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Pharmacological Intervention To Target The Pro-Calcific Phenotypic Drift Of Myeloid Cells: A Pilot Study.

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Abstract:

Introduction: Vascular calcification is prevalent in T2D (T2D) and is closely related to cardiovascular disease. Myeloid calcifying cells (MCCs) are monocytes with an osteoblast-like phenotype expressing osteocalcin (CD14+/OCN+). MCCs have been shown to contribute to cardiovascular disease (CVD) and vascular calcification in diabetes. Several clinic trials demonstrated that metformin, GLP-1 RA and pioglitazone all provide cardiovascular benefits, but mechanisms of CVD protection have been explained only in part. Thus, we hypothesized that metformin, GLP1-RA and pioglitazone can prevent the differentiation of monocytes towards an osteoblast-like phenotype and can reduce the levels of circulating MCCs, which have been identified as a new mechanism of cardiovascular injury, in diabetes.

Study aims: To evaluate the effects of GLP1-RA added on metformin on the levels of MCCs in subjects affected by T2D. We also evaluated *in vitro* the effects of metformin and pioglitazone on the acquisition of an osteoblast-like (CD14+/OCN+) phenotype of cultured human monocytes.

Materials and methods: In this prospective observational study, people with T2D on metformin who started add-on therapy with GLP1-RA (Group A) and people continuing treatment with metformin alone (Group B) were consecutively enrolled at the Diabetes Unit of Umberto I "Policlinico" General Hospital. CD14+/OCN+ MCCs levels were evaluate by flow cytometry before treatment, then one week and three months after treatment. Clinical and biochemical data were collected at baseline and on follow up from Electronic Health Records. Flow-cytometry assay was also used to detect surface expression of osteocalcin of cultured THP-1 monocytes treated for 48 hours with oxidized LDL (oxLDL) alone, or with pioglitazone ± oxLDL, or with metformin ± oxLDL at 48 hours.

Results: Forty-eight have been enrolled, and flow cytometry analyses have been completed for 42 participants (10 in Group A and 34 in Group B). The overall cohort had a median [25^{th}-75^{th} percentiles] age of 64 [55.8-71.3] years, the HbA1c was 6.4 [6-6.8]%, and BMI was 29.3 [26.4-

34.9] kg/m^2. In both groups, a significant reduction in median [25th-75th percentile] MCC CD14+/OCN+ levels was observed already after one treatment week (Group A: 6.4% [1.9; 11.3] at baseline vs 1.8% [1.5; 3.5], p=0.028; Group B: 8.3% [4.5 ; 19.22] at baseline vs 8.2% [4.1; 10.9], p=0.013). The reduction in MCC CD14+/OCN+ levels was significantly higher in the group treated with GLP1-RA compared to metformin alone (p= 0.032). At three-months follow-up 6 participants within Group A and 25 participants within Group B completed the follow-up. The levels of CD14+OCN+ cells tended to be unchanged following metformin treatment during the three-month observation period, whereas they dropped significantly in the GLP1-RA add-on metformin treatment arm. Treatment with oxLDL increased OCN expression by 3-to 10-fold (p<0.05). Metformin ± oxLDL led to a non-statistically significant OCN expression reduction, whereas *in vitro* treatment with pioglitazone led to a significant reduction in OCN expression on THP-1 cells both alone (p=0.03) and after stimulation with oxLDL (p<0.001).

Conclusions: These preliminary study results suggest that both metformin and GLP-1 RA could significantly decrease the circulating levels of CD14+/OCN+ MCCs, with a greater efficacy of GLP1-RAs, consistently with the known cardiovascular benefits of this class of drugs. The in vitro data also suggest possible benefits of pioglitazone in preventing the trans-differentiation of monocytes towards a calcifying phenotype, suggesting a new mechanism of cardiovascular action of these classes of drugs, widely used in people with T2D.

1. Introduction

1.1. Diabetes mellitus: classification and diagnosis

Diabetes mellitus is a chronic, heterogeneous clinical condition determined by a relative or absolute deficiency in insulin production by the β-cells of the pancreas, characterized by increased blood glucose values in the blood. The current classification of diabetes mellitus is based on etiologic criteria defined by the World Health Organization (WHO) and the American Diabetes Association (ADA), also accepted by the Italian Society of Diabetology [1,2], it identifies four main forms of diabetes mellitus [2]:

Type 1 diabetes mellitud (T1D), recognizes autoimmune pathogenesis as its main cause, resulting in the destruction of pancreatic β-cells. Clinical onset is usually acute with onset before the age of 30 years, with a prevalence of 5% to 10% of diabetes mellitus forms. The presence of several autoantibodies characterizes this form of diabetes. In particular, autoantibodies anti-glutamic acid decarboxylase (GAD), anti tyrosine phosphatase (IA-2), anti-insulin (IAA), anti-insulin (ICA) and anti zinc transporter 8 (ZnT8) can be found in these subjects. Often this form of diabetes is associated with other autoimmune diseases such as Hashimoto's thyroiditis, Addison's disease, vitiligo, and celiac disease [2]. Then there is a second form of T1D, called idiopathic, with the same clinical features but in the absence of the biochemical finding of antibodies known to date. Finally, there is a form of autoimmune diabetes (AD) with onset in adulthood, called LADA, (Latent Autoimmune Diabetes in Adults), with a slow progression that does not require insulin treatment at diagnosis [3].

T2D mellitus (T2D) is characterized by a condition of insulin resistance in peripheral tissues and a subsequent reduction of insulin secretion by pancreatic β-cells over time [2]. This is the most common form of diabetes mellitus (90%) and the most common risk factors are family

history, advanced age, obesity, and a sedentary lifestyle. The aetiology of T2D is multifactorial and based on genetic and environmental factors. Insulin resistance and hyperinsulinemia lead first to impaired glucose tolerance and then to overt diabetes with overt symptoms. Given the slow but chronic onset of these conditions, the time of clinical diagnosis of T2D is often delayed by the biological onset of the disease [2].

Gestational diabetes identifies women who develop a form of hyperglycemia in the second or third trimester of pregnancy that often regresses after delivery. This condition is determined by the physiological state of insulin resistance induced by the pregnancy itself, which, however, precipitates a predisposing clinical condition leading to the development of hyperglycaemia. Although transient, gestational diabetes is one of the most important nonmodifiable risk factors for the onset of T2D in women [2].

Fewer than 5% of cases of diabetes mellitus can be ascribed to the category known as "**Other Specific Types of Diabetes**," with different but specific etiologists, different from those that determine the other types, such as T1D, T2D and gestational diabetes. Two main groups could be identified: Monogenic Diabetes, which is due to specific genetic defects that result in impaired insulin secretion or action, and Secondary Diabetes, which as per definition, is secondary to other diseases, particularly other endocrinopathies, other pancreatic diseases, infections, drugs or chemical agents [2].

The clinical diagnosis of diabetes mellitus is made according to the same criteria, regardless of aetiology. A diagnosis of diabetes can be made in the presence of the classic symptoms of the disease, such as polyuria, polydipsia, and weight loss, associated with the finding on one occasion of random blood glucose $\geq$ 200 mg/dL [1,2].

In the absence of typical symptoms of diabetes mellitus, the diagnosis is made according to the confirmed finding on at least two different occasions:

- fasting blood glucose ≥ 126 mg/dL;
- blood glucose ≥ 200 mg/dL 2 hours after oral glucose loading (performed with 75 mg glucose);
- glycated haemoglobin (HbA1c) ≥ 48 mmol/mol (6.5%) with HbA1c assays standardized by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) method [1,2].

1.2. Diabetes mellitus complications

Complications of diabetes mellitus are mainly due to the effects due to high blood glucose concentrations and, according to the speed of onset, are divided into acute and chronic complications.

Acute complications are caused by the abrupt change in blood glucose resulting mainly in electrolyte and metabolic imbalances. These include diabetic ketoacidosis and Hyperosmolar hyperglycemic state. Acute complications of diabetes also include hypoglycemia, an iatrogenic complication, due to a reduction in blood glucose values as a collateral effect of some types of oral or injectable hypoglycemic therapy. For example, one of the main reasons was an increased administration of insulin doses compared to the amount required by the patient. All acute complications of diabetes mellitus are life-threatening if not treated promptly [2].

Otherwise, the development of chronic complications of diabetes mellitus is due to chronic exposure to higher concentrations of blood glucose and the involvement of other factors such as an alteration in lipid profiles, blood pressure values and so on. While sharing some common etiopathogenetic mechanisms, chronic complications manifest themselves in different clinical forms depending on the organs and tissues involved [2].

Chronic complications are classically grouped into microvascular, macrovascular, and mixed complications. In recent years, new and emerging chronic complications have also been proposed that are difficult to classify into the previously reported categories.

Microvascular complications include diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy**. Macrovascular complications** include cardiovascular disease, which is clinically manifested in the forms of ischemic heart disease, stroke, and peripheral vasculopathy. **Mixed complications** consist of the clinical condition called diabetic foot and erectile dysfunction. Finally, emerging complications include diabetic osteopathy manifested by increased bone

fragility and cognitive impairment/dementia [1,2].

1.2.1. Microvascular complications

Diabetes-related microvascular complications represent a notable challenge due to their various presentations and their significant morbidity, recently considered as strong predictors of cardiovascular disease. Prevention and management strategies should focus on lifestyle modification, education and awareness, systematic screening for early complications, and intensive management of modifiable risk factors [4]. Diabetes-related microvascular complications include diabetic neuropathy (eg, diabetic symmetric polyneuropathy (DSPN), cardiac autonomic neuropathy, gastroparesis, enteropathy, erectile dysfunction, female sexual dysfunction, and hypoglycemia unawareness), diabetic kidney disease (DKD), and diabetes-related eye disease (eg, diabetic retinopathy (DR)). Both diabetes duration and degree of glycemic control strongly correlate with the development of microvascular complications [5].

Diabetic neuropathy represents the most prevalent chronic complications of diabetes, present in 10-15% of patients at diagnosis, and it is defined as a loss of sensory function beginning distally in the lower extremities that is also characterized by pain and substantial morbidity. Diabetic neuropathy is a unique neurodegenerative disorder of the peripheral nervous system that preferentially targets sensory axons, autonomic axons and later, to a lesser extent, motor axons. The pathogenesis, still unclear, is certainly multifactorial. Probably, oxidative and inflammatory stress are responsible for direct cell damage in the context of metabolic dysfunction. Several risk factors have been associated with the disease, in particular poor glycaemic control, smoking, hypertension, weight and dyslipidaemia. They are divided according to neurological classifications into diffuse neuropathies, distinguished further between **Symmetric Distal Polyneuropathy (SDPN)** and **Autonomic Neuropathies**, mononeuropathies and radiculopathies. SDPN is defined as the dysfunction of peripheral nerves in patients with diabetes and to the exclusion of other causes. They may contribute to further complications, such as the development of diabetic ulcer, or even evolve with loss of sensitivity, both proprioceptive and thermal and pain. The combination of loss of protopathic sensitivity with loss of proprioception can lead to falls and increased exposure to fractures. The earliest common symptoms are given by the involvement of the thin fibres, and the presence of pain and dysesthesia, with typically aching or electrical perception, and hyperalgesias is common. Involvement of the thick fibres, on the other hand, causes numbness, tingling sensation without pain and loss of the sense of protection, which closely associates the risk of developing diabetic foot. Strict glycaemic control remains mandatory for treatment, as pharmacological strategies pharmacological strategies (Pregabalin, Duloxetine, Gabapentin and sometimes tricyclic antidepressants) represent only a symptomatic approach to therapy [6].

Cardiovascular Autonomic Neuropathy (CAN) is a remarkable risk factor for cardiovascular disease, and has a definite prognostic role for mortality and cardiovascular morbidity. Putative mechanisms for this are tachycardia, QT interval prolongation, orthostatic hypotension, reverse dipping, and impaired heart rate variability, while emerging mechanisms like inflammation support the pervasiveness of autonomic dysfunction. CAN affects at least 20% of patients affected by diabetes, and up to 65% of elderly and those with a long diabetes duration. Early diagnosis is the key to preventing major further complications: CAN is considered a risk factor for cardiovascular mortality, arrhythmias, silent myocardial ischaemia, myocardial dysfunction and major cardiovascular events in general. Clinically CAN is characterized by asthenia, palpitation, fainting and syncope, and when CAN is suspected, an examination of the patient's ability to perceive hypoglycaemias is considered: hypoglycaemia unawareness is one of the serious complications of CAN. Cases of early asymptomatic CAN are not rare, detected by a decrease in the variability of the heart rate on Deep Breathing examination and orthostatic hypotension. In more advanced cases, resting tachycardia and exercise intolerance also occur. The focus of CAN treatment is symptomatic [6,7]. **Gastrointestinal Neuropathy** is a complication typically associated with long-term diabetes, and it is characterized by esophageal dysmotility, gastroparesis, constipation, diarrhea, and fecal incontinence. A small number of cases have been reported in literature, due to the difficulty in making a clinical diagnosis. The clinical picture typically includes a sense of early satiety, bloating, nausea, vomiting, dyspepsia, abdominal pain, and hypoglycemia, but in most cases the symptoms reported by the patient are mute. In any case, the severity of the clinical history may not be proportional to the severity of the condition. Treatment involves a diet consisting of numerous small meals with reduced intake of fat and fiber can improve the condition. Suspension of any drug with an effect on gastrointestinal motility is recommended [6]. **Urogenital neuropathy** is both sexual and

urinary. In diabetes **erectile dysfunction** is three times prevalent that of non-diabetic patients. Erectile dysfunction could be an effect of autonomic neuropathy. Nonetheless, it is considered a multifactorial condition, and additional risk factors (hypertension, hyperlipidemia, obesity, endothelial dysfunction, smoking, cardiovascular disease, drugs and psychogenic factors) should also be evaluated. The diagnosis requires the exclusion of other possible causes (e.g. hypogonadism). Although in type 1 diabetes, good glycemic control is associated with lower incidences of erectile dysfunction, this association is not as strong as in T2D. On the other hand, adequate blood pressure and lipid control can improve the condition. The treatment of erectile dysfunction is based on the administration of phosphodiesterase 5 inhibitors (for example, sildenafil or tadalafil), or, as a second line, the use of transureteral prostaglandins, intracavernous injections or prosthetic devices. **LUTS (Lower Urinary Tract Symptoms)** and female sexual dysfunction represent further urogenital complications associated with diabetes. The characteristic lower urinary tract symptoms are incontinence, bladder dysfunction, while the typical manifestations of female sexual dysfunction are decreased libido, dyspareunia and inadequate lubrication [6].

