Erythrocyte membrane fluidity as a biomarker for a quantitative biological assessment of cardiovascular risk score in type 2 diabetes

Study Code: ***RF-2019-12369293***

PROTOCOL APPROVAL

Summary

1. SYNOPSIS

Title	Erythrocyte membrane fluidity as a biomarker for a quantitative biological assessment of cardiovascular risk score in type 2 diabetes
Study code	RF-2019-12369293
Version	Ver 1.0 of 18/01/2021
Trial Type	Observational
Indication	Subjects affected by Type 2 Diabetes Mellitus with or without CV complications
Patient Involvement in the Study	1 day
Total Study Duration	36 months
Study Rationale	The cardiovascular (CV) risk in type 2 diabetic patients is correlated to various aspects such as an inadequate glucose control, expressed by high HbA1c value, and the presence of hypercholesterolemia and hypertriglyceridemia with an increase in free fatty acids (FFA). The average amount of plasma glucose and plasma lipids in type 2 diabetes patients lead to physical and chemical alterations in cellular membrane properties, modifying its composition, packing and lipid distribution. All these changes determine membrane fluidity variations. Given these we hypothesize that the membrane fluidity could be used as quantitative biological parameter to assess the CV risk in type 2 diabetes patients.
Aim	This project aims to test whether changes in membrane fluidity of red blood cells (RBC) may be associated with cardiovascular risk outcome in a cohort of type 2 diabetic subjects. Membrane fluidity, quantified by a parameter called Generalized Polarization (GP), can be used to compute a sensitive score of T2DM-associated cardiovascular risk in long term disease's management.
Endpoints	**Primary Endpoint:** to underline any difference of membrane fluidity, expressed as GP, in type 2 diabetic subjects with cardiovascular complications compare to ones without previous cardiovascular events. **Secondary Endpoints:** ● To confirm the validity of a new marker of cardiovascular risk (RBC fluidity) in a cohort of type 2 diabetic subjects. ● To verify the ability of the new marker of cardiovascular risk to provide a score independent of HbA1c and other diabetes related risk factors (disease duration, therapy,

	parameters of functional and / or structural vascular disease). ● To compare the efficacy of the new cardiovascular risk marker (RBC fluidity) with that of other systems already in use, such as the UKPDS engine risk score, for the calculation of cardiovascular risk in a cohort of diabetic subjects in primary prevention. This system allows to generate a numerical score deriving from the combination of several risk factors, partly related to diabetes (duration of the disease in years, value of HbA1c) and partly unrelated to diabetes (age, gender ethnicity, cigarette smoking, values of arterial pressure and cholesterol parameters of functional and / or structural vascular disease), able to estimate the risk at 10 years of fatal and not CHD or stroke.
Inclusion Criteria	***For subjects with type 2 diabetes*** **1.** Informed Consent Obtained **2.** Age ≥30 ≤ 70 years old, both male and female **3.** Diagnosis of Type 2 Diabetes Mellitus **4.** Adequate metabolic control (HbA1c <7,5%) in the last 6 months **5.** Peptide C a digiuno> 1 ng / mL **6.** No changes in medical therapy in the last 6 months **7.** For the group with cardiovascular complications is required at least one previous events of Ischemic heart disease (any of the following): − Documented Myocardial Infarction − Percutaneous Coronary Intervention − Coronary Artery Bypass Grafting − Objective Findings of Coronary Stenosis (> 50%) in at least 2 arteries Cerebrovascular disease (any of the following): − Documented ischemic stroke (Known TIA, primary intracerebral or subarachnoid hemorrhage do not qualify) − Carotid stenting or endarterectomy ***For the healthy subjects*** **1.** Signed informed consent **2.** Age ≥30 ≤ 70, years old; both male and female
Exclusion Criteria	***For subjects with type 2 diabetes*** **1.** Previous participation in this trial. Participation is defined as signed informed consent. **2.** Diagnosis of Type 1 Diabetes Mellitus, maturity onset diabetes of the young (MODY). **3.** Inability and not-willingness to adhere to the protocol. **4.** Previous pancreatic surgery or chronic pancreatitis. **5.** Medical history of cancer or treatment for cancer in the last five years prior enrolment.

