, **in which knowledge of the investigational product is critical to the subject's management**, the blind **for that subject** may be broken.

Before breaking the blind of an individual subject's treatment, the investigator should have determined that the information is necessary, i.e., that it will alter the subject's immediate management. In many cases, particularly when the emergency is not investigational product-related, the problem may be properly managed by assuming that the subject is receiving active product without the need for un-blinding.

End of Study Unblinding Protocol

Upon completion of the research study, we will undergo the following protocol in order to unblind the research data.

Unblinding is the process by which the allocation code is broken so that the investigator, clinical staff and the trial statistician becomes aware of which intervention each subject enrolled in the research study was taking.

Unblinding at the end of the study is required in order to make unmasked analysis in accordance with the study analysis plan. It is also conducted in order to inform the participants of which investigational product they were assigned to.

Time to unblind:

Unblinding shall be conducted when all subjects enrolled in the research study have finished treatment, and all follow up visits. There must be no plan to recruit any more subjects in the research study. Additionally, all data points and outcome measures for each research subject must have been collected, and ideally compiled. Prior to unblinding there will be a data lock on clinical outcome measures and basic side outcome measures and associated research data collected for the study.

Procedure to unblind

Once data (from both the clinical and the basic science side) and has been compiled and is data locked, the investigator can choose to unblind. The Principal Investigator must contact the study sponsor, and receive permission to un-blind. If, for unforeseen reasons, at the pre-determined date for full study unblinding the data analysis on the cellular or basic aspects of the study is lagging behind the clinical data outcome measures (though the data has been acquired) the Principal Investigator in consultation with the study sponsor may choose to un-blind the clinical outcome measures before the basic side data has been analyzed but compiled. Upon confirmation from the study sponsor, the principle investigator must make a written request to the designated party to unblind, hereto referred at the "*unblinder*". The unblinder is the bio-statistician of the MFA, **Dr. Richard Amdur**. Upon receipt of an instruction to unblind, the unblinder will sign the request form, indicating their agreement to unblind. This form will then be taken to the pharmacy, **MFA's IDS**, where a member of the IDS staff will take the form, and will give the unblinding study binder to the unblinder. The pharmacy will sign to indicate their release of the binder, and the unblinder will sign to indicate receipt of the binder. At this point in time the chain of custody of the pharmacy unblended binder has been transferred to the designated party to unblind. The form with all of the signatures will be provided to the study coordinator to be kept in the regulatory binder.

Possible Adverse Events, Definitions of Adverse Events and Management

Adverse event

An adverse event (AE) is defined as any untoward medical occurrence, including an exacerbation of a pre-existing condition, in a patient in a clinical investigation who received a pharmaceutical product. The event does not necessarily have to have a causal relationship with this treatment.

Serious adverse event

A serious adverse event (SAE) is defined as any AE which results in death, is immediately life-threatening, results in persistent or significant disability / incapacity, requires or prolongs patient hospitalisation, is a congenital anomaly / birth defect, or is to be deemed serious for any other reason if it is an important medical event when based upon appropriate medical judgement which may jeopardise the patient and may require medical or surgical intervention to prevent one of the other outcomes listed in the above definitions. Patients may be hospitalised for administrative or social reasons during the study (e.g. days on which infusion takes place, long distance from home to site,). These and other hospitalisations planned at the beginning of the study do not need to be reported as a SAE in case they have been reported at screening visit in the source data and have been performed as planned.

"**Adverse Events of Special Interests**" or "**AEs of Special Interest**" shall include:

1. Severe Allergic Reactions
2. Skin changes
3. Angio-edema
4. Heart attack or cerebral attack
5. Neurological manifestations
6. Acute Pancreatitis or acute hepatic failure
7. Acute worsening of renal function
8. Development of malignancy, occult or overt
9. Severe Documented Hypoglycemia (lab blood sugar less than 40mg%)

AEs of special interest are a list of medical topics that are under close surveillance. AE should be reported in an expedited manner similar to Serious Adverse Events even if they do not meet the seriousness criteria.

Please refer to the site master file for the complete list of AESIs (27-30).