Diabetic nephropathy (DN)[8] is one of the most prevalent and severe complications of diabetes mellitus, associated with increased morbidity and mortality in diabetic patients. DN is the major cause of end-stage renal disease worldwide. Chronic hyperglycemia and high blood pressure are the main risk factors for the development of DN. The pathogenesis of DN is very complex and is still not fully understood. There are many pathways and mediators involved in the development and progression of DN [9], including oxidative stress, angiotensin II (Ang-II), and inflammatory processes, which are recently considered to play an important role[10]. Conventionally, the developmental mechanism of DN is the result of abnormal homeostasis, which includes hemodynamic abnormalities, metabolic disorders,

and hormone synthesis such as Ang-II[9]. Renin-angiotensin-aldosterone system (RAAS), advanced glycation end product (AGE) formation, activation of transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), protein kinase C (PKC), mitogen-activated protein kinase (MAPKs), and reactive oxygen species (ROS) are important pathways to the development and progression of DN. Classically, DN is characterized by the appearance of proteinuria followed by a progressive decline in renal function. DN is a clinical syndrome in DM patients characterized by persistent albuminuria (>300 mg/day or >200 μg/min) at 2 out of 3 examinations within 3-6 months, a progressive decrease in GFR, and hypertension. However, some diabetic patients develop decreased renal function and vascular complications without proteinuria, known as nonproteinuric DN (NP-DN). Most of patients with diabetes and CKD have no albuminuria[11]. Based on the most recent data, it was found that the opposite temporal trend in the prevalence of albuminuria and a decrease in eGFR in diabetic patients, i.e., despite regression of microalbuminuria (decreased prevalence of albuminuria), the decline in GFR continued. This increased divergence between albuminuria and decreased eGFR differs from the classic view, that albuminuria always precedes and leads to a progressive decrease in GFR. This suggests that the initiation and progression of decreased renal function may also occur independently of the development of albuminuria. This concept is supported by the emergence of two new phenotypes, i.e., **nonalbuminuric/proteinuric kidney disorders (NP-DN)** and progressive renal decline. Albuminuria and decreased eGFR can occur and continue either together or separately as complementary or "twin" manifestations of DN[12]. So, there are two main pathways for the onset and progression of DN, i.e., **albuminuric** and **nonalbuminuric**. The prevalence of NP-DN in type 2 DM ranges from 45 to 70%, while based on the latest data, its prevalence in type 1 DM is from 50 to 60%[12]. In general, NP-DN is caused by abnormalities in the vascular or predominantly tubulointerstitial abnormalities. There are

several possibilities for this NP-DN, including the accompanying vascular disease (there is an increase in interlobar artery vascular resistance)[13] which causes damage to glomerular and tubular structures and interstitial fibrosis. Regression of albuminuria remains an important therapeutic goal. However, there are problems in diagnosis and treatment of nonproteinuric DN (NP-DN), which does not follow the classic pattern of DN. In fact, the prevalence of DN continues to increase, and additional therapy is needed to prevent or ameliorate the condition. In addition to conventional therapies, vitamin D receptor activators, incretin-related drugs, and therapies that target inflammation may also be promising for the prevention of DN progression. Hyperglycemia, hypertension, obesity, smoking, race, men, dyslipidemia, age, and genetic factors are the main risk factors for the development and progression of DN. In classic DN patients, standard therapy still focuses on glucose and blood pressure control, with the target of halting DN progression and regression of albuminuria. In classic DN patients, standard therapy still focuses on glucose and blood pressure control, with the target of halting DN progression and regression of albuminuria. One of the most important risk factors for the development of kidney disease in diabetic patients is the onset and persistence of proteinuria[14]. In addition to the above approaches, other nonspecific measures must still be implemented, including weight loss, protein restriction, lipid lowering and smoking cessation. In NP-DN patients, the main underlying abnormality is vascular, the principle of targeted therapy as well as cardiovascular risk factors. In classic DN patients, standard therapy still focuses on glucose and blood pressure control, with the target of halting DN progression and regression of albuminuria. Adequate glucose control is a standard foundation in preventing the development and progression of DN. The management of hyperglycemia in CKD, especially those accompanied by a decrease in GFR is a challenge, which requires a more specific understanding, especially in relation to drug choice. In addition to conventional therapies,

vitamin D receptor activators, incretin-related drugs, and therapies that target inflammation may also be promising for the prevention of DN progression. SGLT2 inhibitors are oral hypoglycemic drugs that reduce renal glucose uptake, thereby increaseing urinary glucose excretion and reducing hyperglycemia [8].

Diabetic retinopathy is the major ophthalmic complication of diabetes mellitus with a significant impact in global health. The prevalence and disease burden of DR is expected to increase significantly over the next few decades, from about 103 million individuals in 2020, to 130 million in 2030, and 161 million in 2045. Such projections are due to a variety of factors, including the increasing prevalence of diabetes around the world, lifestyle changes, and increasing lifespans and aging global populations[15]. Several risk factors have been identified, notably hypertension, dyslipidemia, duration of diabetes, gestation, and also ethnicity (with Hispanic and South Asian ethnicity posing a higher risk). It is also associated with numerous other factors, both systemic and lifestyle-related, such as nephropathy, obesity, alcohol consumption, and laboratory evidence of anemia, hypothyroidism, inflammation, and endothelial dysfunction. However, the latter factors have an inconsistent association with the disease. The most obviously related risk factor is hyperglycemia: suffice it to say that a 1% reduction in glycated hemoglobin (HbA1c) corresponds to an approximately 40% reduction in the risk of retinopathy, 25% reduction in the risk of vision threat, 25% reduction in the risk of laser therapy, and 15% reduction in the risk of blindness. The pathophysiology is closely related to chronic exposure to hyperglycemia and other risk factors. In addition to the general molecular mechanisms of complications given by hyperglycemia, seen above, which also lead to increased VEGF, there are other non-VEGF related mechanisms. However, the latter factors have an inconsistent association with disease. The most obviously correlated risk factor is hyperglycemia: suffice it to say that a

1% reduction in glycated hemoglobin (HbA1c) corresponds to an approximately 40% reduction in the risk of retinopathy, 25% reduction in the risk of vision threat, 25% reduction in the risk of laser therapy, and 15% reduction in the risk of blindness. The pathophysiology is closely related to chronic exposure to hyperglycemia and other risk factors. In addition to the general molecular mechanisms of complications given by hyperglycemia, seen above, which also lead to an increase in VEGF, there are other non-VEGF-related mechanisms. Indeed, erythropoietin is a strong ischemia-induced angiogenic factor, and it acts independently of VEGF. An additional role might be played by extracellular carbonic anhydrase, which is greatly increased in diabetic retinopathy. In animal models, inhibition of carbonic anhydrase reduces retinal vascular permeability. However, its use in topical form (typical preparation for glaucoma treatment) has not yet been sufficiently studied for clinical use. Inflammation also plays a role. The response to hyperglycemia and additional stresses triggers a response mediated by multiple inflammatory mediators, which can cause abnormal interactions between leukocytes and endothelium. So the clinically-visible retinal lesions associated with DR, such as microaneurysms, hemorrhages and hard exudates, are primarily the result of retinal microvascular damage. Consequently, the focus on DR pathophysiology, diagnosis and assessment has traditionally always centered around the vascular aspect of the disease. However, with the availability of better structural retinal imaging modalities and functional assessments, evidence has accumulated over the years of significant retinal neural dysfunction as well, which occurs together with, or in some cases precedes, the development of vascular abnormalities. These structural and functional changes have collectively been termed **“diabetic retinal neurodegeneration” (DRN)**[15]**.** In fact, neuroretinal damage may even precede microvascular abnormalities. According to some theories due to the ability of diabetes to be able to reduce the transmission of insulin signal to the retina,

neurodegeneration, accelerated neuronal apoptosis and altered metabolism of neuroretinal support cells occur, qualifying diabetic retinopathy as a true sensory neuropathy of parenchymatous interest, similar to diabetic neuropathy. OCT studies have shown that patients with diabetes demonstrate significant thinning of the inner retinal layers, including the retinal nerve fiber layer (RNFL), and ganglion cell layer (GCL)[16,17]. Retinal thinning is progressive over time, and can precede the development of clinically-visible DR lesions[16,17]. Histological studies on enucleated eyes also corroborate these findings, showing reductions in retinal ganglion cell density in eyes with DR[18]. Functional assessments in diabetes reveal reductions in contrast sensitivity, visual field defects, electrophysiologic deficits, and impaired pupillary responses[19]. There are several typical retinal manifestations of diabetic retinopathy. Increased arteriolar caliber is thought to be strongly associated with the development of retinopathy, and may be an index of microvascular dysfunction. Increased retinal capillary pressure leads to wall dilatation, resulting in microaneurysms, vascular leakage associated with edema or hard exudates, cottony exudates, and vessel rupture resulting in microhemorrhage. Clinical definition requires microvascular retinal signs in patient diagnosed with T2D. Vision loss develops from sequelae regarding maculopathy or retinal neovascularization, although there are cases of severe diabetic retinopathy with preserved vision. The main treatment lies in prevention of the condition and tight control of both blood glucose and blood pressure, and in the case of a condition representing a serious threat to vision, panretinal laser photocoagulation (in the case of proliferative retinopathy) or focal laser photocoagulation (in the case of macular edema) are two techniques capable of stemming the damage. With these techniques, to be weighed carefully against the risks given by the destructive nature of the laser, however, it is not common to reverse the condition. However, laser-induced burns could reduce ischemia-induced VEGF production, arresting the progression of retinal neoangiogenesis. Intravitreal anti-VEGF therapy is the

established first line treatment for center-involved DME, and has also been shown to be a valid treatment option for PDR[20,21]. Observations from the registration trials for anti-VEGF therapy in DME showed that anti-VEGF therapy can also result in significant improvements in DR severity for patients with non-proliferative DR, and this has been confirmed in more recent prospective clinical trials as well[22,23].

1.2.2. Macrovascular complications

T2D causes a variety of macrovascular complications through different pathogenetic pathways that include hyperglycaemia and insulin resistance. Macrovascular complications of T2D include coronary heart disease, cardiomyopathy, arrhythmias and sudden death, cerebrovascular disease and peripheral artery disease. Cardiovascular disease is the primary cause of death in diabetic patients. Many clinical studies have shown a connection between T2D and vascular disease, but almost always other risk factors are present in diabetic patients, such as hypertension, obesity and dyslipidaemia[24]. The resulting prothrombotic state and increase in inflammatory mediators expedite atherosclerotic changes and the development of macrovascular complications. Individuals with diabetes or prediabetes have a higher risk of developing myocardial infarction, stroke, and peripheral artery disease[25].

Atherosclerotic cardiovascular disease (ASCVD)—defined as coronary heart disease (CHD), cerebrovascular disease, or peripheral artery disease (PAD) presumed to be of atherosclerotic origin—is the leading cause of morbidity and mortality for individuals with diabetes. Common conditions coexisting with T2D (e.g., hypertension and dyslipidemia) are risk factors for ASCVD, and diabetes itself confers an independent risk[26], equal to nondiabetic patients cardiovascular risk with a previous acute myocardial ischemia[27]. Mortality for cardiovascular events occur in 52% of T2D patients, and mortality for myocardial infarction are higher than in non-diabetic[28]. The initial manifestations are usually

attributable to peripheral arteriopathy, followed by angina pectoris and nonfatal myocardial infarction, but there are also numerous cases of stroke and Congestive Heart Failure, which could, in the natural history of the disease, affect one in two diabetics. Heart failure is another major cause of morbidity and mortality from cardiovascular disease. Recent studies have found that rates of incident heart failure hospitalization were twofold higher in people with diabetes compared with those without[29,30]. People with diabetes may present with a wide spectrum of heart failure, including heart failure with preserved ejection fraction (HFpEF), heart failure with mildly reduced ejection fraction (HFmEF), or heart failure with reduced ejection fraction (HFrEF). Hypertension is often a precursor of heart failure of either type, and ASCVD can coexist with either type of heart failure[31], whereas prior myocardial infarction (MI) is often a major factor in HFrEF. Recent trials including people with T2D, most of whom also had ASCVD, have shown that rates of heart failure hospitalization significantly decreased with use of sodium–glucose cotransporter 2 (SGLT2) inhibitors[32–34]. Additional major cardiovascular risk factors identified are cigarette smoking, hypertension and elevated serum cholesterol levels, which independently contribute to the development of cardiovascular disease. Additional analytes, such as C-reactive protein, indicate an increased risk of both cardiovascular disease and peripheral arterial disease in the diabetic. Determination of coronary calcification scores is also a reliable index of cardiovascular disease risk. Numerous studies have shown the efficacy of controlling individual cardiovascular risk factors in preventing or slowing ASCVD in people with diabetes. Furthermore, large benefits are seen when multiple cardiovascular risk factors (glycemic, blood pressure, and lipid control) are addressed simultaneously, with evidence for legacy benefits[35,36]. For prevention and management of both ASCVD and heart failure, cardiovascular risk factors should be systematically assessed at least annually in all people with diabetes. These risk factors include duration of diabetes, obesity/ overweight,

hypertension, dyslipidemia, smoking, a family history of premature coronary disease, chronic kidney disease (CKD), and the presence of albuminuria. Management of glycemia, blood pressure, and lipids and the incorporation of specific therapies with cardiovascular and kidney outcomes benefit (as individually appropriate) are considered fundamental elements of global risk reduction in diabetes[26]. Management of the diabetic patient should include monitoring of cardiovascular risk factors on at least an annual basis. Hypertension is a major risk factor for both atherosclerotic cardiovascular disease and microvascular complications, which, together with the risk of heart failure, benefit greatly from tight blood pressure control. It is desirable to achieve pressor values of 130 mmHg (systolic) and 80 mmHg (diastolic) in the high-risk patient. Dyslipidemia is also a strong risk, and control strategies are based on lifestyle improvement, weight loss. Risk stratification can also be determined with several dedicated calculators. The use of invasive or advanced cardiac testing is indicated in cases of typical and atypical cardiac symptoms, or in cases of electrocardiographic abnormalities. Exercise electrocardiogram with or without echocardiography is indicated as an initial second-level test. It is also indicated in the diabetic with at least 40 years of age to measure coronary calcification[26].