	6. Blood dyscrasias or any disorders causing haemolysis or unstable red blood cells (e.g., malaria, babesiosis, haemolytic anaemia). ***For the control group*** **1.** Previous participation in this trial. Participation is defined as signed informed consent. **2.** Diagnosis of Type 1 or Type 2 Diabetes Mellitus, LADA, MODY. **3.** Inability and not-willingness to adhere to the protocol. **4.** Medical history of cancer or treatment for cancer in the last five years prior enrolment. **5.** Blood dyscrasias or any disorders causing haemolysis or unstable red blood cells (e.g., malaria, babesiosis, haemolytic anaemia). **6.** Medical History of previous cardiological problems or cardiovascular events
Study Procedures	**<u>Visit</u>** ● Signed and dated Informed Consent obtained prior to start any procedure scheduled for the study. ● Verification and confirmation of the inclusion/exclusion criteria ● Demographics, medical history ● Physical Examination (Body weight, body mass index, waist/hip ratio) ● Vital Signs (BP, HR) ● Blood Sample ● Few subjects, will undergo to measurement of the ankle-arm index (ABI), standard Doppler-echocardiogram execution and peripheral arterial tonometry of the fingertip (with Endo-PAT 2000 device), for the evaluation of endothelial function; Carotid-femoral PWV for estimating aortic stiffness; bilateral carotid ultrasound examination for IMT and plaque volume assessment using high resolution B-mode ultrasound (MyLab70, ESAOTE) ● Concomitant Medications **<u>Laboratory procedures</u>** ● Samples preparation ● Evaluation of membrane fluidity by confocal microscopy
Statistical Considerations	**<u>Statistical Analysis</u>**. To obtain a statistical power of 0.8 with a two-tailed alpha of 0.05 in a multivariate logistic regression model with presence of CV event as the dependent variable and Hba1C, age, duration of diabetes, smoking habits, sex, Atrial fibrillation, SBP, total cholesterol and HDL levels as the independent variables, we

	will enroll 100 patients with CV events and 100 patients without CV events, following the rule of 10 events for 1 independent predictor. This sample size will also allow us to compare differences in means across groups based on preliminary measurements performed in 9 patients (mean 0.59, SD 0.013 for controls; mean 0.56, SD 0.034 for diabetics) and to detect as statistically significant a correlation of 0.28 between fluidity and a UKPDS ENGINE CV risk score in the group of type 2 diabetic subjects without CV events.
Planned Sample Size	200 (100 with CV events and 100 without CV events); 50 healthy controls
Total Number of Centers	2 in Italy
Data Management	Diabetic subjects and healthy controls will be enrolled in the "Center for endocrine and metabolic diseases" and in Endocrinology and Diabetes Department of Azienda Ospedaliera Pisana, through a dedicated Study Coordinator. The study coordinator will show the aims of the study and enroll them in the study after consensus form is signed. The source data, recorded in the appropriate source documentation, will be reported by the Investigators in a CRF.
Institutions and Units	• U1- IRCCS Fondazione Policlinico Universitario A. Gemelli; Center for endocrine and metabolic disease (Coordinator: Prof. A. Giaccari) • U2- U2- Azienda Ospedaliera Pisana; Endocrinology and Diabetes Department (Coordinatore: Prof. S. Del Prato) • U3- Università Cattolica del Sacro Cuore; Physics Department (Coordinator: Prof. G. Maulucci)
Contacts for Scientific Issues	Prof. Andrea Giaccari, PI

2. INTRODUCTION

2.1. BACKGROUND

Although long-term weighted mean HbA1c is closely associated with the development of severe complications such as cardiovascular disease, nephropathy, neuropathy, and retinopathy in type 2 diabetes (T2D) [1], HbA1c is not representative of all risk factors contributing to cardiovascular risk score in T2D, which include impaired glucose regulation, abdominal obesity, hypertension, atherogenic dyslipidemia (characterized by elevated levels of triglycerides and low levels of high- density lipoprotein cholesterol), microalbuminuria, and specific proinflammatory and prothrombotic abnormalities of endothelial cells and vascular functions [2]. Although each component of the metabolic syndrome brings an individual increased

risk for Cardio-Vascular Disease (CVD), the effect is enhanced by the combination of factors, and risk varies considerably, even within the population with diabetes [3]. Further, despite treatment with intensive, multifactorial therapy, patients still experience CVD related events; therefore, residual risk remains [4,5]. Advances in the management of CVD risk factors have reduced the rate of cardiovascular events, but even intensive therapy for CVD leaves residual CVD risk in patients with diabetes. In the STENO-2 study, death due to CVD was reduced by 62% but not eliminated [6].

However, the residual risk is independent of glucose levels and target HbA1c, suggesting that other factors have an impact on CVD outcomes. This notion provides a compelling rationale for using biomarkers to improve individual risk prediction.