Intensity of adverse event

The intensity of the AE should be judged based on the following:

• Mild: Awareness of sign(s) or symptom(s) which is/are easily tolerated

• Moderate: Enough discomfort to cause interference with usual activity

• Severe: Incapacitating or causing inability to work or to perform usual activities

Causal relationship of adverse event

Medical judgment should be used to determine the relationship, considering all relevant factors, including pattern of reaction, temporal relationship, de-challenge or re-challenge, confounding factors such as concomitant medication, concomitant diseases and relevant history. Assessment of causal relationship should be recorded in the case report forms.

Yes: There is a reasonable causal relationship between the investigational product administered and the AE.

No: There is no reasonable causal relationship between the investigational product administered and the AE.

Worsening of the underlying disease or other pre-existing conditions

Worsening of the underlying disease or of other pre-existing conditions will be recorded as an (S)AE in the (e)CRF.

Changes in vital signs, ECG, physical examination, and laboratory test results

Changes in vital signs, ECG, physical examination and laboratory test results will be recorded as an (S)AE in the (e)CRF , if they are judged clinically relevant by the investigator.

Responsibilities for SAE reporting: The Sponsor shall report (i.e., from signing the informed consent onwards through the trial defined follow-up period) all SAEs and non-serious AEs which are relevant for a reported SAE and Adverse Events of Special Interest (AESI) by fax or other secure method using BI IIS SAE form to the BI Unique Entry Print in accordance with timeline specified below.

• within five (5) calendar days upon receipt of initial and follow-up SAEs containing at least one fatal or immediately life-threatening event;

• Within ten (10) calendar days upon receipt of any other initial and follow-up SAEs.

Address for contact:

BIPI Unique Entry Point:

Boehringer Ingelheim Pharmaceuticals, Inc.

900 Ridgebury Road Ridgefield, CT

Fax: 1-203-837-4329

E-mail: PV_global_casemanagement@boehringer-ingelheim.com.

For each adverse event, the investigator will determine the expectedness of the investigational drug to the AEs as defined in the Listed Adverse Events section of the Boehringer Ingelheim's (BI's Summary of Product Characteristics (SmPC) or Product Information (PI) for the authorised Study Drug provided by BI.

4.5 Study Schematic. Time and Events Schedule

Time and Events Schedule for Protocol

Procedure	Screening Visit (Week -4)	Baseline/ Randomization Visit 1 (Week 0)	During Treatment Visit 2 (Week 6)	End-of-Treatment Visit 3 (Week 12)	Follow up phone call (week 16)
Eligibility Assessments					
Informed Consent	X				
Inclusion/Exclusion Criteria	X				
Medical History	X				
Safety Assessments					
Physical Examination	X			X	
Targeted Physical Examination (as needed)		X	X	X	
Vital Signs	X	X	X	X	
Assessment of Signs and Symptoms		X	X	X	
Adverse Events Assessment	X	X	X	X	X
Laboratory Tests (Biochemical)	screening	X	X	X	
Urine Pregnancy (If needed)	X	X	X	X	
Spot Urine Sample	X	X	X	X	
Tanita Scale Body Composition		X	X	X	
Waist / Hip		X	X	X	
Efficacy Assessments					
Peripheral blood draw for CD34+cell harvest		X	X	X	
Pulse wave analysis and Pulse wave velocity assessment		X	X	X	
RMR		X	X	X	
Clinical Drug Supplies					
Randomize		X			
Dispense Study Treatment		X	X		

Screening visit= week-4; Visit 1= week 0, Visit 2= week 6 and Visit 3= week 12.

Research Study Design and Methods:

Patient Definition and Selection: Adults aged 40-70 years will be recruited from the GWU MFA research patient database provided by MFA Information Technology, and then contacted via email and phone, from the endocrinology and other provider clinics at GWU and through referrals from the Veterans Affairs (VA) clinics. Suitable advertisements for the study patient enrollment will be posted. Patients will be included in the study if they have been diagnosed with type 2 diabetes within the previous 15 years using criteria of the American Diabetes Association (2), and are currently treated with a stable dose of Insulin, Metformin, or both. We plan to enroll subjects with HbA1C between 6.5% and 10% (both inclusive) and with BMI 25-39.9 kg/m^2 (both inclusive). Post enrollment, patients will be randomized to Linagliptin 5 mg or placebo as detailed in Study Design for direct comparison between the two regimens.