The risk of **cerebral ischemic disease** is an important factor in the management of the diabetic patient. About 20% of diabetic patients die as a result of a **stroke**. Of the patients who survive the event, however, approximately one in two will develop long-term disability. The cerebrovascular diseases most associated with diabetes mellitus are acute ischemic stroke, transient ischemic stroke, and intracerebral hemorrhage, most affecting the posterior cerebral circulation. Lacunar infarcts are also more common in diabetics than in nondiabetics. The duration of diabetes mellitus correlates directly with the risk of ischemic

stroke, but the same conditions of impaired fasting blood glucose (IFG) and impaired carbohydrate tolerance (IGT), which will be discussed in the section on T2D diagnosis, are also a risk factor for the condition. The diabetic patient with stroke has a worse outcome[37]. In addition, the strong association of T2D with hypertension, coronary artery disease, obesity, and dyslipidemia results in an aggravation of risk given by the latter factors. A degree of recurrence of ischemia in the diabetic has also been demonstrated, and hyperglycemia and insulin resistance, together with additional as yet unclarified factors, contribute to the recurrence of episodes. Major modifiable risk factors for stroke include hypertension, diabetes, smoking and dyslipidemia. Diabetes is a well-established risk factor for stroke. It can cause pathologic changes in blood vessels at various locations and can lead to stroke if cerebral vessels are directly affected. Additionally, mortality is higher and poststroke outcomes are poorer in patients with stroke with uncontrolled glucose levels. Controlling diabetes and other associated risk factors are effective ways to prevent initial strokes as well as stroke recurrence. Epidemiologic studies have shown that diabetes is a well-established independent but modifiable risk factor for stroke, both ischemic and hemorrhagic stroke[38–40]. Risk for stroke is actually higher in the young population with diabetes. There are several possible mechanisms wherein diabetes leads to stroke. These include vascular endothelial dysfunction, increased early-age arterial stiffness, systemic inflammation and thickening of the capillary basal membrane. Vascular endothelial function is critical for maintaining structural and functional integrity of the vessel walls as well as the vasomotor control. Nitric oxide (NO) mediates vasodilation, and its decreased availability can cause endothelial dysfunction and trigger a cascade of atherosclerosis. For example, NO-mediated vasodilation is impaired in individuals with diabetes, possibly due to increased inactivation of NO or decreased reactivity of the smooth muscle to NO. Individuals with type II diabetes have stiffer arteries and decreased elasticity compared with subjects having

normal glucose level. . An increased inflammatory response is frequently seen in individuals with diabetes, inflammation plays an important role in the development of the atherosclerotic plaque. The C-reactive protein, cytokines and adiponectin are the main serum markers of inflammation. The C-reactive protein and the plasma levels of these cytokines including interleukin-1, interleukin-6 and tumor necrosis factor-α are independent predictors of cardiovascular risk. Uncontrolled diabetes puts subjects at risk for both ischemic and hemorrhagic strokes. Patients with diabetes had higher relative prevalence of subcortical infarction and lower relative prevalence of intracerebral hemorrhage (ICH)[41]. Hyperglycemia is a common phenomenon presented in the early acute stroke phase. The initial level of plasma glucose is highly correlated with poor poststroke outcomes[39]. Hyperglycemia further aggravates the stroke consequences through augmented reperfusion injury by increasing oxidative stress, stimulating systemic inflammation and increasing barrier permeability. Patients with acute ischemic stroke with both diabetes and hyperglycemia have an increase in aggregation and adhesion of platelets to the endothelium. The mechanisms of diabetes leading to aggravated risk remain to be further investigated, but it is clear that the disease is a cause of accelerated atherosclerosis, risk of atrial fibrillation, and disease of small-caliber penetrating branches of cerebral arteries. Mechanisms facilitating atherosclerosis include hyperglycemia, hyperinsulinemia, impaired endothelial function, hypercoagulability, vascular inflammation, dyslipidemia, and hypertension. In addition, in experimental models of diabetes, an increased frequency of cerebral edema, revascularization phenomena, and expression of certain proteases, which pose a threat to endothelial integrity, have been found. Both acute hyperglycemia and hyperinsulinemia have been shown to increase plasminogen activator inhibitor type 1 and decrease free tissue plasminogen activator (tPA) activities by decreasing plasma fibrinolytic activity in animal model[42].16 In tPA-treated patients, acute hyperglycemia delays reperfusion of the ischemic

penumbra and decreases tPA-induced recanalization rates. Among patients with stroke who were treated with intravenous thrombolysis, hyperglycemia was associated with significantly lower rates of desirable clinical outcomes, higher rates of symptomatic ICH and reduced benefits from recanalization with thrombolytic therapy. The influence of hyperglycemia to patients with ICH is similar to that of ischemic stroke. The effect of hyperglycemia on patients with ICH leading to poor outcomes may be related to exacerbation of hematoma expansion and perihematoma edema. As hyperglycemia is associated with poor outcomes, proper management of poststroke hyperglycemia is critical for improving outcomes. Aggressive glucose control through lifestyle change or medications and modification of other associated risk factors (such as BP and dyslipidemia) are critical steps toward effective stroke prevention.

Peripheral arteriopathy (PAD) is a condition in which, mainly due to atherosclerosis, chronic arterial occlusive disease of the lower extremities occurs. Diabetes mellitus increases both the incidence of peripheral arterial disease and the progression and severity of the disease, the risk of ischemic events, and the risk of amputation. Important modifiable risk factors include smoking, dyslipidemia and diabetes. Smoking, in particular, doubles the risk of peripheral arterial disease. Chronic ischemia with limb risk (CLTI) is a complication in about 11% of patients with PAD, with an increased mortality and amputation risk, respectively estimated of 15-20% and 15-40%[43]. In the case of the patient with diabetes, PAD, and ulceration requiring amputation, 5-year survival tends not to exceed 50%[44]. Coordination with prevention teams and treatment teams is vital to avoid progression of PAD to CTLI, ulceration, and amputation. Many cases of PAD may remain undiagnosed until the patient presents with severe tissue loss or claudication, underscoring the importance of screening for early diagnosis. Diagnostic tests may be less reliable, however, because of

the possible concomitance of peripheral neuropathy, arterial calcification of the tonaca media and peripheral edema. But, especially in the patient who already has a diabetic ulcer, even at the earliest stage, PAD associates with an increased risk of major amputation, infection, and ulcers. Foot examination and arterial pulses, measurement of the Ankle Brachial Index are recommended. Imaging examinations indicated are echocolordoppler, angioTC, angioRM, or digital subtraction intrarterial angiography. In the case of severe impairment of ankle brachial index (<0.5) or ulcers being treated with no improvement in 4-6 weeks, urgent imaging with evaluation of urgent revascularization is indicated[44].

The **diabetic foot** is a crossroads of micro- and macrovascular complications. Infections, ulcers, and gangrene of the foot are a strong and frequent indication for hospitalization of the diabetic patient, who risks, annually, 2.5% of getting an ulcer, which can even lead to amputation. The prevalence is increasing due to an aging population and obesity. In the patient with a foot ulcer, clinically relevant peripheral arteriopathy is often present, so the presence of clinic suggestible to the diabetic foot is an important red flag. The pathophysiology is based on three main pillars: neuropathy, trauma (resulting in infection), and peripheral arteriopathy. Peripheral neuropathy produces muscle atrophy, leading to anatomical changes with hammer toe formation, and development of areas of high pressure load, especially at the plantar and metatarsal levels. Repeated trauma, e.g., walking, predisposes to skin lesions, unknown to the patient due to poor sensory and proprioceptive perception. Injuries evolve with atrophy and dislocation of fat pads, predisposing to ulcerative and infective evolution. The major predisposing factors for the development of the condition are inadequate skin protection, inadequate footwear, and poor attention to early recognition of skin trauma. The coexistence of neuropathy, foot deformity, or prior digital amputation carries a 32-fold increased risk of ulceration. Ulceration may evolve, with

soft tissue invasion. If the patient continues to ambulate, tissue damage increases. The natural history of the disease is deep involvement of fascia, musculature, tendon sheaths, and joints. As discussed earlier, neuropathy in T2D is typically symmetrical, with motor, sensory, and autonomic interest. In some patients, the first nerves affected are the longer nerves, and this interest leads to typical sock neuropathy. The first clinical warning sign is loss of the achilles reflex. This is followed by atrophy of the lumbar and intraosseous muscles, with increased arch of the foot and relative increase in the extensor tendons of the toes, resulting in claw toe. The latter alteration contributes to depression of the metatarsal heads and ankle equinus. In addition, loss of sensory, proprioceptive, and vibratory perception (A-type myelin fibers) and loss of pain perception (C-fibers) can render silent not only the previously described microtrauma but even possible metatarsal fractures. Charcot foot is the ultimate evolution of neuroarthropathy. The transition for the inflammatory phase of neuroarthropathy, with swelling and calor of the area, invite differential diagnosis with infectious complication. In addition, the development of the arch-like appearance, produced by subluxation or dislocation of the tarsal bones, makes the foot even more susceptible to pressure ulceration. Loss of autonomic function, with impaired microvascular thermoregulation and anhydrosis, makes the skin dry and further prone to injury, rendering it inadequate in its protective role against microorganisms and dermal infection, with development of cellulitis. Arterial insufficiency, given by endothelial damage, hyperlipidemia, increased viscosity, and atherosclerosis, mainly affects the anterior and posterior tibial arteries, and less commonly the superficial femoral and popliteal arteries. The anatomical location of the affected arteries accounts for the poor perfusion of the foot, which is reportedly predisposed to skin lesions, ischemic ulcers, and gangrene. Diabetic foot infections range from uncomplicated cellulitis to necrotizing fasciitis capable of threatening both the limb and the patient's life. Hyperglycemia causes immune dysfunction, a condition

that facilitates the development of tissue-invasive infections. Infection is commonly polymicrobial, typically mediated by staphylococci, streptococci, enterococci, E. coli, and additional GRAM-negative bacteria. Resistant strains, particularly methicillin-resistant S. Aureus, are not uncommon, increasing the risk of amputation. Evaluation of the lower extremities for skin trauma is essential, remaining (as analyzed in the discussion of peripheral arteriopathy) mandated by palpation of the peripheral wrists. If they are not palpable, flow detection with imaging techniques is indicated. Clinically useful is a search for crepitus or tendon sheath tenderness, signs suggestive of involvement of deep structures. Neurologic examination of the foot includes tests for vibratory sensitivity, sensitivity to superficial touch, painful and thermal sensitivity, and especially evaluation of the reflexes patellar and achilles reflexes. Treatment of the complicated diabetic foot is multidisciplinary, and includes both antibacterial therapy and evaluation for debridement and amputation, with strong indication for reduction of pressure relief until healing is achieved. But prevention is the real cornerstone of the diabetic foot: the at-risk patient needs at least annual (or even quarterly in the high-risk patient) foot checkups by primary care physicians, podiatrists, or angiology specialists. The patient and caregivers should be trained to perform a home assessment of the skin and foot, educated in proper skin hygiene, and instructed in appropriate footwear for prevention[45].

1.2.3. Newly diabetes mellitus complications

Bone Fragility

Fragility fractures are increasingly recognized as a complication of both type 1 and T2D, with fracture risk that increases with disease duration and poor glycemic control. Bone fragility is a serious yet under-recognised complication of diabetes mellitus (DM) that is associated with significant morbidity and mortality. Multiple complex pathophysiological

mechanisms mediating bone fragility amongst DM patients have been proposed and identified. It was found that only 31% of patients with diabetes were being assessed for their bone health[46]. Impaired bone quality in T1D and T2D has distinct pathophysiologic bases that give rise to the different alterations in bone microarchitecture and fracture risk. In addition, T1D and T2D patients are at a significantly elevated risk of falls[47], which further contributes to the risk of fragility events and compounds the morbidities and mortality already associated with DM. Additionally, fractures in patients with DM are known to be associated with complications such as delayed healing, non-union, re-dislocation and wound infections[48], which can contribute to longer lengths of hospital stay[49]. The increased fracture risk faced by patients with DM has various aetiologies, including aberrations in bone density, microarchitecture and bone matrix. An increased fall risk associated with DM also contributes to the increased fracture risk[50,51]. Multiple investigators have found T2D patients to have a higher risk of hip fracture. In contrast to that found in T1D, both men and women with T2D have higher BMD than age-matched controls[52–54]. Yet paradoxically, despite the higher BMD, T2D patients are at a higher risk of vertebral and non-vertebral fractures. It is evident that the increased fracture risk in people with DM is not solely a function of bone density but is further defined by other factors impairing bone quality that drive 'diabetic osteopathy'. T1D and T2D are associated with low bone turnover compared with controls without DM. The bones of patients with T1D and T2D exhibit low bone turnover in bone biopsy studies with reduced osteoblast counts, reduced number of osteoid, and reduced mineralising surfaces prominently in the bone cortex[55]. Biochemical studies have reported decreased markers of bone formation and bone resorption in T1D and T2D[52,55]; lower circulating IGF-1 levels; high blood levels of advanced glycation end-products (AGEs); high levels of sclerostin that exerts inhibitory effects on osteoblastic differentiation and activity via the Wnt/β-catenin signalling cascade; high levels of pro-inflammatory cytokines,

such as interleukin (IL)-1, IL-6, IL-18 and tissue necrotic factor alpha (TNF-α) released in hyperglycaemic state, that can impair differentiation and survival of osteoblasts, osteoclasts and osteocytes via the generation of ROS[56]. T1D and T2D patients have been found to have increased cortical porosity, that appears most prevalent in patients with microvascular disease[57] and peripheral neuropathy[58]. Mechanisms of fractures in T1D and T2D are multi-factorial and are broadly contributed by abnormal bone resistance to mechanical stress and increased fall risk in DM patients. Traditional risk factors for fracture, such as BMI, continue to be proposed as a determinant in fracture prediction models for T2D. Low BMI in older T2D patients may be associated with frailty and a fragility event[59]. The effect of age on fracture risk continues to be observed in patients with T1D and T2D. Longer DM duration was associated with a higher risk of fractures compared with age-matched controls. DM control, hypoglycaemia and the presence of DM complications are also well-established as significant determinants of fracture risk[46]. Longitudinal studies have established that FRAX and BMD T-score predict fracture risk in those with T2D but both require adjustment for diabetes to avoid underestimation of risk. An increasing amount of evidence has assured us of the osteo-pharmacological efficacy of bisphosphonates, denosumab, teriparatide and abaloparatide in T2D. Data would suggest that if a patient has indication for therapy based on criteria developed for non-diabetes patients, these patients should be treated with osteoporosis drugs. In absence of established osteoporosis though, these medications may be used with caution though, as the effects of these drugs in situations where bone fragility is mainly due to alterations in bone quality remain to be thoroughly evaluated[60].

Cognitive impairment/Dementia

Patients with T2D frequently develop neurological complications. Peripheral neuropathy and cardiac autonomic neuropathy are long known neurologic complications[61]. However,

increasingly, evidence indicates that T2D may also cause injury to the brain, possibly through similar pathological processes as occurring in peripheral nerves, which would manifest as cognitive impairment and, eventually, dementia. Indeed, clinical studies underscore a correlation between presence of peripheral neuropathy with the development of cognitive impairement[62,63]. There are also important and shared pathological features between T2D and dementia, which are both characterized by metabolic perturbations in the brain, e.g., insulin resistance, altered glucose uptake and utilization[64]. These similarities in pathology are reflected in clinical studies that demonstrate an increased risk of dementia in individuals with T2D and dementia[65,66]. Cognitive dysfunction is increasingly recognized as an important comorbidity of diabetes mellitus. Different stages of diabetes-associated cognitive dysfunction can be discerned, with different cognitive features, affected age groups, prognosis, and likely also different underlying mechanisms. The onset of dementia in diabetes patients is gradual. It starts with subtle cognitive impairment, which, in progressive patients, develops into mild cognitive impairment followed by frank dementia, oftentimes as Alzheimer's disease[67]. Relatively subtle, slowly progressive cognitive decrements occur in all age groups. More severe stages, particularly mild cognitive impairment and dementia, with progressive deficits, occur primarily in older individuals. Epidemiological studies have established an increased risk of dementia among individuals with diabetes[67]. Diabetes is also linked to less severe forms of cognitive dysfunction[68]. This has important implications for patient management, particularly in older individuals where dementia and pre-dementia stages of cognitive impairment most commonly occur. Manifestations and prognosis of diabetes-associated cognitive dysfunction vary depending on diabetes type and age[69]. Adults with T1D also present subtle decrements in cognitive performance relative to age-matched controls, particularly affecting the domains intelligence, psychomotor efficiency, and cognitive flexibility, particularly those with

advanced microvascular complications. In adults with T2D, deficits in cognitive functioning can roughly be divided in three different stages, according to severity: diabetes-associated cognitive decrements, mild cognitive impairment (MCI), and dementia. The subtle cognitive changes may concern one or several domains, including processing speed, executive function, and memory. These decrements are likely to have an onset in pre-diabetic stages[68,70]15 and evolve only very slowly over the course of many years, at a rate that is up to 50% faster than that of normal cognitive ageing[70–72]. Converging evidence shows that higher A1C levels are associated with diabetes-associated cognitive decrements. Vascular risk factors, in particular hypertension and dyslipidemia, may be associated with cognitive decrements among people with T2D. It is also clear that patients with manifestations of microvascular (e.g. diabetic retinopathy) or macrovascular disease (e.g. myocardial infarction, stroke) are more likely to have worse cognitive performance[70,73] and are at increased dementia risk[67].