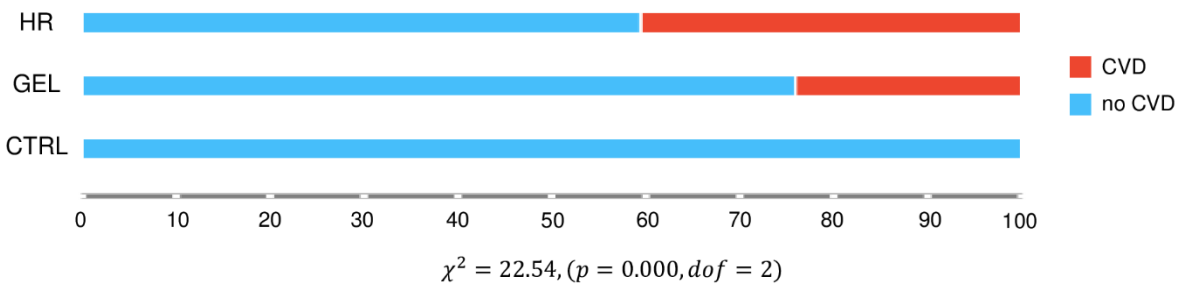
Hypertriglyceridemia has been unfairly neglected for long time as a major cardiovascular risk factor, however epidemiological data suggest that high plasma triglyceride (TG) levels are associated with a substantial increase in long- term total mortality and CVD risk, and recently the REDUCE-IT trial has demonstrated that the risk of ischemic events, including cardiovascular death, was reduced by treating patients with 2 g of icosapent ethyl twice daily [7-9]. Triglyceride- rich lipoprotein lipolysis releases oxidized free fatty acids (FFA) that induce endothelial activation, inflammation, and thrombosis, which may initiate early vascular abnormalities that promote atherosclerosis [10-11]. The use of HbA1C is limited, since this assay is specific for glucose levels, and cannot account for plasma triglyceride and FFA levels, which are impaired in type 2 diabetes and modified with nutritional interventions on dietary lipid composition. Further, changes in circulating FFA levels could directly impact CVD risk by modifying the fluidity of the cell membrane, which is determined by its phospholipid composition [12]. Cell culture studies have shown that membrane fluidity affects cellular functions, i.e. glucose transport across membranes, as well as the properties of the insulin receptor [13,14]. Recently, the estimation of lipophilic index, in plasma and adipose tissue, has been proposed as a measure of overall FA fluidity, which is associated with a higher risk of coronary heart disease (CHD) [15,16], while a lower fluidity of erythrocyte membranes is associated with a higher risk of type 2 diabetes. Our group monitored changes in membrane viscoelastic properties of circulating red blood cells (RBC) in type 2 diabetes using atomic force microscopy [17], and showed that the physical state of the RBC membrane, which shows pronounced alterations in Type 1 diabetes mellitus, could be an ideal biomarker for monitoring disease progression and treatment [18,19], especially because it is able to integrate physical modifications occurring in the three months prior to measurement.

2.2. PRELIMINARY DATA

Our group has extensively analyzed, in in vitro cell cultures and in ex-vivo extracted tissues, how glycosylation, oxidation and other post-translational modifications of the membrane and transmembrane proteins can alter lipid density, packing, and interactions, which in turn affect fluidity in membranes [20- 24]. We have provided evidence that the physical state of the RBC membrane, determined using a functional two-photon microscopy approach, shows pronounced alterations in Type 1 diabetes mellitus (T1DM), and could be an ideal candidate for monitoring disease progression and the effects of treatment [19, 25]. On these grounds, we performed the same analysis on T2DM patients with and without cardiovascular events. We collected blood samples from 32 non-diabetic subjects (Controls) and 234 T2D subjects, 148 were diabetics with no former major cardiovascular events, while the remaining 86 subjects had a history of major cardiovascular events (any of the following: ischemic heart disease, documented myocardial infarction; percutaneous coronary intervention; coronary artery bypass grafting; objective findings of coronary stenosis (> 50%) in at least 2 arteries; documented ischemic stroke; carotid stenting or endarterectomy).