Before any study procedures are performed, subjects will have the details of the study described to them, and they will be given a written informed consent document to read. Then, if subjects consent to participate in the study, they will indicate that consent by signing and dating the informed consent document in the presence of study personnel.

Recruitment: Subjects will be recruited from the physician clinics at GWU, Medical Faculty Associates, where Dr. Sen is a clinician, as well as through referral from the VA. All patients will receive counseling regarding the study aims and methods.

Study Design Overview: We propose a 2-arm randomized, double-masked, placebo-controlled, parallel group, longitudinal study of 12-weeks duration. (See study design below), recruited over 3 years. The 12 week time interval has been previously shown adequate to observe changes in endothelial progenitor stem cells, biochemistry and importantly, PWV changes [20, 36]. Patients will be randomized to 2 groups:

Control, (n=20), Insulin/Metformin/Both + Lifestyle + Placebo

Treatment, (n=20) Insulin/Metformin/Both + Lifestyle + Linagliptin 5mg

This proposed study is based on patients with type 2 diabetes of ≤15 years duration, who are on stable dose of insulin, Metformin, or both for at least 3 months but still remain poorly controlled with HbA1C between 6.5% and 10% (both inclusive), but not overtly out of control (> 10% of HbA1C). Our study design is similar to that of Fadini et al [20] where Sitagliptin was compared to placebo with endothelial progenitor cells as an outcome measure in patients with type 2 diabetes over 4 weeks.

Though our study is over 12 weeks we do not anticipate significant drop in HbA1C in between the two groups in this relatively short time period to act as a confounding factor on CD34+ cell number, function and gene expression.

Most clinical studies looking at HbA1C reduction were of a much longer duration of 6 months or above and most DPP-4 inhibitor studies are with animal EPCs where comparison with human CD34+ population is difficult to make. In one study (58) where saxagliptin was used for **12 weeks** in drug-naïve type 2 diabetes patients there was placebo-subtracted HbA1c reduction noted of 0.45-0.63%. It showed mean placebo-subtracted reductions in fasting serum glucose of 20 mg/dL. From our laboratory studies of in vitro human EPC studies we do not think glucose level change of 20 mg/dL is significant enough to account for changes in EPC number, function and gene expression. However we will take the possibility of glycemia change/ reduction in experimental arm into account during statistical analysis of the results between the Linagliptin and placebo groups.

As per recent clinical guidelines (2), all patients at entry will receive dietary advice by study coordinator, diabetes educator, or registered dietician specifically geared towards type 2 diabetes patients. They will be advised to adhere to 150 minutes of weekly aerobic exercise (50-70% of maximal heart rate). Patient's activity will be monitored using accelerometers and will be downloaded at visit 2 and visit 3 by the study staff.

Subjects will be taking 5.0 mg of Linagliptin or placebo. Dose titration of insulin will be allowed so that the HbA1C is maintained below 10%.

Visits with outcome measurements will be scheduled for the morning while overnight fasting (except water) to reduce diurnal variability, and clinical parameters will be obtained at scheduled office visits as depicted in the study schema and will be measured at each outcome visit as follows:

Week -4: Subjects will be screened and eligibility will be verified based on physical and laboratory tests. Equal numbers of male and female patients will be recruited. All patients will be educated on the recommended lifestyle changes of maintaining a level of aerobic activity of 150 minutes/ week (non-supervised) and adherence to a healthy diet as advised by the study coordinator, a diabetes educator, or a dietitian as part of the type 2 diabetes patient management plan [2]. We will allow 4 weeks for the patients to reach a steady state while implementing diet and exercise changes.

Week 0: At this stage patients will be randomized into the study. Subjects will have the first set of measurements. We will make sure that patients still meet the inclusion criteria. They will be started on medication or placebo and will be encouraged to continue 150 minutes/week of moderate physical exercise and dietary management for the remainder of the study.

Weeks 0, 6 and 12: Subjects will all have the same measurements and will continue on study medication or placebo. All primary and secondary measures as outlined before will be carried out.