1.3. Diabetes mellitus and Vascular calcification: pathophysiology mechanisms and clinical aspects

Vascular calcification is an independent risk factor for cardiovascular disease (CVD). Calcium deposits in coronary arteries can weaken vasomotor responses and alter the stability of atherosclerotic plaques, increasing the risk of cardiovascular events. Vascular calcification has historically been considered as a passive process caused by cellular death; recently however, the hypothesis that calcium deposition in arteries may be an active process of extracellular matrix mineralisation is gaining traction. In this regard, molecular markers of osteogenic activity can be found in all arterial calcified segments[74]. Interestingly, a strict relationship between vascular and bone diseases has been described. Advances in our understanding of the pathophysiology of osteoporosis and vascular calcifications indicate

that these two processes share common pathogenetic mechanisms, suggesting the existence of a bone–vascular axis. Factors implicated in the pathogenesis of both osteoporosis and vascular calcification include proteins, hormones, lipids, vitamins, and cellular activities[75]. Bone morphogenetic proteins participate in osteoblast differentiation while simultaneously producing reactive oxygen species and increasing the adhesiveness of monocytes on the vascular wall. Pro-calcifying conditions, e.g., inflammation, oxidative stress, uraemia, increased oxidised low-density lipoprotein (oxLDL), decreased high-density lipoprotein, and apoptosis trigger the cellular reprogramming and phenotype switching of vascular smooth muscle cells (SMC) from a contractile to bone-forming state. Reprogrammed vascular SMC express bone related proteins such as RUNX2 that act in a paracrine way on all vessel pluripotent mesenchymal cells with concomitant downregulation of SMC contractile proteins, generate mineralised matrix vesicles which initiate the mineralisation process, and form bone matrix within the vessel wall. Vascular calcification is caused by the deposition of hydroxyapatite crystals in the arterial wall, both in the tunica intima and tunica media. Arterial intimal calcification is associated with the development of atherosclerotic plaques. Intimal calcification is characterised by microcalcification deposits within the fibrous caps of the atherosclerotic plaque, weakening the structure of the arterial wall and increasing the risk of plaque rupture[76]. Microcalcifications originate from the apoptotic SMC or mineralising vesicles that are released by these cells[77].17 Arterial medial calcification (Monckeberg's medial sclerosis) is a concentric process distinguished by macrocalcification, medial fibrosis, and arterial stiffness. This occurs in the absence of lipid accumulation and inflammatory cell infiltration, similar to intimal calcification[74]. Although a variety of mechanisms have been proposed for vascular calcification, the transdifferentiation of vascular SMC from a contractile to an osteochondrogenic phenotype seems to play a key role. This phenotype is characterised by the loss of SMC markers (SM22 α and α-SMA) and

the gain of osteochondrogenic markers (RUNX2, SP7, OPN, OCN, alkaline phosphatase, SOX9, Type II/X collagen), accompanied by the down-regulation of mineralisation inhibitory molecules and the production of a calcification matrix. The differentiation of vascular SMC towards an osteoblast-like phenotype has been confirmed in vitro and in vivo. In a transgenic mouse model, it has been demonstrated that vascular SMC in the tunica media are able to differentiate into chondrocytes and osteoblasts if exposed to oxLDL and reactive oxygen species which up-regulate the expression of the bonerelated transcription factor RUNX2[78,79]. RUNX2 induces osteoblastogenesis from mesenchymal stem cells, an essential pathway in the ossification process of the extracellular matrix. In particular, RUNX2 increases expression of the bone-related proteins OCN, sclerostin, and RANKL. Therefore, oxLDL in the arterial wall leads to arterial intimal calcification by vascular SMC differentiation into osteoblast-like cells, regulated by molecules that initiate and regulate osteoblastic and chondrocytic differentiation. OxLDL have also been shown to promote trans-differentiation of circulating monocytes towards a pro-calcific phenotype[80]. Monocytes are well known for playing a key role in the development of atherosclerotic plaques through their transmigration to the arterial wall. Here, they differentiate into macrophages and contribute to the pro-inflammatory milieu, and phagocyte lipid droplets can give rise to foam cells. Recent evidence also strongly supports a role for monocytes in the active calcification process of arterial wall. Circulating osteoblastic progenitors were initially described in a landmark paper by Eghbali-Fatourechi et al.,[81] in which mononuclear circulating OCN+ cells were able to form mineralised nodules when cultured in osteoblast-differentiating medium and cause ectopic calcification when transplanted in mice. Subsequently, monocytes have been described as a source of mesenchymal progenitors which can differentiate into osteoblast-like cells[82] and contribute to atherosclerotic calcification[83]. Some reports have suggested that circulating myeloid cells with osteogenic

potential may affect CVD in the general population and in diabetes[81,84,85]. Chronic inflammation seems to have a central role in the pathogenesis of medial calcification, which is often independent of atherosclerosis: this suggests different processes drive vascular SMC differentiation[86]. Hyperglycaemia and dyslipidaemia cause the production of pro-inflammatory cytokines, such as TNFα primarily released by monocytes and macrophages. TNFα was found to be the main cytokine activating osteogenic programmes of vascular SMC via Msx2–Wnt signalling[87] MSX2 is a gene coding for an osteochondrogenic transcription factor associated with intramembranous ossification. Wnt signalling is also implicated in osteoblast maturation, medial calcification, and fibrosis. Therefore, vascular SMC of the medial layer respond to different osteogenic stimuli, including inflammation or prolonged uraemia, and trans-differentiate into osteoblast-like cells, which can cause vessel wall stiffening through calcium deposits[74].

Initially, vascular calcification was thought to be a passive, degenerative process analogous to a form of ageing. However, evidence suggests that in reality the mechanism is very complex, active and cell-mediated, characterised by an important interaction between genetic, environmental and vascular components. Moreover, it is a mechanism particularly more common in diabetes mellitus and in renal failure. Calcification can be classified according to the location of the site of deposition at the vascular level, affecting for instance the intima or media tonaca, but also the heart valves or the myocardium. Calcification of the intima is typically associated with atherosclerosis, and is involved in inflammatory, necrotic and dyslipidemic processes, and typically originates from microcalcifications that may progress to macrocalcifications. Calcification of the middle tonaca, on the other hand, hardens the arterial wall altering blood flow, and is, of the two forms, the more characteristic of chronic renal failure, diabetes mellitus, hypertension and osteoporosis. It is independent of atherosclerosis and has similar mechanisms to intramembranous bone deposition[88]. The

mechanism typically begins with deposition of calcium phosphate complexes. In particular, there is a process of differentiation of the smooth muscle cell into an osteoblastoid cell, related to both genes such as BMP2 or MSX2 and alkaline phosphatase (ALP). Cell differentiation begins with smooth muscle cell deposition of a calcium-rich intercellular matrix. There is well-established evidence that excess hyperphosphatemia (well associated, for example, with kidney failure) triggers complexes that activate certain pro-calcium cell signalling mechanisms, but further promoting factors will be listed below. The mechanism of initiation and progression is then analogous to physiological osteogenesis: high phosphate levels promote Pit1, which increases intracellular levels of inorganic phosphate, which in turn promotes the transcription factor RUNX2. This promotes osteogenic differentiation of vascular smooth muscle cells. In addition, hyperphosphatemia induces remodelling of the extracellular matrix by metalloproteinases and cysteinaproteinases, which cause matrix degradation giving rise to bioactive elastin peptides, and promoting the production of collagen, which further alters the composition of the matrix. In addition, hyperphosphatemia eventually leads to necrosis or apoptosis of smooth muscle cells, which may form nuclei for the precipitation of calcium phosphate[89].

Other additional factors promote calcification: **extracellular vesicles** are cell-produced structures composed of soluble proteins, lipids and non-coding RNA, and are capable of transferring functional transcribed material and lipids to other cells, even changing their phenotype. They have been found in calcified human aortic valves, in the middle tonaca of the aorta and in atherosclerotic plaques of the intima. Smooth muscle cells are used physiologically to secrete matrix vesicles to maintain homeostasis, but are capable of calcifying target cells under pathological conditions. They may induce osteogenic (RUNX2, SMAD1, SP7, TNAP) and proinflammatory genes, or they may simply be rich in calcium in an attempt, by extrusion of the cation, to reduce intracellular accumulation[89].

Autophagy is an important mechanism for the removal of damaged cell organs or unwanted metabolites, and is a crucial process in maintaining normal smooth muscle cell function. Under the stress conditions already described, including excess ROS, hypoxia, infection or protein aggregation, the mechanism is accelerated. In particular, Platelet Derived Growth Factor (PDGF), which has the ultimate autophagic effect of degrading the proteins required to maintain the contractile phenotype of muscle cells, appears to play an important role in promoting it. In addition, hyperglycaemia has a direct role in accelerating the mechanism, as it induces activation of AKT/mTOR, signalling pathways that lead to altered autophagic activity resulting in vascular calcification[89].

Molecular inducers of vascular calcification: **Cathepsin K** is a typical marker of osteoclasts, is a major lysosomal cysteine protease that degrades organic matrix, and is increased in vascular calcification as well as in osteoporosis and cardiovascular disease. Its deletion is associated with reduced differentiation of muscle cells, blocking in particular the Wnt3a and osteoprotegerin (OPG) pathways.

FGF23 is produced by osteocytes in response to increases in phosphates, and acts both in the kidney (reducing phosphorous reabsorption and decreasing calcitriol synthesis) and intestine (reducing absorption of dietary phosphorus). In the absence of the membrane protein Klotho, which acts as a cofactor, FGF23 is unable to regulate phosphate homeostasis, so elevated FGF23 levels are indicative of a response to hyperphosphoremia, and should be considered an alarm signal for vascular calcification. FGF23 is also able to reduce calcidiol activation (by blocking its conversion to 1,25(OH)-Vit.D). It correlates positively with parathormone and phosphate levels, and negatively with both 1,25(OH)-Vit.D and with eGFR and tubular phosphate reabsorption.

BMP2 (Bone Morphogenic Protein 2) is a protein that binds to a heterodimeric complex of transmembrane receptors, which transduce a signal to transdifferentiate smooth muscle cells into osteochondrogenic cells, and is strongly associated, along with BMP4, with calcific arteriopathy. It is also capable of suppressing smooth muscle cell proliferation by inhibiting p21 and arresting the cell cycle, or it can stimulate apoptosis, ROS production or inflammation at the cellular level.

ALP (alkaline phosphatase) is a typical marker associated with hydroxyapatite formation, a classic mineral of calcification.

RUNX2 is a master regulator of osteoblastic differentiation, and is also present in osteoblastic differentiation of smooth muscle cells. It is selectively expressed in calcified arterial tissue, and is stimulated (especially in the development of calcification with atherogenesis) by hydrogen peroxides, which promote its activation of the Akt-mediated signalling pathway and thus phenotypic change of muscle cells. Inorganic phosphate also induces the expression of RUNX2, acting as a transcription factor for osteogenic genes (OC, OPN, Osterix), of which osterix in particular is essential for the activation of the calcifying phenotype induced by RUNX2.

Osteocalcin (OCN) is the most expressed non-collagenous protein in the bone matrix, synthesised by osteoblasts. Several studies have shown an association between OCN and cardiovascular risk factors such as insulin resistance and dyslipidaemia[90]. The relationship between OCN and atherosclerosis in humans has been suggested in different studies. In a study conducted in healthy post-menopausal women, there was an increased prevalence of

carotid atherosclerosis in subjects with OCN levels above the median and low bone mineral density[91]. Moreover, some evidence has shown that OCN is expressed in the calcific atherosclerotic lesions and in the vascular SMC of the vessel wall, suggesting a potential role in the differentiation of vascular SMC into osteogenic cells[92]. Circulating osteoprogenitor cells, a member of the monocytic family, express OCN on their surface and exhibit increased abundance in patients with CVD[93]. Because of their procalcific phenotype, these cells contribute to the development of vascular calcification and atherosclerosis under normal conditions[94]. Patients with diabetes show elevated levels of OCN+ circulant monocytes compared to controls, especially in the presence of atherosclerotic CVD[93]. Moreover, OCN+ cells have been found in coronaries of subjects with coronary artery disease, of which levels are associated with the presence of vascular calcifications and instability of the plaques[85].

Sclerostin is a soluble factor secreted by osteocytes. It regulates bone turnover by inhibiting Wnt/β-catenin signalling, which promotes the differentiation and proliferation of osteoblasts. Sclerostin is also able to stimulate osteoblast apoptosis through the activation of caspases[95]. Therefore, when sclerostin action is suppressed, osteogenesis is indirectly stimulated[96]. Loss of sclerostin gene function is related to diseases characterised by a hyperostosis process[95]. Elevated serum sclerostin has also been associated with the presence of atherosclerosis and is a proposed marker of CVD, particularly in subjects with diabetes[97].45 Kuiper et al.[98] showed that increased levels of sclerostin are associated with an augmented risk of having coronary artery calcifications. Sclerostin can also play a role in the development of valve calcification. Moreover, a study conducted by Leto et al.[99] in 2018 showed that high levels of sclerostin were present in vascular smooth muscle cells of the atherosclerotic plaques in a cohort of patients who had undergone carotid endarterectomy

suggesting a potential role of this molecule in the development of atherosclerosis. Koos et al.[100] showed that patients with aortic valve calcification have increased sclerostin levels compared to healthy controls, and that the severity of calcification is directly proportional with its serum levels. Eventually, the presence of calcification in the abdominal aorta seems to be associated with elevated serum sclerostin levels, as emerged in a study conducted by Hampson et al.[101] in 2013. This study showed that subjects with higher aortic pulse wave velocity, an indicator of arterial stiffness, had significantly higher serum sclerostin levels compared to subjects with normal pulse wave velocity. Of note, it has been evidenced that the use of monoclonal anti-sclerostin antibodies for the treatment of post-menopausal osteoporosis can result in increased risk of cardiovascular adverse events[102], suggesting a possible protective role of sclerostin on the vasculature.

Klotho is known as a membrane protein and as a circulating peptide (the extracellular domain is secreted into the blood), with peculiar antiageing properties. The membrane protein form is predominantly expressed in the distal tubule of the kidney, but also in multiple other tissues. In the kidney, it acts as a co-receptor for FGF-23, a bone-derived growth factor that mainly inhibits renal activation of vitamin D and induces tubular excretion of phosphatemoe[103]. Klotho is also supposed to be involved in the pathogenesis of arterial calcification[103]. Mice with targeted deletion of klotho display severe osteoporosis and progressive atherosclerosis, as described in a study conducted by Kuro et al.[104] in 1997. The relationship between levels of soluble klotho and the occurrence and severity of CVD has been shown in several clinical studies[105], and some polymorphisms of the gene have been related to the incidence of cardiovascular events[106]. Lim et al.[107] demonstrated for the first time that klotho and FGFR are expressed in human arteries, with downregulation in response to phosphorus and TNF. They also found that decreased klotho levels lead to

increased calcification and demonstrated that upregulation of klotho (through vitamin D receptor activation by calcitriol) restored klotho/FGF-23 signalling, inhibiting vascular calcification.[107]

Molecular inhibitors of vascular calcification: Osteoprotegerin (OPG) is a molecule that interferes with RANK-RANKL binding, which is fundamental in the maturational differentiation of osteoclasts. Its role in vascular calcification is controversial, partly because of the molecule's presence in human and murine atherosclerotic lesions and because of the direct correlation between its 100 increase and carotid thickening in the intima and media. Although, as mentioned, it neutralises RANK and RANKL binding, it is ineffective at high RANKL concentrations.