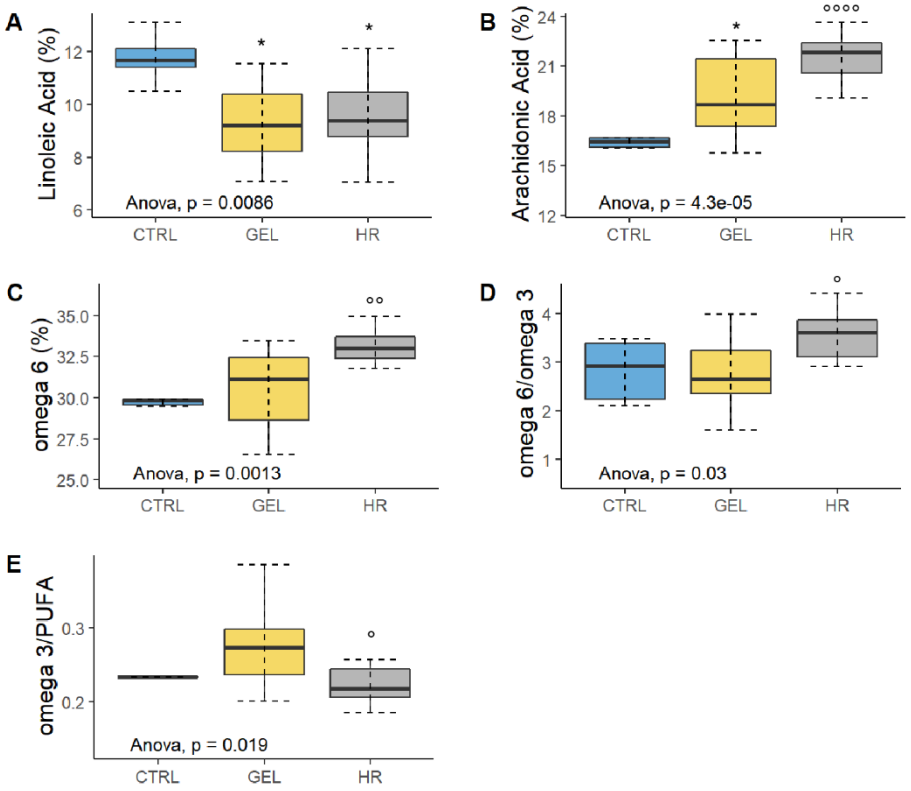
Diabetic subjects were carefully selected among those in stable therapy with HbA1c lower than 7.5%. Then on the basis of GP value, we were able to dived diabetic subjects into 2 groups: GEL, characterized by low membrane fluidity, (GP value: mean±sd =0.460±0.012) and HR, expressing high membrane fluidity (GP value: mean±sd =0.398±0.031). While GEL and HR groups presented different GP values, they matched Age, Duration, HbA1C values, BMI, and lipidic profile (total cholesterol, RBC cholesterol, TG, HDL), as well as a matched gender distribution, UKPDS risk score, smoking habits and treatments controlling dyslipidaemia (STATIN/OMEGA3). Interestingly, GEL and HR differed in the percentage of patients affected by macrovascular complications (GEL: 24.07%; HR: 40.56%) as reported in Figure 1. We can infer that if the patient belongs to the HR cluster, the odds of developing a macrovascular complication increase by 1.68 times (p=0.045).

Fig 1. Percentage of subjects with prior CV events in each group



Then to investigate if this difference in membrane fluidity was due to a different fatty acid composition of the membrane, some samples were further analysed to characterize RBC membrane FA composition, and we were able to compare erythrocyte FA profiles among groups in a sample of 26 patients (n=6 CTRL, n=10 GEL and n=10 HR). What we found is that the percentage of arachidonic acid (AA, 20:4), linoleic acid (LA, 18:2) and the total percentage of omega 6 were significantly different among the 3 groups. The omega 3/PUFA index, which is more sensitive in detecting an alteration in the metabolic fluxes of PUFA, showed a 22% decrease in HR group compared to GEL (p= 0.041). This decrease reflects an altered omega6/omega3 ratio, considered a proinflammatory risk index, which increases in the HR group with respect to GEL (p= 0.041). Conversely, the GEL group displays the same value of omega 6, omega 3/PUFA and omega 6/omega 3 indexes as the CTRL group. (Fig 2)

Fig. 2 Representative PUFA levels and lipidomic indexes. Levels of linoleic acid (A), arachidonic acid (B), total content of omega 6 (C), inflammatory risk index omega 6/omega 3 (D), and omega 3/PUFA ratio (E) obtained from the fatty acid-based red blood cell membrane lipidomic analysis are shown in the box plots for CTRL (in blue), GEL (in yellow), and HR (in gray) clusters, respectively. FA content is reported as a fraction of the total RBC membrane composition (on the y-axis). p-values obtained from the ANOVA are indicated along with the box plots, and post-hoc comparisons are reported (* stands for p-value < 0.05 vs CTRL; ° stands for p-value < 0.05 vs GEL; °° stand for p-value < 0.01 vs GEL; °°°° stand for p-value < 0.0001 vs GEL).