The cellular outcome measures are as follows:

From MNC population (pre magnetic column sorting):
A. FACS Analysis, 1 million cells in each tube/assay column. 12 tubes-- total 12 million. (All labelling antibodies are obtained from Miltenyi Biotec except Sytox blue-Invitrogen and anti-VEGEFR2/KDR-R&D systems)
1. Unlabelled
2. CD34
3. CD309 (VEGFR2-KDR)
4. CD184 (CXCR4), receptor substrate for SDF1 alpha
5. CD31 (PECAM-1, a mature circulating endothelial cell marker)
6. CD133 (a progenitor marker)
7. CD144 (Vascular Endothelial cadherin)
8. Sytox Blue OR Propidium Iodide staining (to detect apoptotic cells)
9. Combinations of 2+3+4+8, to assay percentage of double or triple positive cells
10. (3+4+5+8), to assay percentage of double or triple positive cells
11. 500,000 CD34+ cells, post column will be stained with – PE labelled + Sytox Blue (as a quality control measure, post column)

B. Plate 5 million cells in each well of a 6 well or 12 well plates (2 million) BD Biosciences Human Fibronectin coated plate, total 2 wells, total 10 million mononuclear cells (prior to CD34 magnetic bead sorting) using CFU Hill Colony forming unit (Stem Cell Technologies- Cat#05900) media for Colony Forming unit (CFU) Assay.
2 days post plating, harvest non-adherent cells from each well, counted and will be re-plated at 1.0 x 10^6 cells/well in 12-well fibronectin coated plate. It is important than cell number of 1.0 x10e6 is maintained in each well.
CFU needs to be counted on Day 7 post plating (from initial plating).

From CD 34+ cell population (Post Sorting):
A. Migration Assay using SDF1 Alpha concentrations of 0, 10, 100ng/ml using 100,000 cells in 300ul serum free cell suspension media per insert. If sufficient cells are available we will note migration in response to VEGF-A (0, 20, 50 ng/mL).
SDF1 Assay alone will need 100,000*9 = 0.9 million cells. Use triplicates.
We will use 24 well plate using 3 micron pore migration membrane inserts from BD or Corning (354575).

Genes to be assessed on CD34 positive, CD34 negative cells:

Endothelial lineage cell surface markers: CD34, VEGFR2 (KDR), CD31, CD144
For Anti-oxidant gene expression: Superoxide dismutase (SOD) 1, 2 and 3, catalase, glutathione- peroxidase.
Apoptosis pathway: p53, p21, Bcl2, caspase-3

Endothelial Function Assay Gene: eNOS
Genes associated with progenitor cell chemotaxis: VEGF-A, SDF1 alpha, CXCR4

Secondary Measures:

- Highly selective C-reactive protein (hs-CRP), IL-6, IL-10, TNF-alpha, and fasting lipid profile including ApoA1 and ApoB (3, 6)
- Serum SOD activity and SDF1 alpha (ELISA), VEGF-A (ELISA) and GLP-1 (ELISA) levels will also be assessed (14, 20, 24, 39).
- Assessment of insulin sensitivity using the HOMA-IR (32), calculated from individual serum measures (fasting glucose (mg/dl)* insulin (μU/mL)/405) [37].
- Adiposity will be measured using the Tanita Body Composition Analyzer scale, measured as percentage body fat, BMI, waist-hip measurements (5, 55)
- Fasting lipid profile will be checked as a marker of insulin resistance and lipo-toxicity at weeks 0, 6 and 12.
- We also intend to acquire Pulse wave analysis and Vascular Flow using SphygmoCor CP system from ATCOR to measure central arterial pressure and arterial stiffness (38-42). The secondary measures are indirect measures of endothelial inflammation in early type 2 diabetes patients. (30, 31, 55, 56, 57).
- Obtain indirect calometric measurements of basal metabolic rate using machine obtained from Korrs Medical. This is a non invasive procedure. We plan to measure the oxygen that the body consumes. It will help us calculate a patient's Resting Energy Expenditure (REE), commonly referred to as a Resting Metabolic Rate (RMR). As our protocol is directed to overweight and obese individuals using incretin therapy, it will be beneficial to note if the treatment modifies RMR. Description: The machine ReeVue by KORR directly measures the concentration of oxygen breathed out by each patient. The patient breathes through a simple mouthpiece as all the exhaled air is collected and analyzed. Because there is a direct correlation between oxygen consumed and calories burned (4.813 calories for every milliliter of oxygen consumed), an accurate measurement of oxygen consumption is an effective measurement of calorie consumption. The machine uses a one-way valve in the disposable (MetaBreather) mouthpiece. This draws in fresh room air with each inspiratory breath and eliminates concerns about cross contamination. The disposable mouthpiece is thrown away. It is un-necessary to clean the equipment or the tubing between each patient. This

equipment is FDA approved and has been used in physician offices as an oxygen uptake test with associated CPT code #94690. (58)

- Urine pregnancy testing for women of child bearing potential
- Creatinine Clearance and proteinuria estimation via random spot urine.