Osteoprotegerin (OPG) is a member of the TNF superfamily that acts by binding RANKL and TRAIL[108]. Its role in bone metabolism is to inhibit osteoclastic bone resorption, a role it enacts through its presentation as a 'decoy receptor' for RANKL, preventing its binding to RANK and stimulating osteoclast activation. Indeed, elevated serum OPG levels are associated with higher BMD[109]. Some evidence has shown that OPG could have a protective influence on the vascular system. OPG-knockout mice have concomitantly shown early onset osteoporosis and increased vascular calcifications. However, observational studies in humans suggest that OPG may be a marker of CVD. High levels of serum OPG have been associated with the presence of vascular calcifications[110], coronary artery disease[111], carotid atherosclerotic plaques[112], peripheral artery disease[113], and cardiovascular mortality. Furthermore, OPG also binds TRAIL, which is involved in the regulation and modulation of apoptosis[108]. This binding also seems to play a direct role in the development of atherosclerotic plaques, with pleiotropic effects on the vasculature. Some evidence has

shown increased apoptosis to occur in TRAIL-treated endothelial cells, while other studies have described increased cellular survival and proliferation in response to TRAIL[114].41 Furthermore, it seems that high levels of TRAIL are expressed at the vulnerable plaque sites[108],33 and it is known that TRAIL stimulates the production of nitric oxide, which plays a protective role within the endothelium[115]. Overall, the debate remains open as to whether increased OPG serum levels attenuate CVD or are instead representative of counter-regulatory protective pathway activation in the context of vascular calcification processes.

Osteopontin (OPN) is a strongly related phosphoprotein for hydroxyapatite, due to its negatively charged phosphoserines. It is able to prevent calcium crystal growth and accelerate osteoclastic function. Its inhibitory role in vascular calcification has been widely demonstrated, and in fact its phosphorylated form is important in inhibiting vascular calcification. OPN is a structural glycoprotein of the bone matrix. Because of its high number of aspartic acid residues, it can bind calcium and hydroxyapatite ions, inhibiting crystal formation. Additionally, it can bind several integrin receptors, especially integrin β3 on the surface of osteoclasts. This binding leads to a decrease in calcium concentration and to the activation of carbonic anhydrase II, both required for osteoclastic activation[116]. These actions are important for the resorption of ectopic calcifications. OPN is also a regulator of calcification in the vessel wall. In a murine model, OPN has demonstrated to be an inhibitor of vascular calcification. In fact, Speer et al.[117] showed that mice deficient in matrix Gla protein (a factor involved in the inhibition of bone mineralisation) and with deleterious OPN mutations are more prone to develop extensive vascular calcifications than mice deficient for matrix Gla protein alone. Studies conducted in humans confirmed that OPN could be a marker of vascular disease. The FinnDiane study showed that OPN is a strong predictor of cardiovascular events in subjects with T1D[118], whereas another study has shown an

association between elevated OPN serum levels and the presence of coronary artery calcification in patients with T2D mellitus. Interestingly, some evidence has shown OPN to potentially be an inhibitor of vascular calcification, and may stimulate the dissolution of calcifications by inducing macrophages to express carbonic anhydrase. In this context, elevated serum levels of OPN may reflect a compensatory mechanism against calcification.

Matrix Gla Protein (MGP) is a vitamin K-dependent inhibitor of calcification. The absence of MGP is lethal in the mouse model due to pathological arterial calcification[89]. Its overexpression, however, suppresses BMP2 activity, reducing atherosclerosis, calcification of intima and media, and inflammation[119]. It also binds to calcium crystals, preventing their further growth. It is highly expressed in pathological conditions as a mechanism to balance calcification.

Fetuin A (Fet-A) is a strong inhibitor of calcification, binding early to calcium phosphate crystals and causing their clearance with a reduction in inflammatory response. Its plasma concentrations are inversely proportional to vascular calcification.

BMP7 is a protein of the BMP family, but unlike BMP2, it reduces the phenotypic change of smooth muscle cells to osteogenic cells. In addition, it is able to reduce plasma phosphorus, and is able to ameliorate bone diseases characterised by both excess and deficient tissue renewal[89].

1.3.1. Myeloid Calcifying Cells (MCCs)

The phenomenon of vascular calcification is not only related to a phenotypic alteration of resident cells, but certain circulating cells belonging to the monocytic lineage have also been

described as being associated with calcification. These are cells characterised by the membrane expression of OCN (osteocalcin), and are present in particular concentration both in pathophysiological conditions of increased bone deposition (e.g. pubertal growth age, but also following fracture) and in the pathological condition of the T2D patient. In the adolescent patient, in particular, a much greater amount of OCN+ cells, about five times greater than in simple cells, was detected positive for alkaline phosphatase[81]. Further studies underlined the importance of the pathological interest of these cells in vascular calcification[120]. In particular, recalling the physiological migratory and diapedetic capacity aimed at vascular and angiogenic repair typical of both monocytic elements and endothelial progenitor cells (EPCs), both considered among the possible actors capable of producing substantial calcifying changes in the vascular walls. The same study also emphasised the increased expression of these circulating CD34-positive cells in T2D patients with coronary artery disease, emphasising a pro-calcific drift of cell populations in this context as well, and suggesting that these cells, normally reparative and protective of arteries, may under conditions of over-activation cause a worsening of cardiovascular risk, especially in the case of epithelium-mesenchyme transition and subsequent osteoblastic redifferentiation[84,121]. Additional bone marrow-derived cells, in particular cells belonging to the monocyte-macrophage lineage, also demonstrated a similar mesenchymal re-differentiation capacity, and indeed also associated, albeit electively at the level of the intima rather than media tonaca, with vascular calcification[121]. A higher concentration of these cells has demonstrated in patients with both T2D (with positive correlation with both glycated haemoglobin levels and the presence of cardiovascular atherosclerosis)[84]. Further studies supported the possible role of monocyte-derived cells in coronary atherosclerosis, in the phenomena of vascular calcification and in the complication of plaques[85]. Immune and flogistic mechanisms, as well as hyperglycaemia, hypoxia and the various molecular markers mentioned above, also

play an important role in cell differentiation, and further development in studies of the pathways involved may lead to effective therapeutic approaches.

1.4. Diabetes mellitus treatments

Early treatment of T2D is recommended by the most recent and important guidelines[122]. The ADA/EASD consensus[122] recommends a holistic, multifaceted, person-centered approach accounting for the complexity of managing T2D. Person-specific factors that affect choice of treatment include individualized glycemic goals, individualized weight goals, the individual's risk for hypoglycemia, and the individual's history of or risk factors for cardiovascular, kidney, liver, and other comorbidities and complications of diabetes. The consensus on glycaemic control recommends a glycaemic haemoglobin target of 7% or less for adults with sufficient life expectancy to have long-term benefits, especially microvascular benefits, but even lower targets are permissible if there are no real risks of hypoglycaemia or adverse effects from therapy[123]. Lifestyle modifications and health behaviors that improve health should be emphasized along with any pharmacologic therapy. Weight reduction is associated with improved glycated haemoglobin levels and reduced complications and comorbidities. The primary goal for the management of a large number of T2D patients should be a weight loss of 5 to 15%, considering that losses of more than 10 per cent could lead not only to metabolic and preventive benefits against complications, but also to remission of the disease[124]. Physical activity significantly improves cardiometabolic balance in patients with T2D.

Pharmacologic therapy should be guided by person-centered treatment factors, including comorbidities and treatment goals. In adults with T2D and established/high risk of atherosclerotic cardiovascular disease (ASCVD), HF, and/or chronic kidney disease (CKD), the treatment plan should include agents that reduce cardiovascular and kidney disease.

Weight management is a distinct treatment goal, along with glycemic management, in individuals with T2D, as it has multifaceted benefits, including improved glycemic management, reduction in hepatic steatosis, and improvement in cardiovascular risk factors[125,126]. When A1C is >1.5% above the individualized glycemic goal, many individuals will require dual-combination therapy or a more potent glucose-lowering agent to achieve and maintain their goal A1C level. Traditional recommendations have been to use stepwise addition of medications to metformin to maintain goal A1C. However, there are data to support initial combination therapy for more rapid attainment of glycemic goals[127] and later combination therapy for longer durability of glycemic effect. The VERIFY (Vildagliptin Efficacy in combination with metformin For earlY treatment of T2D) trial demonstrated that initial combination therapy—in this case of metformin and the dipeptidyl peptidase 4 (DPP-4) inhibitor vildagliptin—is superior to sequential addition of medications for extending primary and secondary failure (100). Initial combination therapy should be considered in people presenting with A1C levels 1.5–2.0% above goal. Finally, incorporation of high-glycemic-efficacy therapies or therapies for cardiovascular and kidney disease risk reduction (e.g., GLP-1 RAs, dual GIP and GLP-1 RA, and SGLT2 inhibitors) may allow for weaning of the current medication plan. Thus, treatment intensification may not necessarily follow a pure sequential addition of therapy but instead reflect a tailoring of the medication plan in alignment with person-centered treatment goals and pursuit of multifaceted treatment goals. Important clinical characteristics include the presence of overweight or obesity, established ASCVD or indicators of high ASCVD risk, HF, CKD, obesity, nonalcoholic fatty liver disease or nonalcoholic steatohepatitis, hypoglycemia, and risk for specific adverse drug effects[122].

Metformin is the most widely prescribed drug for the treatment of T2D, effective, safe, and may reduce risk of cardiovascular events and death. It represents the first line therapy in T2D. The pharmacodynamic mechanism is still a matter of debate. According to studies conducted using the hyperinsulinemic euglycaemic clamp in T2D patients before and after chronic metformin treatment, a reduction in hepatic glucose production has been systematically demonstrated, while it has been associated peripherally with increased insulin-mediated glucose uptake. Although the specific mechanisms are still unknown, several hypotheses have been proposed, including hypotheses on the transcriptional alteration of certain factors (considered a possible mechanism but insufficient to justify metformin's action, given the extremely rapid onset of action of the drug), or direct mechanisms on certain factors. In particular, the inhibition of complex I activity is the most studied and most widely accepted. This complex is the site through which NADH makes its contribution to the genesis of the mitochondrial proton gradient, and, given the energy cost of gluconeogenesis, its inhibition has been correlated with reduced hepatic glucose production. At mM concentrations of metformin, an inhibition of complex I has been found to be transversely accepted by various studies. Thus, a down-regulation effect on the electron transport chain has been proposed, resulting in a cellular alteration of ATP/ADP and ATP/AMP ratios, which could indeed mediate anti-diabetic effects. But another proposed mechanism is the activation of AMPK, a kinase allosterically activated by an alteration in ATP/ADP/AMP ratios (an alteration that also occurs in prolonged fasting and intense exercise). This activation would lead to a down-regulation of genes involved in gluconeogenesis, but also to an immediate phosphorylation effect of AcetylCoA carboxylase 1 and 2 (ACC1 and 2), which reduce lipogenesis and promote mitochondrial oxidation in the liver, increasing the liver's sensitivity to insulin. Increased cytosolic oxidoreduction, mediated by inhibition of glycerol-3-phosphate dehydrogenase 2 (GPD2), has also been

suggested as a further mechanism, GPD2 being another enzyme required for glycerol utilisation in the gluconeogenetic pathway. Muscle mechanisms, however, are still unclear, and are probably more indirectly related to metformin action. Studies, in fact, associate the increased muscle uptake following insulin stimulation in metformin-treated patients more with a reduction in glucose toxicity than with any metabolic pathway involved[128]. Metformin is available in an immediate-release form for twice-daily dosing or as an extended-release form that can be given once daily. Compared with sulfonylureas, metformin as first line therapy has beneficial effects on A1C, is weight neutral, does not cause hypoglycemia, and reduces cardiovascular mortality. The principal side effects of metformin are gastrointestinal intolerance due to bloating, abdominal discomfort, and diarrhea; these can be mitigated by gradual dose titration and/or using extended release formulation. The drug is cleared by renal filtration, and very high circulating levels have been associated with lactic acidosis. However, the occurrence of this complication is now known to be very rare, and metformin may be safely used in people with estimated glomerular filtration rate >30 mL/min/1.73 m2. Metformin use is associated with vitamin B12 deficiency and worsening of symptoms of neuropathy. It is also contraindicated in patients suffering from any type of acute metabolic acidosis, prediabetic coma and other chronic conditions capable of leading to alterations in tissue perfusion or aggravation of renal function.

For people with T2D and established ASCVD or indicators of high ASCVD risk, HF, or CKD, an SGLT2 inhibitor and/or GLP-1 RA with demonstrated cardiovascular benefit is recommended as part of the glucose-lowering plan independent of A1C, independent of metformin use, and in consideration of person-specific factors. Individuals with these comorbidities already achieving their individualized glycemic goals with other medications may benefit from switching to these preferred medications, if possible, to reduce risk of

ASCVD, HF, and/or CKD in addition to achieving glycemic goals. This is particularly important as SGLT2 inhibitors and GLP-1 RA are associated with lower risk of hypoglycemia and individuals with ASCVD, HF, and CKD experience heightened hypoglycemia risk[129]. Cardiovascular Outcomes Trials There are now multiple large randomized controlled trials reporting statistically significant reductions in cardiovascular events in adults with T2D treated with an SGLT2 inhibitor or GLP-1 RA. Emerging data suggest that use of both classes of drugs will provide additional cardiovascular and kidney outcomes benefit; thus, combination therapy with an SGLT2 inhibitor and a GLP-1 RA may be considered to provide the complementary outcomes benefits associated with these classes of medication. In cardiovascular outcomes trials, empagliflozin, canagliflozin, dapagliflozin, liraglutide, semaglutide, and dulaglutide all had beneficial effects on indices of CKD, while dedicated renal outcomes studies have demonstrated benefit of specific SGLT2 inhibitors. Individuals at low risk for ASCVD may benefit from GLP-1 RA therapy to reduce their risk of future ASCVD events, although the evidence is currently limited.