Summarizing the membrane of HR patients presented a pro-inflammatory profile that probably exposed them to a greater residual cardiovascular risk, which is mainly drive by diet. Therefore, we believe that the increase in fluidity observed in the HR cluster with respect to GEL may be ascribed to the alteration in the quantity and quality of PUFA, and that changes in diet composition, possibly with an increase in omega 3 amount, could rebalance membrane FA composition and therefore its fluidity, consequently reducing the residual cardiovascular risk in type 2 diabetic subjects.

3. PURPOSES AND OBJECTIVES OF THE CLINICAL TRIAL

Our working hypothesis is that RBC membrane fluidity, reflecting the state of a more complex network of regulatory processes activated by the disease, can provide a sensitive score of T2DM progression to complement HbA1c in defining the strategy for the long-term management of diabetes. This project aims to test whether changes in RBC membrane fluidity could be associated with cardiovascular risk outcomes in a cohort of type 2 diabetic subjects. We aim to develop a novel high spatial resolution bioimaging system capable of measuring erythrocyte plasma membrane modifications occurring in the three months prior to measurement. The average amount of plasma glucose and plasma lipids in T2DM patients leads to physical and chemical alterations in the cellular membrane properties, modifying the composition, packing and lipid distribution on the erythrocyte membrane. All these changes determine membrane fluidity variations, whose values, quantified by a parameter called Generalized Polarization (GP), can be used to compute a sensitive score of T2DM- associated cardiovascular risk to complement HbA1c in establishing the strategy for long-term management of diabetes.

The measurement of the GP value with a high-throughput, high-resolution microscope, will allow us to determine, with enhanced sensitivity and specificity, the number and the configurations of fluid microdomains.

Specific Aim 1:

To confirm the validity of a new marker of cardiovascular risk (RBC fluidity) in a cohort of type 2 diabetic subjects in predicting the continuum of CV risk, as compared with other risk estimands.

Specific Aim 2:

To verify the ability of the proposed new marker of cardiovascular risk to provide a score independent of HbA1c but associated with other diabetes-related risk factors as accurately measured by several vascular non-invasive diagnostic tools.

Specific Aim 3:

To compare the new marker of cardiovascular risk with more classic risk calculators, such as the UKPDS engine.

4. EXPERIMENTAL DESIGN

Experimental Design Aim 1:

To pursue the objectives proposed in this project, we will perform a cross-sectional, observational study.

Study subjects: Units 1 and 2 will enroll 200 type 2 diabetic patients (aged 30-70 years) with stable metabolic control (HbA1c <7.5 % for at least 6 months), fasting C-peptide > 1 ng/mL and stable medical therapy (including all anti- hyperglycemic agents and insulin treatment) for at least 6 months. Subjects will be stratified into 2 groups: with (DM+CV) and without (DM) previous cardiovascular events. An age and weight-matched control group of non-diabetic subjects without cardiovascular events (controls) will be enrolled from our large population of euthyroid, tiroxine-treated patients with primary hypothyroidism (but otherwise healthy) attending our Centre for Endocrine Diseases for thyroid hormonal monitoring.

The experimental design will be divided into four tasks (T1-T4).

T1. Metabolic evaluation (Unit 1-2). The UKPDS Risk Engine will be utilized to estimate CV risk in all type 2 diabetes subjects without previous CV events. This score can be calculated for any given duration of type 2

diabetes based on current age, sex, ethnicity, smoking status, presence or absence of atrial fibrillation and levels of HbA1c, systolic blood pressure, total cholesterol and HDL cholesterol [26-27]. All the data will be collected in a common database developed in T3 (Milestone M1.1). A selected group of patients enrolled from Unit 2, including 10 DM, 10 DM+CV and 10 controls will also undergo a non-invasive state-of-the-art technique able to assess functional and structural vascular abnormalities at the macrovascular levels.

T2. Measurement of PM fluidity by using confocal microscopy and analysis of PM heterogeneity (Unit 1-2-3) (details in Methodologies).