We will obtain a total of 85 mL of peripheral blood per visit. Of these 85 mL, 60 mL will be used to obtain CD34+ cells from MNC population and 25 mL for biochemistry and serum ELISA assays.

We may obtain 95mL of peripheral blood at visits 2 and 3 if necessary, and the patient agrees. Of this 95mL, 75mL will be used to obtain CD34+ cells from MNC population and 20mL for biochemistry and serum ELISA assays.

4.7 Study Materials

BI, manufacturers of Linagliptin are expected to provide Linagliptin and matching placebo at no cost for this study.

4.8 Safety Assessments

Study drug toxicities will be assessed continuously. Adverse events will be evaluated on a continuous basis while the patient is on study and until 30 days after the last dose of study drug. Patients should be followed until all treatment-related adverse events have recovered to baseline or are deemed irreversible by the principal investigator.

5 STATISTICAL CONSIDERATIONS

Statistical Analysis:

Sample Size Estimation:

The total sample size requested, accounting for attrition over the 12-week period, is 20 subjects per group or 40 subjects total. We plan to allow for 25% attrition (drop out), therefore plan to screen 55 patients over three years.

Sample size estimates were based on the effects of exercise on CD34+/KDR+ cells and VEGF, as described in the literature (47-50).

This is a pilot study and accurate power calculation is not feasible.

The effect of a single session, as well as extended training, on healthy subjects or those with existing cardiovascular conditions appears to increase the CD34+/KDR+ cells and VEGF.

To compute sample size we used the approach suggested by Diggle, Liang, & Zeger (1994)(51) which compares the rates of change in the two study groups over time. This approach incorporates the number and interval of time points and the correlation among repeated measures. For this study, we will employ one baseline and two follow-up measures at 6 and 12 weeks. Further, we will assume a correlation 0.60 among repeated measures of the outcome. We consider this a conservative estimate since Frison and Pocock (1992) (52) suggest a correlation of 0.65 as reasonable in the absence of an existing estimate. We also note that as this correlation increases, statistical power also increases.

The results in the table below show the expected mean difference in study groups at the end of follow-up, as well as the average rate of change in the two groups at 80% power and 90% power. To estimate the effect of Linagliptin on the CD34+/KDR+ cells, we expect that the effect would be at least 25% greater than the effect seen for exercise alone. Using the results from Sandri et al. (2005)[49] for the rate of change and the variability, the CD34+/KDR+ cells increased an average rate of about 4/wk with a standard deviation of about 15. Thus, for a 25% increase in the rate of change for the CD34+/KDR+ cells due to Linagliptin, a sample size of 18 subjects per group would provide about 84% power, assuming measures taken at baseline and 2 equally-spaced time points over 12 weeks. At the conclusion of follow-up, we would expect study groups to differ by an average of 12 cells. If the effect of Linagliptin is only 20%, a sample of 18 would provide about 70% power, whereas a sample of 20 would provide about 73% power.

For VEGF, the results from Sandri et al. (2005)(49) were mixed with one study group showing a .6 pg/mL per week increase and another showing a 0.5 pg/mL per week decrease after 4 weeks of training. If we assume that Linagliptin will increase VEGF consistently at 0.3 pg/mL per week difference in slopes compared with exercise alone, then assuming a standard deviation of 5 and correlation or 0.60 among repeated measures, a sample size of 18 per group would provide about 77% power to detect a difference in slopes of 0.3 pg/mL per week or a mean difference in the groups at the end of the study of 3.6 pg/mL. A sample size of 27 would provide about 90% power to detect the same effects.

Thus, we feel that a sample size of at least 18 subjects per group with complete data would provide sufficient power for the study outcomes. In order to insure that we will have 18 per group who complete the study, we will enroll 20 subjects per group in order to account for attrition over the 12-week intervention period.