Glucagon Peptide Like 1' (GLP-1) is a molecule that is part of the intestinal incretins, hormones released by the gastrointestinal system following food intake, with the ability to increase the insulin secretory response during periods characterised by hyperglycaemia[130]. As early as 1993 there was an initial study suggestive of the possibility of using exogenous GLP-1 in normalising hyperglycaemia, also providing evidence of useful pleiotropic effects[131], while another study from the same year showed an effect of stimulating insulin secretion and inhibiting glucagon from truncated GLP-1. A peptide called exendin-4 homologous to human GLP-1 was identified, capable of binding the receptor for GLP-1 and giving the aforementioned normoglycaemic effects. Production of synthetic exendin-4 was initiated, and the peptide, renamed ‘Exenatide’, was approved as the first GLP-1 RA for

human use. The mechanisms of the drug that lead to the control of hyperglycaemia are multiple. Firstly, the insulinotropic effect, which only occurs under hyperglycaemic conditions; secondly, it leads to suppression of glucagon secretion; thirdly, it reduces gastric transit, leading to changes in appetite, a sense of satiety and reduced calorie intake. The effects on appetite control are mediated by specific brain circuits at a central level, which are involved in both the physiology of appetite for the achievement of energy needs and the hedonistic role of food. In particular, the arcuate nucleus, hypothalamus, postrema area and nucleus tractus solitarius are all structures directly involved in the process. GLP-1 receptor agonists prevent the stimulation of appetite by suppressing the activity of neurons in the arcuate nucleus, which produce NPY/agouti-related peptide (AgRP), and by inducing neurons in the parabrachial nucleus associated with the cessation of eating. There are, however, some differences in the brain pharmacodynamics of the various GLP-1 RAs, which may account for the difference between different GLP-1 RAs in changing body weight. In addition to their hypoglycaemic effects, GLP-1 RAs have also proved to be important drugs due to their pleiotropic effects, first and foremost their cardiovascular effects[130]. Several studies have been conducted on the effect of GLP-1 RA on cardiovascular prevention, which resulted in a meta-analysis showing a reduction in major cardiovascular events (Hazard Ratio 0.88, IC 95% 0.82-0.94), cardiovascular death (HR 0. 88, CI 95% 0.81-0.96), fatal and non-fatal stroke (HR 0.84, CI 0.76-0.93), and also a protective effect for myocardial infarction (HR 0.91, CI 95% 0.84-1.00), which, however, as can be seen from the confidence interval, is at the limit of statistical significance[132]. Several mechanisms have been proposed to underlie this protection, including the reduction of progression and complication of atherosclerosis. In particular, GLP-1 RAs reduce ROS production, reduce activation of monocyte-macrophage migration mediated by oxidised LDL-cholesterol and promote differentiation of macrophages into M2 phenotype, which, however, due to the

reduction of ROS, will not progress to Foam Cells and will not contribute to plaque growth. The renal system also benefits from the use of GLP-1 RA. In particular, prevention of the onset of macroalbuminuria, reduction of renal albuminuria and reduction of glomerular filtrate degradation (eGFR) over time have been described. The pharmacokinetic profile varies considerably depending on the active ingredient used. In general, as mentioned, GLP-1 is rapidly degraded by DPP-4, leading to a very short half-life. This has led to the need to devise strategies to lengthen the half-life of the drug, leading to the production of 'short acting' GLP-1 RA (characterised by rapid peaks in plasma concentrations with periods between administration and the other, of no drug in circulation) and 'long acting' (with a constantly high drug concentration once steady state is reached, with minimal fluctuations between injections). Due to the real risk of common adverse effects (e.g. nausea and vomiting), especially at the start of therapy or dose escalation, it has been recognised as an effective preventive strategy dose titration, which therefore involves a gradual increase of the administered dose in order to reach steady state gradually and reduce gastrointestinal adverse effects. The decision on the titration strategy should be made considering the pharmacokinetic properties of the formulation. In terms of clinical use, treatment with GLP-1 RAs has been shown to be comparable in efficacy to insulin therapy. Unlike insulin therapy, however, GLP-1 RAs also led to weight reduction, and did not cause hypoglycemia unless combined with sulfonylureas or insulin. Another advantage of GLP-1 RAs is the standardization of the dose, with minimal effort to perform the previously treated titration, while insulin requires an extremely personalized approach[130]. Given the difficulty given by the side effects of hypoglycemia, and given the considerable evidence supporting the use of GLP-1 RAs even in the critically ill hospitalized patient with hyperglycemia, it is reasonable to consider the possibility of using these drugs also in the hospitalized patient, although further scientific evidence in this regard is desirable[133]. GLP-1 RAs are not free from

adverse effects. The most common are nausea, vomiting and diarrhea, typical of the beginning of therapy or of dose increases. The effects typically occur in the fasting state, suggesting a direct interaction with the GLP-1 receptors at the brain level, located in the area postrema of the brainstem which is actually responsible for controlling vomiting. The probability of an adverse effect of this type is proportional to the speed of reaching the maximum concentration, further explaining the need to titrate the dose to prevent ADRs. Indeed, the appearance of the adverse effect is very rare in cases of co-administration of basal insulin and GLP-1 RA (formulation that requires extremely slow titration)[130]. The safety profile has long been debated, with studies highlighting a risk of developing pancreatitis, pancreatic and thyroid neoplasms[134], but thanks to the trials performed using the various available databases, a Hazard Ratio of 1.05 (95% CI 0.78-1.41) for the development of pancreatitis, HR 1.14 (95% CI 0.77-1.70) for the development of pancreatic cancer and HR 0.99 (CI 0.90-1.08) for the development of neoplasms in general has been demonstrated, denoting an absence of statistical significance in the association between therapy and the development of the complications mentioned[135]. The only contraindication at present is hypersensitivity to the active ingredient.

There are few randomized clinical trials that have explored the efficacy and safety of drugs for the treatment of T2D in patients with renal insufficiency. Therefore, the recommendation on which drugs to use as first or second choice derives solely from (indirect) data on the metabolic, cardio- and renoprotective effects and on the cost-effectiveness of individual molecules, which show that SGLT-2i have a better profile than other molecules. Numerous randomized clinical trials support the use of metformin, SGLT-2i or GLP-1 RA as first-choice drugs in the treatment of patients with T2D due to their efficacy in terms of reducing HbA1c without causing hypoglycemia and with beneficial effects on major cardiovascular events and all-cause mortality. In particular, SGLT-2i also have, compared to metformin and

GLP-1 RA, favorable effects on the risk of hospitalization for heart failure. Furthermore, GLP-1 RA and SGLT-2i have favorable effects on body weight. The use of SGLT-2 inhibitors is recommended as first-line drugs for the long-term treatment of patients with T2D with heart failure. Emerging data suggest that use of both classes of drugs will provide additional cardiovascular and kidney outcomes benefit; thus, combination therapy with an SGLT2 inhibitor and a GLP-1 RA may be considered to provide the complementary outcomes benefits associated with these classes of medication. In cardiovascular outcomes trials, empagliflozin, canagliflozin, dapagliflozin, liraglutide, semaglutide, and dulaglutide all had beneficial effects on indices of CKD, while dedicated renal outcomes studies have demonstrated benefit of specific **SGLT2 inhibitors**.

Normally, glucose is freely filtered by the glomerular capillary membrane, and is reabsorbed with a virtual threshold of 375 mg/min mainly thanks to the Na+/D-glucose co-transporter system at the level of the luminal membrane of the tubular cells[136]. The effective threshold, however, is 180 mg/dl, a concentration starting from which glycosuria appears. These transporters use a Na+/K+ ATPase to extrude sodium cations, depleting cellular reserves and creating a negative potential, which is then exploited for the symport necessary to internalize glucose. SGLT1 and SGLT2 are the two most studied transporters. SGLT2 is located in the S1 segment of the proximal convoluted tubule, while SGLT1, located in the S3 segment, is also responsible (with a much lower reabsorption capacity) for the recovery of glucose not reabsorbed by SGLT2[136].

The first inhibitor of sodium/glucose co-transporters was isolated in 1835 by French chemists from apple bark, and was called phlorizin. In the 1970s, the connection between phlorizin and glucose reabsorption was better understood, and in the 1990s, the description of SGLTs and the prospect of a therapeutic use of their inhibitors began (excluding phlorizin

due to poor intestinal absorption and poor selectivity for SGLT2)[136]. Unfortunately, all **SGLT2i** have a glucosuric power proportional to the glomerular filtration rate, associating a decline in their efficacy with the reduction of eGFR[136]. But the effects of these extraordinary drugs are not limited to glycemic control. A meta-analysis by McGuire et al. highlighted an overall reduction in major cardiovascular events corresponding to a HR 0.90 (95% CI 0.85-0.95), and a reduction in overall cardiovascular mortality of 15% (HR 0.85, 95% CI 0.78-0.93). Furthermore, it significantly reduces hospitalization in patients with heart failure (HR 0.68, 95% CI 0.61-0.76), with almost superimposable effects among patients with cardiovascular atherosclerosis (HR 0.70, 95% CI 0.62-0.78) and without (HR 0.63, CI 0.50-0.80)[137]. These extraordinary results have allowed the inclusion in the most up-to-date guidelines on heart failure, SGLTi in the treatment of chronic patients94. The renal protection results are also remarkable, with a general improvement in renal outcomes (worsening eGFR, progression to chronic renal failure, renal death), with significant results both globally (HR 0.62, 95% CI 0.56-0.70) and stratifying between patients with atherosclerotic disease (HR 0.64, 95% CI 0.56-0.72) and without (HR 0.60, 95% CI 0.50-0.73)[137]. The cardiovascular protection mechanisms are probably independent of traditional risk factors. First of all, changes in glycated hemoglobin, blood pressure and cholesterol alone do not justify the global cardiovascular benefit of SGLT2i. Furthermore, despite the decrease in glycosuric power with the reduction of filtration, the benefits on the prevention of hospitalization of the patient with heart failure and the reduction of cardiovascular deaths were observed in all possible ranges of glomerular filtration, suggesting the presence of mechanisms dissociated from the antidiabetic mechanism or in any case of a different dose/response curve. Finally, even non-diabetics can derive the same benefits as patients with T2D. They improve the conditions of ventricular filling, in particular by lowering the preload through the diuretic and natriuretic effect, and considering the steep Frank Starling

curve usually characteristic of T2D, the proposed mechanism partly justifies the beneficial effect. Furthermore, 50% of the cardiovascular benefit was ascribed to the hemoconcentration capacity. At renal level, SGLT2i significantly reduced the worsening of filtration, end-stage renal failure or renal death by 45% (HR 0.55, 95% CI 0.48-0.64), and although the results are very favorable, the precise mechanism of renal protection is still unknown96. Probably, inhibition of tubulorenal feedback could play a central role in delaying the progression of renal disease[138]. Some adverse effects associated with SGLT2i are described. In particular, the increased risk of urogenital infections is a clear class effect. This effect is dose-dependent, and is more frequent. A final very rare but very dangerous adverse effect of SGLT2i is euglycaemic diabetic ketoacidosis. The pathogenesis is quite similar to diabetic ketoacidosis typical of type 1 diabetes, and is based on a reduction in glucose use with promotion of lipolysis. Euglycaemic ketoacidosis is traditionally defined with the same criteria as diabetic ketoacidosis, but with plasma glucose levels below 300 mg/dl. The real difference, however, between diabetic ketoacidosis and euglycemic ketoacidosis is glycosuria: the loss of glucose mediated by SGLT2i artificially reduces blood glucose, predisposing to ketogenesis. Patients, therefore, must be aware of the risk, especially if they have predisposing conditions such as marked beta-cell failure. Some factors may increase the risk of hyperketonemia, such as stress, illness or alcohol use. In these circumstances, general malaise and mild nausea may occur. Contraindications are typically related to renal filtration. Dapagliflozin and Empagliflozin are indicated in patients with chronic renal disease.

The idea of inhibiting DiPeptidyl Peptidase 4 (DPP4) for the treatment of T2D was born in 1995, and since then **DPP4 inhibitors (DPP4i)** have become an integral part of the algorithms for the treatment of T2D mellitus. Pharmacodynamically, the active ingredients

do not have a directly hypoglycemic power, but the effect is secondary to the action of endogenous substrates (mainly GLP1), whose levels are altered as a result of the inhibition of the catalytic activity of DPP4. Some studies conducted with exendin 9-39 (GLP-1 RA) have demonstrated a reduction in the efficacy of DPP4i in case of elimination of GLP1-mediated signaling. Inhibition of DPP4 helps preserve the glucagon-mediated regulatory response following hypoglycemia. A number of additional hormones and chemokines may be susceptible to cleavage by DPP4, but there is no evidence to support clinically harmful effects of inhibition of these molecules. Adverse effects are uncommon. The possibility of an influence on immunity, mediated by a role of DPP4 in the regulation of T lymphocytes, is not supported by sufficient scientific evidence, and other studies, stating the absence of an enzymatic interaction of DPP4 with these cells, further discredit the theory. Furthermore, no altered infection rates were detected in the various trials aimed at investigating cardiovascular outcomes. The effect on weight is neutral, probably due to a reduced conversion of the intestinal peptide tyrosine-tyrosine (PYY) to its metabolite with an anorectic effect (thereby compensating for the weight loss theoretically mediated by the increase in GLP1). The correlation with the risk of acute pancreatitis is controversial, and there is no strong scientific evidence to support a direct causal correlation, except for small increases that are not statistically significant reported in trials aimed at cardiovascular outcome. Other adverse effects reported are arthralgia and bullous pemphigoid, however rare[139].

Thiazolidinediones are oral drugs that improve insulin sensitivity and are highly effective in reducing glucose. They have a long-lasting glucose control response, probably through a strong beta cell preservation effect. The drug is also indicated in patients with Non-Alcoholic Fatty Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH),

having demonstrated positive effects in disease progression. Born in 1982, these drugs immediately demonstrated promising effects in controlling lipid and glucose profiles. The pharmacodynamic mechanisms are mediated by the modulation of PPARs, nuclear receptors that can be controlled through binding to small lipophilic compounds that involve numerous downstream metabolic pathways. In particular, the binding of thiazolidinediones to PPAR-γ causes a transactivation or transrepression of target genes. The insulin-sensitizing actions of thiazolidinediones follow two possible theories. The first theory proposes a direct mechanism of "fatty acid theft". Thiazolidinediones promote fatty acid uptake and storage in adipose tissue. As a result, especially at the liver, muscle and pancreatic levels, a reduction in lipid content occurs, protecting these tissues. The genes involved in this mechanism are related to lipoprotein lipase, fatty acid transport protein and oxidized LDL receptor 1. A second theory predicts, indirectly, insulin sensitization through effects on the modulation of adiponectins, which mediate a molecular dialogue between fat, muscle and liver tissue. Unfortunately, the drug has some adverse effects that must be carefully evaluated in the management of therapy. It is associated with fluid retention and congestive heart failure, weight gain and bone fractures (effects that can however be mitigated by using lower doses or combinations with other drugs). A higher frequency of bladder cancer in association with pioglitazone has also been reported in a meta-analysis, and epidemiological studies also suggest increases (although not statistically significant) in the number of cases. Consequently, prior evaluation of bladder cancer risk factors is indicated before starting therapy with pioglitazone. The drug is contraindicated in patients with heart failure, liver failure, diabetic ketoacidosis, active bladder cancer or a history thereof, or gross hematuria of unspecified nature[140].

Insulin has the advantage of being effective where other agents are not and should be considered as part of any combination medication plan when hyperglycemia is severe, especially if catabolic features (weight loss, hypertriglyceridemia, ketosis) are present. It is common practice to initiate insulin therapy for people who present with blood glucose levels >300 mg/dL or A1C >10% or if the individual has symptoms of hyperglycemia (i.e., polyuria or polydipsia) or evidence of catabolism (unexpected weight loss)[122]. The advantages of insulin therapy consist of a dose-dependent glycemic reduction, and it is capable of controlling almost any level of glycemia. However, its efficacy and safety are largely dependent on patient education. New formulations have allowed insulins to be marketed with release patterns very similar to physiological secretion. The challenges of insulin therapy remain centered on weight control, patient education and adequate titration of the drug. They present a significant risk of hypoglycemia, which justifies the need for frequent monitoring of glycemia. Rapid-acting insulins can also be added to basal therapy to intensify therapy in conjunction with the prandial period. The most dangerous adverse effect of insulin therapy is hypoglycemia (considered as blood glucose <70 mg/dl). Although it is generally a much more frequent effect in type 1 diabetes than in T2D, restricting the case study to patients on insulin therapy shows that it is an extremely frequent effect in T2D patients on insulin therapy.