T3. Development of a multiplatform analysis software and database building (Unit 3). A multiplatform analysis software will be built, to ensure the diffusion of the analysis method in conventional microscopy facilities available in hospitals. The software will be independent of the image format, to make the analysis platform microscope-independent (Deliverable D3.1). The input of the software will consist in the acquired images. The fluidity map will then be built, and the statistical quantities calculated. GP outcomes, as well as the data from metabolic evaluations (i.e. Hba1C values, lipid profiles, medical records) will be collected in an ad-hoc developed database (Microsoft Azure). (Deliverable D3.2).

The developed database will provide a subject-specific profile for the patient and will form the basis for the work of T4. The database and the analysis software will be integrated in a single package (Deliverable D3.3) and loaded on a web-service for a centralized management of measurement outcomes (Deliverable D3.4)

T4. Data Analysis and statistical assessment of membrane fluidity as a potential tool to assess CVD risk (Unit 1-2). On the basis of the database built in T3, an extensive statistical analysis will be performed to evaluate any significant difference in the GP value in the DM and DM+CV groups respectively, compared to controls.

EXPERIMENTAL DESIGN AIM 2:

The correlation between GP value and CV risk in type 2 diabetes will be evaluated in a multivariate logistic regression model with presence of CV event as the dependent variable and Hba1C, age, duration of diabetes, smoking habits, sex, atrial fibrillation, SBP, total cholesterol and HDL levels; these results will be further compared to central pulse pressure, segmental arterial stiffness, forward and backward energy (wave intensity) and pressure waves timing/amplitude, impedance mismatch between proximal elastic arteries and distal muscular arteries as the independent variables.

Experimental Design Aim 3:

An assessment of existing correlation between fluidity and the UKPDS ENGINE CV risk score in type 2 diabetic subjects without CV events (DM) will be performed. Further, we will also verify the correlation between fluidity and functional and structural vascular abnormalities at the macrovascular levels in a selected group. (MILESTONE M4.1).

4.1 FLOW CHART

StudyProcedures	Visit 1
Informed Consent	**X**
Inclusion and Exclusion criteria (verification/confirmation)	**X**
Demographics / Medical History	**X**
Physical Examination	**X**
Vital Signs (BP, HR)	**X**
Blood Sample	**X**
Concomitant Medications	**X**
	Laboratory assessment
Sample preparation	**X**
Measure PM fluidity by using confocal microscopy	**X**
	Data analysis
Correlation between GP value and the CV risk	**X**
Validation of GP value as a biomarker of CV risk	**X**

5. STUDY POPULATION

Sample size (200 patients) 100 with CV events and 100 without CV events, 50 healthy subjects as control will be enrolled from those attending the Centre for Endocrine and Metabolic Diseases at Policlinico A.

Gemelli – Università Cattolica del Sacro Cuore (Rome, Italy). Signed informed consent will be obtained from the potential subject before any study-specific procedures are performed. Among diabetic patients, those with stable metabolic control (HbA1c <7.5 % for at least 6 months), Fasting C-peptide > 1 ng/mL and stable medical therapy (including all anti-hyperglycemic agents and insulin treatment) for at least 6 months prior to the screening visit will be included in the study.

Exclusion criteria: Type 1 diabetes (as assessed by medical history); history of diabetic ketoacidosis or hyperosmolar non ketotic coma; severe liver dysfunction; asthma; uncontrolled blood pressure; symptomatic tachy- or bradyarrhythmia; treatment with PCSK9 inhibitors; severe/uncontrolled medical conditions; chronic pancreatitis, acute diseases, moderate-severe hepatic disease, severe chronic kidney disease (CKD-EPI eGFR<30 ml/min per 1.73 m2), active thyroid disorders, recent or ongoing infections, inability or unwillingness to provide informed consent.

Diabetic subjects will be stratified into 2 groups: DM including diabetic subjects without cardiovascular events and DM+CV including patients with major cardiovascular events

- Ischemic heart disease (any of the following): − Documented Myocardial Infarction − Percutaneous Coronary Intervention − Coronary Artery Bypass Grafting − Objective Findings of Coronary Stenosis (> 50%) in at least 2 arteries
- Cerebrovascular disease (any of the following): − Documented ischemic stroke (Known TIA, primary intracerebral or subarachnoid haemorrhage do not qualify) − Carotid stenting or endoarterectomy

An age and weight-matched control group of non-diabetic subjects without cardiovascular events (controls) will be enrolled from those attending our centre for endocrine diseases, selecting euthyroid patients whose thyroid hormones are monitored after surgical thyroidectomy for goitre.