Biochemical Measure	Mean difference at end of 12 weeks	Sample Size per group	Power
CD34+/KDR+	12 cells	18	0.84

cells	(25% increase; 4/wk vs. 5/wk)	22	0.90
VEGF, pg/mL	3.6 pg/mL (50% increase; (0.6/wk vs. 0.9/wk)	18	0.77
		27	0.90

Sub-Group Subject Number Breakdown:

To estimate the effect of linagliptin on CD34+/KDR+ cells, we originally proposed a sample size of 20 per group in 2 groups, giving us power = .73, assuming alpha=.05, between time points of .60 and equal allocation between groups. Thus, the group x time interaction effect size we were modeling was an interaction that explained 3% of total variance, or a partial eta-square = .0291. Using 3 groups (Insulin only, metformin only, metformin + insulin), with the same effect size, we would achieve this same level of power using a total sample size of 51 (17 per group). However, given the relatively small numbers of potential participants with insulin-only (n=21) and insulin+metformin (n=54), and the relative large number with Metformin only (260), we will need to change the allocation ratio between groups. Maintaining the same total sample size as originally proposed (40), we will allocate patients from each group in the following numbers**: Metformin only (22), Metformin+insulin (12), Insulin only (6).** We recognize that we will be underpowered for comparisons between the Metformin + insulin versus Insulin groups. However, comparisons with the Metformin-only group will still have reasonable power. Given the small number of potential patients with Insulin-only, this is a feasible strategy for including this group in the analysis.

Data Analysis.

The distributional assumptions of all measures will be examined. Means and standard deviations will be computed for continuous measures and proportions for categorical variables. Graphical representations of the mean group slopes and individual slopes will be generated and inspected. Study groups will be compared to determine whether any imbalance between the groups on patient characteristics remain after randomization. If imbalance is evident, by inspection, all models will be adjusted for the unbalanced covariates. Change in outcome over time will be examined using a multilevel approach with linear mixed models (LMM) (53, 54). The longitudinal multilevel modeling approach will enable us to examine characteristics of within-person change, as well as between-group differences that may influence change. LMMs handle missing data more efficiently than traditional ANOVA designs. In the linear mixed models, the patient is considered a random effect and the outcome measured at specific time points is nested within patient. In an initial model containing only the variable TIME, we will use a likelihood ratio test to determine whether a random effect for time should be modeled.

The model below represents a random intercept for patient and a random coefficient for TIME.

$$Y_{ij} = \beta_0 + \beta_1 Time_{ij} + u_{i0} + u_{i1} Time_{ij} + \varepsilon_{ij}$$

We will examine whether TIME may be modeled linearly by testing the added contribution of TIME (represented with two indicator variables) to a model with TIME as a single continuous variable. Assuming time may be modeled as a continuous variable, subsequent analyses will add study group and other patient characteristics as covariates. We are particularly interested in whether there is a significant interaction between study group and time, adjusting for other patient level covariates. A significant interaction would indicate that the slopes in outcome over time differ by study group. If the global test of interaction is significant, subsequent significance testing of the pair wise comparisons of group slopes group will be adjusted for multiple comparisons. The model also includes coefficients for the group-by-time interactions and random effects for patient and time.

$$Y_{ij} = \beta_0 + \beta_1 Time_{ij} + \beta_2 Group_i + \beta_3 (Time_{ij} \times Group_i) + u_{i0} + u_{i1} Time_{ij} + \varepsilon_{ij}$$

In the event that TIME is modeled using indicator variables, two indicator variables will be used to represent the three time points, with baseline serving as the reference category. The interaction between GROUP and TIME will be modeled in a similar fashion using two additional indicator variables. The above multivariable model enables us to adjust for time-varying covariates, such as glucose. Since glucose will be measured contemporaneously with the outcomes at each visit, however, causal interpretations of glucose and any outcome are suspect. As such, we will consider glucose as a nuisance covariate, and consider any relationship between glucose and an outcome as merely associative.

APPENDIX A: OPTIONAL URINE EXOSOME STUDY

A urine sample is already required as a part of Dr. Sen's Linagliptin protocol. This urine will be used for urine pregnancy tests (if applicable), and labcorp tests for microalbumin/creatinine ratio. There will likely be excess urine.