2. PRO-DRIFT STUDY

2.1. Background and rationale of the study

In patients with diabetes a complex interplay of different pathological mechanisms causes several end-organ damages and cardiovascular disease (CVD) is still the major cause of morbidity and mortality in subjects affected by T2D (T2D)[28,141] though during the last decades a reduction of cardiovascular-related mortality was observed driven by multifactorial intervention, such as improvement of metabolic control, hypoglycemia events and development in newer medications with positive cardiovascular effects[142]. According to the National Diabetes Statistics Report 2020 released by the American Diabetes Association, hospitalization rates for ischemic heart disease and for stroke were 18.9 and 13.6 per 1,000 adults with diabetes, respectively, and death rate for CVD was 3.5 per 1,000 adults affected by T2D compared with nondiabetic individuals[143]. Several factors contribute to the pathogenesis of CVD in T2D: hyperglycemia, oxidative stress and inflammation play pivotal roles in the development of atherosclerotic disease; dyslipidemia and hypertension often coexist in subjects with diabetes. Overall, an unbalance between mechanisms of injury and protective factors brings to the development of vascular complications in diabetes[144]. Circulating osteoprogenitor cells, defined as circulating cells co-expressing the bone-related markers osteocalcin (OCN) and/or bone alkaline phosphatase (BAP) together with the progenitor stem cell antigen CD34, were shown to be increased in subjects with CVD with and without diabetes[93]. Because of their pro-calcific phenotype, these cells are thought to contribute to the development of vascular calcification and atherosclerosis[94]. Recently, a more abundant type of circulating calcifying cells, named myeloid calcifying cells (MCC), has been described[84,85]. These cells are monocytes (usually identified as CD45+ CD14+ cells) expressing bone surface markers that are able to calcify in vitro and in vivo[84]. MCCs have been associated to plaque vulnerability in patients with early coronary

atherosclerosis10. In subjects with T2D, MCCs were associated to CVD and ectopic vascular calcification and their levels correlated with changes in HbA1c[84]. We also confirmed the association between CVD and MCC levels in ageing subjects with extreme duration type 1 diabetes[80]. In the same study, we also showed that the expression of osteocalcin can be induced in cultures of human monocytes by treatment with OxLDL, a known activator of monocytes involved in the pathogenesis of atherosclerosis, at the concentration of 40 μg/ml OxLDL for 12, 24, 48 and 72 hours. In particular, the percentage of monocytes expressing osteocalcin increased in a time-dependent fashion up three to tenfolds at 72 hours treatment with OxLDL. As well, also the osteocalcin content in cell lysates significantly increased after 48 hours 3 treatment with OxLDL, suggesting an increased production of osteocalcin by monocytes stimulated by OxLDL. However, despite the increasing knowledge and interest about the role of circulating osteoprogenitor cells in the pathogenesis of vascular calcification, to date no pharmacological intervention has been proposed to target MCCs in diabetes. Pioglitazone is a PPARγ agonist approved and widely used in clinical practice for the treatment of T2D. Accumulating evidence demonstrates that PPARγ functions as a key regulator of the differentiation of bone cells[145]. In particular, PPARγ activation suppresses β-catenin protein levels and function, which are required for commitment of stem cells to the osteoblast lineage, resulting in inhibition of osteoblastogenesis. Consistently, PPARγ-null embryonic stem cells spontaneously differentiate into osteoblasts and PPARγ agonists inhibit osteoblast differentiation[146,147]. Of note, PPARγ has an important role in the regulation of immune cells, including monocytes and macrophages[148]. In particular, PPARγ agonists act on monocytes causing the inhibition of inflammatory cytokines production. Interestingly, PPARγ activation has been shown to counteract vascular and valvular calcification and prevent plaque disruption, but the protective mechanism has not been completely elucidated yet[149–151]. Moreover, results of the

PROactive study, a phase III randomized controlled clinical trial, suggest that the addition of pioglitazone to standard therapy for T2D reduces the composite end-point of all-cause mortality, myocardial infarction and stroke[152]. Consistent with these observations, subsequent studies demonstrated that pioglitazone reduced coronary atherosclerotic plaque volume[153] and decreased carotid intima-media thickness[154]. Based on the described effects of PPARγ agonists in inhibiting osteoblast differentiation and monocyte activation, we hypothesize that pioglitazone could prevent the differentiation of monocytes towards and osteoblast-like phenotype. Three GLP1-RAs, liraglutide[155], semaglutide[156] and dulaglutide[157], have been shown reduce cardiovascular outcomes in individuals affected by T2D with either previous cardiovascular disease or at high cardiovascular risk. While GLP1-RA anti-atherogenic effect could be promoted by and improvement of many cardiovascular risk factors (obesity, hypertension, dyslipidemia, inflammation, visceral/ hepatic fat, hyperglycemia)[158], it not yet clear whether GLP1-RA may directly act on pro-atherogenic pathways. Indeed, the molecular mechanisms responsible for GLP1-RA anti-atherogenic effects needed to be better elucidated yet. In this regard, experimental data detected that GLP1 could shift the polarization profile of macrophages from the M1 phenotype (pro-inflammatory) towards the M2 phenotype[159], which may contribute to the 4 protective effects of GLP1-RA cardiovascular diseases and their anti-atherosclerotic proprieties. It has been showed that the GLP1 receptor genes are expressed in human monocytes and downregulated when monocytes differentiate to macrophages or foam cells[160]. As previously mentioned, GLP1 receptors are also present on bone tissue, but the effects of GLP1 on bone metabolism is still debated. Recent in vitro and in vivo experiments showed that GLP1-RA can improve bone metabolism, even though the relationship between GLP1-RA use and fracture risk is controversial[161,162]. GLP1 could affect the fat-bone axis by promoting osteogenic differentiation and inhibiting adipogenic differentiation of bone mesenchymal

precursor cells (BMSCs), which express the GLP1 receptor[163]. BMSCs can differentiate both into osteoblasts and adipocytes and the balance between osteoblast/adipocyte differentiation of BMSC plays a key role in bone homeostasis[164]. Previous in vitro observations revealed exenatide can promote the osteogenic differentiation and inhibit the adipogenic direction of differentiation of BMSCs[165], suggesting GLP1 acts directly on BMSCs. GLP-1 could also stabilize β-catenin by binding to its receptor and increasing the level of intracellular cAMP[166] and, consequently, the expression of bone formation regulatory genes including osteocalcin[92,167], the most expressed non-collagenous protein in the bone matrix, synthetized by osteoblasts involved in the development of vascular calcification and atherosclerosis36. Therefore, in consideration of the described effects of GLP1 in driving BMSCs differentiation towards an osteogenic profile and promoting the expression of bone-related markers as osteocalcin, the emerging effects of GLP1-RA on bone health and on cardiovascular system, we hypothesize that semaglutide, a weekly GLP1-RA, could also regulate BMSCs differentiation of monocytes towards an osteoblast-like phenotype. Metformin, the cornerstone drug for treatment of T2D, has also been shown to have beneficial effects in reducing cardiovascular complications. The pleiotropic effects of metformin are partly mediated by the activation of 5' Adenosine Monophosphate-activated Protein Kinase (AMPK)[168]. Activation of the AMPK signaling pathway suppresses proinflammatory responses and promotes macrophage polarization to an anti-inflammatory functional phenotype in macrophages[169]. Recently, Vasamsetti et al. specifically investigated the effects of metformin on a human monocytic leukemia cell line, showing that metformin inhibits monocyte-to-macrophage differentiation and attenuates atheromatus plaque formation by reducing monocyte infiltrates[170]. Therefore, we will also investigate in vitro whether metformin could influence the differentiation of monocytes towards and osteoblast-like phenotype.

2.2. Hypothesis and study aims

We hypothesize that the differentiation of monocytes towards an osteoblast-like phenotype can be prevented by pharmacological intervention. In consideration of the anti-osteoblastogenesis properties of thiazoledinediones, we individuated in pioglitazone a candidate treatment to be tested to reduce MCCs in T2D. Therefore, we aim to evaluate the effects of pioglitazone, a peroxisome proliferator-activated receptor-γ (PPARγ) agonist on the levels of MCCs in subjects affected by T2D and, in vitro, on the pro-calcific phenotypic drift of human monocytes. Moreover, we will also test in vivo and in vitro whether metformin, the cornerstone of diabetes treatment with inhibitory effects on monocytes differentiation, affects the acquisition of an osteoblast-like phenotype of human monocytes. Furthermore, in consideration of the increasing interest in vascular disease and bone markers and in consideration of a possible favorable effect of GLP1-RA on bone metabolism, we also individuated in this drug class a further candidate treatment to be tested to regulate monocytes' differentiation towards an osteoblast-like phenotype in T2D subjects. Thus, we decided to evaluate also the effects of GLP1-RA on the levels MCCs in subjects affected by T2D and, in vitro, on pro-calcifying differentiation of human monocytes, with or without metformin addition.

Specific aims

Specific Aim 1: To evaluate the effects of the addition of pioglitazone and of GLP1RA both at the recommended therapeutic doses and on top of metformin vs metformin alone on MCC levels, defined as the percentage of circulating Peripheral Blood Mononuclear Cells (PBMCs) expressing all CD45, CD14 and osteocalcin as surface markers (CD45+CD14+OCN+) on the total number of CD45 PBMCs, in subjects affected by T2D.

Specific Aim 2: To evaluate the expression of osteoblast-related genes and of osteocalcin protein in cultures of human monocytes (THP-1) treated with OxLDL ± pioglitazone or ± GLP1-RA exendin-4 or metformin.

2.3. Materials and methods

2.3.1. Study design

PRO-DRIFT is diveded in two sub-studies.

2.3.1.1. Study A: Observational retrospective pilot study

This is an observational prospective study. Two cohorts of patients will be included: those initiating any GLP1-RA at the recommended therapeutic doses in addition to with metformin (2000 mg or max tolerated dose) [Group A]; those on metformin monotherapy (2000 mg or max tolerated dose) [Group B]. Power calculation results in an estimated sample of a total of 150 subjects fulfilling the pre-specified inclusion/exclusion criteria. An index date at baseline will be set as the date patients received for the first time prescription of GLP1-RA plus metformin or metformin alone. Baseline characteristics at the time of first prescription of GLP1-RA plus metformin or metformin alone will be recorded in a database. Updated values of endpoint variables will be recorded at each follow-up visit up. Recommendation about diet and lifestyle according to current guidelines for treatment of T2D will be provided to subjects enrolled in both groups. A sufficient number of subjects will be screened to achieve this number of enrolled subjects. After enrolment subject will be followed up for 3 months. Four study visits will take place at Time 0 and Week 1, Month 1 and 3. Unscheduled visits will occur as medically necessary. Patients will ask to collect once a week 3-point profiles before morning, midday and evening meals, needed for glycemic control evaluation. The following is a detailed explanation of the procedures scheduled in each visit:

V0 (Time 0):

- Admission with diagnosis of T2D, inclusion/exclusion criteria and consent process
- Clinical examination (body weight, height, blood pressure, heart rate) and medical history comprehensive of history of diabetes-related complications
- Blood sample to isolate PBMCs
- Blood sample to measure: HbA1c, creatinine, total cholesterol, HDL, triglycerides. LDL cholesterol will be calculated according to the Friedewald formula.
- Urine samples for visual examination, dipstick test (to measure specific gravity; pH; protein; glucose; ketones; hemoglobin; leukocyte esterase; nitrite; bilirubin; urobilinogen) and microscopic exam (to measure leukocytes, erythrocytes, epithelial cells, bacteria, casts and crystals).
- Educating patients to collect the 3-point Sugar Blood Monitoring Glucose (SBMG)

V1 (1 week):

- Blood sample to isolate PBMCs

V2 (1 month):

- Clinical examination (body weight, height, blood pressure, heart rate)
- Check the 3-point SBMG
- Registration of hypoglycaemic events
- Evaluation of compliance to anti-diabetes treatment

V3/End of the Study (3 months):

- Clinical examination (body weight, height, blood pressure, heart rate) and medical history comprehensive of history of diabetes-related complications
- Blood sample to isolate PBMCs

- Blood sample to measure: HbA1c, creatinine, total cholesterol, HDL, triglycerides. LDL cholesterol will be calculated according to the Friedewald formula
- Urine samples for visual examination, dipstick test (to measure specific gravity; pH; protein; glucose; ketones; hemoglobin; leukocyte esterase; nitrite; bilirubin; urobilinogen) and microscopic exam (to measure leukocytes, erythrocytes, epithelial cells, bacteria, casts and crystals)
- Check the 3-point SBMG
- Registration of hypoglycaemic events
- Evaluation of compliance to anti-diabetes treatment.

Primary End-Point: Difference in the percentage of circulating CD45+CD14+OCN+ (MCC) cells after one month initiating home treatment with GLP1-RA+metformin (Group A) vs metformin alone (Group B).

Secondary End-Points: I) Relationship between MCC levels and history of CVD, diabetic nephropathy, retinopathy and neuropathy. II) Relationship between MCC levels and body mass index, total cholesterol, triglycerides, HDL, LDL. III) Relationship between changes in body mass index, total cholesterol, triglycerides, HDL, LDL, and changes in MCC levels after 3 months treatment with the study drugs IV) HbA1c, hypoglycemic events, weight changes in Group A vs Group B.

Assessments: *clinical evaluation and blood/urine samples*. Subjects will be assessed at the recruitment site (Unit of Diabetology, Department of Medicine "La Sapienza" Rome University, Italy). Briefly, subjects will be asked about their medical history comprehensive of history of diabetes-related complications (history of CVD, diabetic retinopathy, nephropathy, and neuropathy). Vital signs, will be recorded at each visit. Vital signs will be assessed with the subject in a sitting position. Blood pressure and heart rate will be measured at each study while patient in a sitting position and after the patient has rest for 10

minutes. Subjects must be fasting for at least 9 hours before each study visit where fasting laboratory samples are obtained. At visit 0 and visit 2 blood samples will be drawn to isolate PBMCs and to measure HbA1c, creatinine, total cholesterol, HDL, triglycerides. The volume of blood required for these laboratory tests (with the exception of PBMCs isolation and HbA1c and hsCRP measurement) will be 10 ml subdivided into one 5 ml tube for serum (Red cap SST) and one 5ml Lithium-heparine tube (green cap SST). For the isolation of PBMCs 8 ml blood will be collected in one 8ml BD Vacutainer® CPT tubes will be drawn. For measurement of HbA1c 2 ml blood will be drawn in one EDTA tube (lavender cap SST). At visit 1 blood samples will be drawn to measure HbA1c and creatinine. The volume of blood required for these laboratory tests will be 2 ml for HbA1c and 2 ml for creatinine.

Self-reported hypoglycaemia. Subjects will be asked to report on a paper diary each hypoglycemic event defined as blood glucose levels < 70mg/dl. Subject will be also asked to report eventual symptoms accompanying the event. A new paper diary for the record of hypoglycemic events will be provided by the investigators at each scheduled visit. Subjects will be asked to return it to the study center at the following scheduled visit.

Laboratory analysis. All laboratory evaluations (with the exception of PBMCs isolation and measurement of MCC levels) will be performed at the laboratory of the "La Sapienza" Rome University, Italy. HbA1c will be measured by immunoturbidimetric method. Creatinine, total cholesterol, triglycerides, HDL by specthrophotometry (LDL will be calculated according to Friedewald formula).