5.1 WITHDRAWAL PROCEDURES

Patients have the right to withdraw from the trial on their request or at the discretion of the Principal Investigator and/or his delegates.

The discontinuation from the study is foreseen in the following conditions:

- patient's request (also not motivated);
- Principal Investigator's decision (motivated) if considered as necessary, in own judgment, for the interest of the patient;
- occurrence of particularly important adverse reactions;

- occurrence, in the course of the study, of one or more of the situations listed in the "exclusion criteria".

6. METHODS

Sample preparation: RBC will be isolated from blood by density gradient centrifugation, counted, seeded in an uncoated two well dish (RPMI-1640 5% FCS) and labeled with Laurdan.

Vascular multimodal assessment: ultrasound-based methods for estimating local arterial stiffness, arterial wave separation, wave reflection and central pulsatile pressure [28, 29], and arterial wall material properties by means of simultaneously acquired local pressure (applanation tonometry) and arterial distension (high-resolution, radiofrequency-based, vascular ultrasound) will be performed in Unit 2. To this end, we will perform: ankle-brachial index (ABI), and standard Doppler- echocardiogram; fingertip peripheral arterial tonometry (by Endo-PAT 2000 device) to assess endothelial function. [30]; carotid-femoral PWV for aortic stiffness estimate, by gold standard technique (Complior System, ALAM Medical, Paris) [31]; carotid ultrasound examination, bilaterally, for IMT and plaque volume assessment, by high-resolution B-mode ultrasound by a 7.5-12 MHz linear array transducer (MyLab70, ESAOTE).

Image acquisition, preprocessing and analysis: The multiwell will be placed on an inverted confocal microscope (Nikon A1 MP), equipped with an on-stage incubator (T=37°C, %5 CO2, OKOLAB) and images will be obtained using a 60× objective lens (NA 1.4) under 402 nm excitation for Laurdan. Two emission channels (Filter cubes 440/50 and 525/50) will allow the measurement of fluidity outcomes. A GaAsP detector will collect 16-bit, unsigned images at 0.25 ms dwell time. Calculation of the Generalized Polarization (GP) index: The GP will be calculated for each pixel using the two Laurdan intensity images using Ratiometric Image processor program [33]. GP images (as eight-bit unsigned images) will be pseudo colored in ImageJ. GP measurements at pixel resolution will be performed: RBC seeded on a multiwell will be imaged with confocal microscopy and fluidity maps will be generated as in [6]. The method, relying on an internal reference (ratiometric probe), is not sensitive to different instrumental setups, laser intensity, probe concentration, uneven illuminations [34]. To determine fluidity sub-micron structure, polarized light will photoselect laurdan molecules associated with high GP values [33]. With this method it is possible to distinguish and measure size and number of lipid domains with respect to pixel size (100 nm). The number and configuration of fluid microdomains will be determined, to further

enhance sensitivity and specificity of the method. All the acquired data will be stored in the database developed.

7. STATISTICAL ANALYSIS

To obtain a statistical power of 0.8 with a two-tailed alpha of 0.05 in a multivariate logistic regression model with presence of CV event as the dependent variable and Hba1C, age, duration of diabetes, smoking habits, sex, Atrial fibrillation, SBP, total cholesterol and HDL levels as the independent variables, we will enroll 100 patients with CV event and 100 patients without CV event, following the rule of 10 events for 1 independent predictor. This sample size will also allow us to compare differences in means across groups based on preliminary measurements performed in 9 patients (mean 0.59, SD 0.013 for controls; mean 0.56, SD 0.034 for diabetics) and to detect as statistically significant a correlation of 0.28 between fluidity and a UKPDS ENGINE CV risk score in the group of type 2 diabetic subjects without CV events.

8. EXPECTED RESULTS

We will find differences in the GP value means between diabetic groups with and without CV events, well matched for Hba1C values or other plasma parameters. This will allow us to identify whether the GP value could be a sensitive marker to distinguish T2D patients with low risk from those with very high cardiovascular risk. Based on preliminary measurements, we will also establish a correlation between fluidity and a UKPDS ENGINE CV risk score in the group of type 2 diabetic subjects without CV events.

9. ETHICAL ISSUES

This trial must be carried out in compliance with the protocol, designed to ensure adherence to Good Clinical Practice, as described in:

1. ICH Harmonised Tripartite Guidelines for Good Clinical Practice, 1996. Note for Guidance on Good Clinical Practice CPMP/ICH/135/95
2. EU Directive 2001/20/EC, 2005/28/EC
3. Declaration of Helsinki (1964, and its amendments and subsequent clarification)

The Principal Investigator or his delegates agrees, when signing the protocol, to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice that it conforms to.