Subjects will have the option to opt in or out of a research study in which the urine will undergo a urine exosome analysis.

Purpose of this Study: **Exosomes** are cell-derived vesicles that are present in many and perhaps all biological fluids, including blood, urine, and cultured medium of cell cultures. The reported diameter of **exosomes** is between 30 and 100 nm, which is larger than LDL, but much smaller than for example, red blood cells. Exosomes are either released from the cell when multivesicular bodies fuse with the plasma membrane or they are released directly from the plasma membrane.[3] Evidence is accumulating that exosomes have specialized functions and play a key role in, for example, coagulation, intercellular signaling, and waste management.[1] Consequently, there is a growing interest in the clinical applications of exosomes. Exosomes can potentially be used for prognosis, therapy, and biomarkers for health and disease. In this study we are particularly interested

in exosomes released from podocytes as a marker of podocyte function and podocyte inflammation. Podocytes are often considered a modified endothelium and one of its more commonly used parameter is microalbuminuria.
In this study we are using urine exosomes as an added parameter of kidney function other than microalbuminuria and GFR calculation.

The procedure for this study is as follows:
From this sample, 10-20 mL of the fresh spot urine (or as much as possible) is placed into a urine cup containing 1 tab of cOmplete Ultra - protease inhibitor cocktail (Roche) for exosome analysis. The specimen can be refrigerated at 4ºC for ~ 1 week or frozen at -80ºC until assay, or will be processed for exosomes as follows;

Spot urine for exosomes isolation has been successfully employed in our laboratories from ~15 ml urine using a differential centrifugation method[2,3]. The supernatant will be subjected to ultracentrifugation, with final centrifugation at 200,000g for 2 hr at 4ºC to obtain the urinary exosome pellet. The pellet will be tested for exosomes markers, namely CD63, CD9, CD81, Hsp70, and WT-1, nephrin and podocalyxin, using one of two methods. Preferably mRNA will be extracted and then RT-PCR will be run. Alternative analysis will be done using gel electrophoresis followed by Western blot techniques.

Method 1: Urine exosome mRNA extraction
mRNA extraction from exosomes will be accomplished by using commercial kits (Norgen Biotek Corp., Catalog # 47200). Thereafter, a pre-amplification step will take place, this is a necessary step to measure mRNA in exosomes and will be followed by quantification of specific endothelium and podocyte proteins such as CD9, WT-1, nephrin and podocalyxin by RT-PCR.

Total urinary protein content, albumin and creatinine will be measured via Lapcorp for the same urine samples that have been collected.

Method 2: Gel electrophoresis and Western blot
Exosome pellet will be dissolved in Laemli buffer and proteins will be resolved by gel electrophoresis (4-12% polyacrylamide gel) under denaturing conditions. Subsequently, proteins will be transferred to a PVDF membrane by Western blotting using a dry blotting method (iBlot). The blot will be incubated with blocking solution for 1h, and subsequently exposed to a solution with the first antibody overnight, at 4ºC. After washing (5 x 5 min), the membrane will be exposed to a secondary antibody (horseradish peroxidase [HRP]-conjugated) for 1h at room temperature. Finally, the membrane will be incubated with an HRP substrate reagent to reveal the proteins bound to the antibodies tested. Quantitative analysis will be performed by densitometry readings of the positive signals corrected to creatinine concentration of the urine aliquot equivalent to each exosome sample.

End-point: Exploratory but in conjunction with urine albumin/creatinine ratio will help to establish impact of Linagliptin on renal function in diabetes related CKD population.

Guidance document for Adverse Events of Special Interest

Adverse events of special interest are events that we are keeping under close surveillance and we would be very grateful if non-serious adverse events relating to the following medical topics can be reported using the SAE form so that the Global Pharmacovigilance Team receives it as soon as possible:

Hepatic adverse events
Renal adverse events
Hypersensitivity reactions
Skin lesions
Pancreatitis
Pancreatic cancer
Thyroid neoplasia or cancer
Cardiac failure

The following information is key in assisting case evaluation. If possible please provide the following when reporting an Adverse Event of Special Interest:

Hepatic adverse events

- Alcohol intake (e.g. grams or units per week) present and past
- Jaundice (personal or family history)
- Hepatobiliary gallstone disease
- Known hepatic disease- eg metabolic, autoimmmune, malignancy or -metastatic disease
- Metabolic-induced liver disease, non-alcoholic steatohepatitis (NASH)
- Recent administration of other drugs with known hepatic toxicity- please state reason if these are not recorded as co-suspect drugs
- Environmental exposure to liver toxins (e.g. CCL_4, death cap mushroom, vinyl chloride)
- Substance abuse/Intoxications/Intravenous drug use
- History of drug allergy/hypersensitivity reaction
- Infectious diseases:
  - Bacterial, Protozoic/parasitic hepatic infection
  - Viral hepatic serology
- Travel history
- Blood transfusions history
- Site, radiation and character of pain
- Biopsy results &/or hepatology consult reports
- Radiological investigations eg sonography

Renal adverse events

- Clarity on whether the patient has pre-renal, renal-renal or post-renal failure and the cause of this, eg hypovolaemia due to diarrhoea, glomerulonephritis or bladder obstruction
- Fluid balance if possible
- Baseline renal function and past history of renal insufficiency
- Renal and bladder stone disease- if possible with type of stone (eg Ca oxalate, etc)
- Site, radiation and character of pain
- Biopsy results &/or nephrology consult reports
- Known renal or urinary tract disease
- All BUN / U&Es results
- All urine investigation results including microscopy and culture
- Creatinine clearance
- Radiological investigation reports, eg Kidney-Ureter-Bladder X-ray, Intravenous pyelogram

Hypersensitivity reactions

- Clarity on whether the patient has a diagnosed condition- eg angioedema or anaphylaxis as opposed to reporting symptom of tongue swelling
- For systemic hypersensitivity syndromes, all laboratory and radiological investigations and differential diagnoses
- Clear description of clinical presentation, time course and treatments
- If there are consults from other specialities, please include a copy of the report if possible

Skin lesions

- Clarity on whether the patient has a diagnosed condition as opposed to reporting symptoms
- If a dermatology consult has occurred, please send a copy of the report
- Results of any biopsies
- Estimate of the percentage of the total body surface area that is involved
- Clearly state whether or not there is mouth ulceration
- Clearly state whether or not the rash has blisters
- Please provide any evidence of systemic reaction, laboratory abnormalities

Pancreatitis

- Pancreatic enzymes and liver enzyme values, if possible on presentation and during course of the event
- Baseline and current lipid investigations
- Report of Radiological diagnostic procedures, eg sonography, CT, ERCP
- Clinical symptoms with description of site radiation and character of pain
- Clarity on the diagnosis in the verbatim reported term- eg whether the patient had clinical pancreatitis or assymptomatic enzyme rise
- Permission to contact treating physician for follow up information
- All relevant abnormalities in the pancreaticobiliary tree, eg gallstones, stenosis, CBD thickening
- Alcohol intake

- Clarity on whether the patient has chronic pancreatitis with a known aetiology

Pancreatic cancer

- Please clearly state the start date of the study drug, the start date of the presenting symptoms and the date of diagnosis of cancer
- Please state method of diagnosis, staging, grading and (if available) histology
- Family history of cancer or pancreatitis
- BMI of the patient
- If previous radiology had not shown abnormalities, please confirm if vascular imaging techniques had been requested
- Please state all previous diabetic medication
- Has the patient had previous chemo/radiotherapy? If so please provide details
- Please provide permission to contact pancreatic oncologist

Thyroid neoplasia or cancer

- Please clearly state the start date of the study drug, the start date of the presenting symptoms and the date of diagnosis of cancer
- Please state method of diagnosis, staging, grading and (if available) histology
- Family history of cancer
- Please state all previous diabetic medication
- Has the patient had previous chemo/radiotherapy? If so please provide details
- Please provide permission to contact oncologist
- Please clarify whether the lesion is benign or not
- Please list previous medications used to treat thyroid disease

Cardiac failure

- Please clearly state the start date of the study drug, the start date of the presenting symptoms and the date of diagnosis of heart failure
- Did the patient have a history of heart failure, coronary artery disease, renal failure, arrhythmia, of heart valve abnormality?
- If EchoCG was done, please provide the report
- Provide NYHA class of CHF

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