9.1 ETHICAL AUTHORIZATIONS

Taking into account the study design of the trial protocol, the proposed informed consent form and other information to the subjects, should be submitted to the referent Ethics Committee.

9.2 PROTOCOL AMENDEMENTS

All significant deviations from the protocol should be documented. Any changes made after data analysis has begun should be documented as such and the rationale provided. Amendments significantly affecting the scope of the investigation or the scientific quality of the study and require additional approval by the Independent Ethics Committee. If an approval is not required, the amendment must be reported to the Ethics Committee for its information.

9.3 INFORMED CONSENT

It is the responsibility of the Principal Investigator or his delegates to obtain a written informed consent from each subject. The Principal Investigator or his delegates will explain the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail and provide the subject with a copy of the information sheet. The subject will be given sufficient time to consider the study before deciding whether to participate. Each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the trial at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician. All patients are to provide written informed consent in accordance with applicable laws of the country. The patient will sign and date the informed consent form before he/she enters the study (i.e. before any study related activity). In the case of an amendment that would directly affect the patient's participation in the study, the patient must provide new written informed consent indicating that he/she re-consent to participate in the study.

10. **BIBLIOGRAPHY**

1 The Diabetes Control and Complications Trial Research Group, Diabetes 45, 1289 (1996).
2 National Cholesterol Education Program (NCEP) Circulation 106, 3143 (2002).
3 E.J. Boyko and J.B. Meigs, Diabetes Care 34, 782 (2011).
4 P. Gæde, et al. Diabetologia 59, 2298 (2016).

5 K.M. Borow et al. Atherosclerosis. Sep;242(1):357-66 (2015).
6 P. Gæde, et al. N. Engl. J. Med. 348, 383 (2003).
7 W.B. Kannel and R.S. Vasan, Curr. Opin. Cardiol. 24, 345 (2009).
8 Tenenbaum, et al. Cardiovasc. Diabetol. 13, 159(2014).
9 D.L. Bhatt et al. N Engl J Med. 380(1):11-22. (2019)
10 S. Pilz, et al. J. Clin. Endocrinol. Metab. 91, 2542 (2006).
11 A. Hirasawa, K. et al. Nat. Med. 11, 90 (2005).
12 U. Risérus, et al. Prog. Lipid Res. 48, 44 (2009).
13 P.F. Pilch, P.A.et al. Proc. Natl. Acad. Sci. U. S. A. 77, 915 (1980).
14 C.M. Vazquez, et al. Am. J. Physiol. Integr. Comp. Physiol. 273, R1086 (1997).
15 E. Toledo, et al. Am. J. Epidemiol. 178, 392 (2013).
16 H. Wu, et al. t Br. J. Nutr. 110, 466 (2013).
17 G. Ciasca, et al. Nanoscale 7, 17030 (2015).
18 G. Maulucci, et al. PLoS One 12, e0184109 (2017).
19 E. Cordelli, et al. IEEE 29th Int. Symp. Comput. Med. Syst. (2016).
20 C. Angelucci, et al. Br. J. Cancer 112, 1675 (2015).
21 G. Balogh, et al. PLoS One (2011).
22 G. Maulucci, et al. Free Radic. Res. 50, (2016).
23 C. Angelucci, et al. PLoS One 7, e50804 (2012).
24 G. Maulucci, et al. Biochim. Biophys. Acta 1840, 2192 (2014).
25 G. Maulucci, E. et al. PLoS One 12, (2017).
26 A.M. Kanaya, et al. 162, 1737 (n.d.).
27 R.J. Stevens, V. et al.Clin. Sci. (Lond). 101, 671 (2001).
28 Kozakova M et al. J Hypertens. 36:2362-2368 (2018).
29 Rakebrandt F et al. Ultrasound Med Biol. 35:266-277(2009).
30 Bruno et al. Nutr Metab Cardiovasc Dis. 27:121-128 (2017).
31 Kozakova M et al. Cardiovasc Diabetol 16:78 (2017).
32 Palombo C et al. Cardiovasc Diabetol 10:88 (2011).
33 G. Maulucci, V. at al. Sci. Signal. 1, (2008).
34 L.A. Bagatolli and E. Gratton, Biophys. J. 78, 290 (2000).