Measurement of circulating myeloid calcifying cells. Identification and quantification of circulating myeloid calcifying cells will be performed using polychromatic flow cytometry. Blood samples will be collected and Peripheral Blood Monocluear Cells (PBMCs) will be isolated within two hours of collection using Ficoll density gradient. Freshly isolated PBMC will be washed three times in PBS+1%FBS and then will be incubated for 45 min at 4°C in

the dark with BrilliantViolet421-conjugated anti-human CD45 (BioLegend, San Diego, CA), PE/Dazzle594- conjugated anti-human CD14 (BioLegend, San Diego, CA) and AlexFluor488-conjugated anti human osteocalcin [R&D Systems, Minneapolis, MN], according to manufacturers' instructions. All antibodies will be titrated to achieve working concentrations. After incubation samples will be washed other three times in PBS+1%FBS and then assessed by flow cytometry (FACSCalibur, Becton Dickinson). Ten minutes before cell counts, cells will be stained for viability with 7- aminoactinomycin D (7AAD). Data will be analyzed with FlowJo software (Tree Star, Ashland, OR). Doublets will be excluded by a FSC-W vs FSC-A scatter. A side and forward scatter will be used for a first identification of lymphocytes and monocytes. After exclusion of nonviable cells by gating cells negative for 7AAD, we will gate CD45 bright cells and then will examine the expression of CD14 and osteocalcin. Fluorescence minus one (FMO) controls will be used to optimize the gating strategy. MCC will be quantified as cells positive for CD45, CD14 and osteocalcin Patient samples were performed in duplicate; the mean of two runs will be used as MCC levels.

2.3.1.2. Study B: in vitro study

Study B is an in vitro study. THP-1 cell will be treated with 40 μg/ml Ox-LDL (AlfaAesar, ThermoFisher Scientific, Waltham, MA, USA), to induce osteocalcin expression ± 10nM pioglitazone (Takeda Chemical, Osaka, Japan) or 1 mmol metformin for 48 hours. After treatments, cells will be assessed for surface expression of osteocalcin by flow cytometry.

Flow cytometry analysis: Flow cytometry will be used to assess osteocalcin expression in THP-1 cells. After the appropriate treatment, cells will be washed three times in PBS+1%FBS and then incubated for 45 minutes at 4°C in the dark with AF488-conjugated anti-human osteocalcin (R&D System, Minneapolis, MN, USA) according to

manufacturer's instructions. After incubation samples will be washed other three times in PBS+1%FBS and then assessed by flow cytometry. Ten minutes before cell counts, cells will be stained for viability with 7-aminoactinomycin D (7AAD). Osteocalcin positive cells will be gated on the morphologic mononuclear cell fraction according to FMO controls.

2.3.2. Study population

This proposal is to conduct an observational study [Study A] in subjects affected by T2D starting pioglitazone or any GLP1-RA on top of metformin vs metformin alone on the number of circulating MCCs. Moreover, an in vitro study [Study B] will be conducted to evaluate the effect of pioglitazone and GLP1-RA exendin-4 on the differentiation of cultured human monocytes towards an osteoblast-like phenotype. Subjects affected by T2D attending the outpatient clinic of the Unit of Diabetology, Department of Medicine "La Sapienza" Rome University, will be consecutively screened for this study. According to the sample size calculation, screening will terminate when n=41 subjects per group will be recruited in the study. The Investigators anticipate a screen failure rate of up to 20%. Thus, a total of 150 subjects will be screened. Subjects will be enrolled in the study according to the following inclusion/exclusion criteria.

Inclusion Criteria

1. Males and females aged >18 years and < 80 years affected by T2D
2. Stable therapy with metformin for at least 6 months before randomization
3. HbA1c <7.5%
4. Body mass index lower than 45 Kg/m2

Exclusion Criteria

1. Neoplastic disease in progression

2. History of hematologic disease or hematologic disease in progression

3. Estimated glomerular filtration rate < 30 mL/min/1.73m2

4. History of hypo- hyperparathyroidism, anorexia, alcoholism, hypogonadism

5. Inflammatory bowel disease

6. Diabetic gastroparesis

7. Previous acute pancreatitis

8. Trauma or chronic immobilization

9. Pregnancy

10. Lactation

2.3.3. Statistical analysis

Descriptive statistics will be presented for efficacy variables. For continuous variables, descriptive statistics will include the mean, median, standard deviation, minimum, maximum, number of available observations, and number of missing observations. For ordinal and nominal variables, descriptive statistics will include numbers and percentages for each score or category, number of available observations, and number of missing observations. Values that lie outside the interquartile range (IQR), using the IQR multiplier value k=1.5, were considered as outliers and excluded from the analysis. Discrete numeric variables (counts) will be described through frequency counts (grouping counts as needed for clarity), mean, median, variance, minimum, maximum, number of available observations, and number of missing observations. Descriptive statistics will be provided for each treatment group. Complete data listings will be provided. Graphical displays will be presented when they add to understanding of the data. Results of hypothesis tests will be considered statistically significant if p values are less than or equal to 0.025, and will be deemed not significant if p values are greater than 0.025.

Primary end-point (Study A). The primary efficacy end-point of the study A is the percentage of MCC, defined as CD45+CD14+OCN+ cells, on the total number of CD45+ cells. The primary efficacy analysis is to compare these outcomes between Group A, Group B and Group C. The primary test for efficacy will then be a Student's unpaired t-test. A multivariate analysis will be conducted by performing linear and logistic regression analyses to adjust results for confounding factors.

Secondary end points (Study A). The associations to be evaluated for the secondary end-points of the study A will be tested by general linear models. To evaluate differences HbA1c, hypoglycemic events, weight changes between Group A, Group B and Group C. Student's unpaired t-test or Kruskal Wallis tests will be used, depending on distribution. Linear models will be used for multivariable analyses to adjust for covariates, with $p < 0.1$ considered significant for testing in the final model with main effect and outcome.

Study B. All analyses will be conducted with Student's unpaired t test with a two-tailed distribution.

Sample size/ power: Based on published data, the sample size for the study A has been calculated for the primary end-point. A sample size of 41 patients per group will provide 80% power to identify a difference of 7% in the percentage of MCC in a 2- way, 2-tailed comparison at a Bonferroni adjusted alpha level of 0.025. To allow a 20% drop-out rate, we anticipate the enrolment of a total of 150 patients.

2.3.4. Ethics

The study was performed in accordance with the Declaration of Helsinki, and the study procedures were approved by the institutions' ethics committees. The local ethics committees approved this retrospective observational study as minimal-risk research using

data collected for routine clinical practice and waived the requirement for informed consent, ensuring that the new privacy policy was followed.

2.4. Results

2.4.1. Results *Study A*

Forty-eight patients with T2D have been enrolled in order to estimate the percentage of CD14+OCN+ circulating cells in the sera of study participants, which were stratified as follows: 10 subjects within the GLP1-RA Arm (Group A), 34 subjects within the MET arm (Group B), and 4 people within the PIO Arm (Group C). Of these, two individuals in the Group B dropped the study before the study visit V1 and were excluded from the follow-up analysis. We decided also to exclude the Group C from the analysis due to the small number of enrolled participants. The flow cytometry analyses have been completed for 42 participants (10 in Group A and 34 in Group B). The overall cohort had a median [25^{th}-75^{th} percentiles] age of 64 [55.8-71.3] years, the HbA1c was 6.4% [6-6.8], and BMI was 29.3 [26.4-34.9] kg/m^2.

At V0, patients in monotherapy had less than 30% CD14+OCN+ cells, with three outliers exceeding this threshold value (for information on the evaluation of outliers, see Statistical Analysis). The median [IQR] CD14+OCN+ cell percentages for the overall population at V0 was 7.0 [2.8-14.6] %. **Table 1** shows the clinical, anthropometric and biochemical feature of the study cohort stratified by anti-hyperglycaemic treatment. Participants in the GLP1-RA arm (Group A) showed higher HbA1c (6.2 [6.0-6.5] *vs* 7 [6.7-7.2]; $p<0.001$), and lower HDL cholesterol (39.0 [33.8-45.5] vs 50.0 [42-56]; $p=0.012$) compared to those on metformin alone. In addition, GLP1-RA group had a numerically higher systemic blood pressure ($p=0.099$) and triglycerides ($p=0.092$) than Group B. The study groups did not

	Metformin n = 34	**GLP1-RA** n = 10	**p-value**
Females, n (%)	10 (29.4)	3 (30)	0.654
Age, years	61 [55-72.5]	65 [59.5-70.5]	0.763
Diabetes duration, years	[-]	[-]	
BMI, kg/m^2	28.6 [26.3-32.9]	33.2 [26.9-38.0]	0.105
Waist circumference, cm	101.0 [93.5-109.0]	106 [100.8-116.5]	0.118
Diastolic blood pressure, mmHg	80.0 [70.0-84.5]	84.0 [71.5-94.0]	0.371
Systolic blood pressure, mmHg	130.0 [121.0-140.0]	142.5 [120.0-168.5]	0.099
Heart rate, bpm	74.5 [70.3-80.8]	79.0 [66.8-87.8]	0.590
HbA_{1c}, %	6.2 [6.0-6.5]	7 [6.7-7.2]	**<0.001**
Total Cholesterol, mg/dL	165.5 [137-191.8]	157.0 [121.3-176]	0.314
LDL-cholesterol, mg/dL	86.0 [71.8-101.8]	77.0 [54.5-100.5]	0.396
HDL-cholesterol, mg/dL	50.0 [42-56]	39.0 [33.8-45.5]	**0.012**
Triglycerides, mg/dL	92.0 [75.0-136.0]	140.0 [63.5-101.5]	0.092
eGFR, $ml/min/1.73m^2$	88.0 [71.8-97.6]	81.5 [63.5-101.5]	0.457
History of CVD, n (%)	7 (20.6)	1 (10)	0.387
History of hypertension, n (%)	19 (55.9)	7 (70)	0.288
History of dyslipidemia, n (%)	23 (67.6)	8 (80)	0.330
History of nephropathy, n (%)	0 (0)	0 (0)	1
History of retinopathy, n (%)	0 (0)	1 (10)	0.130
History of neuropathy, n (%)	0 (0)	1 (10)	0.130

Table 1. Population features by anti-hyperglycemic treatment. Values are median [25-75th percentile] for continuous variables and number (percentages) for categorical variables.

differ in terms of age, sex, BMI and prevalence of comorbidities and cardiovascular complications (p>0.05 for all). In addition, the levels of CD14+OCN+ circulating cells were similar between groups (p=0.33).

The overall median [IQR] CD14+OCN+ cell percentages at study V1 (one-week follow-up) were 5.1 [1.9-9.0]. At V1, CD14+OCN+ cell percentages were generally reduced or otherwise retained at same levels when compared to those at V0, independently from the ongoing anti-hyperglycaemic treatment, with only a few detected cases of increase (p-value for the difference V1-V0 in terms of CD14+OCN+ cells between groups=0.037).

At one-week follow-up visit (V1), a reduction in CD14+OCN+ cell percentages was registered in both study groups (Group A: 6.4% [1.9; 11.3] at baseline vs 1.8% [1.5; 3.5] at one-week follow-up, p=0.028; Group B: 8.3% [4.5; 19.22] at baseline vs 8.2% [4.1; 10.9] at one-week follow-up, p=0.013), with a greater effect in those patients with a higher percentage of CD14+OCN+ cells at baseline.

The difference between the reduction shown in the GLP1-RA + metformin (Group A) arm and in the metformin monotherapy arm was also evaluated. The reduction in the levels of CD14+OCN+ MCC was significantly higher in the group treated with GLP1-RA compared to metformin alone (p= 0.032, **Figure 1**).

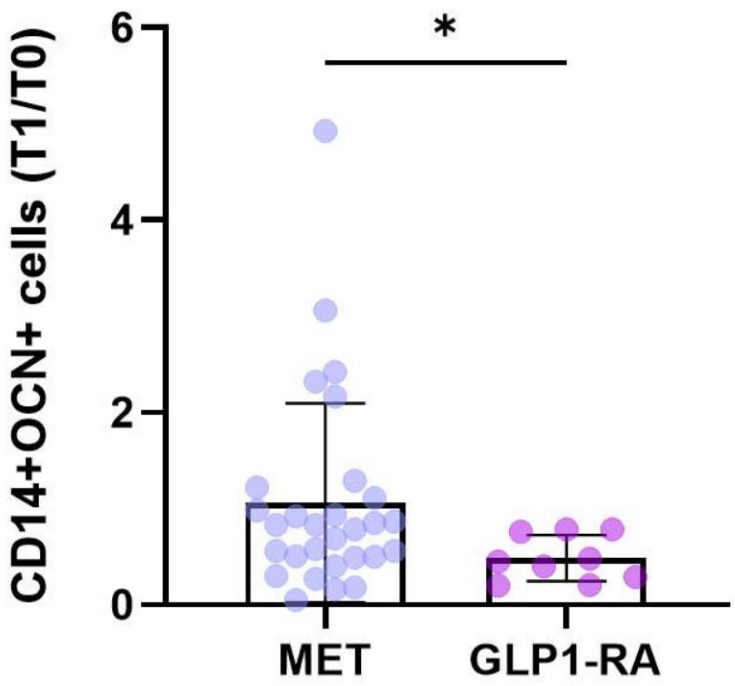

Figure 1. Difference in ONC+ cells reduction in GLP1-RA+metformin vs metformin monotherapy in one week (T1). *p-value (<0.05).

At three-months follow-up, only subjects for whom we had data for all time points were included in the analyses: 6 participants within Group A and 25 participants within Group B completed the follow-up. The levels of CD14+OCN+ cells tended to be unchanged following metformin treatment during the three-month observation period (p=0.27, **Figure 2**), whereas they dropped significantly in the GLP1-RA add-on metformin treatment arm (p=0.012, **Figure 3**).

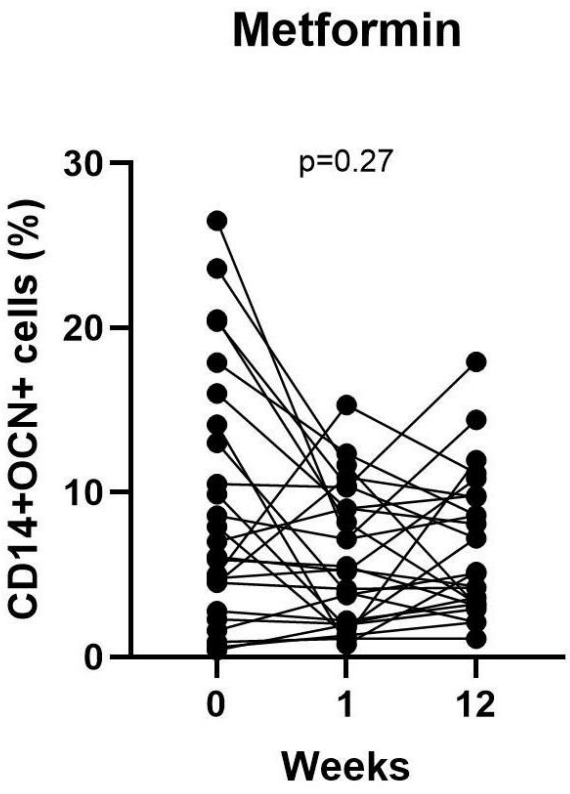

Figure 2. Levels of CD14+OCN+ cells in metformin group in three months.

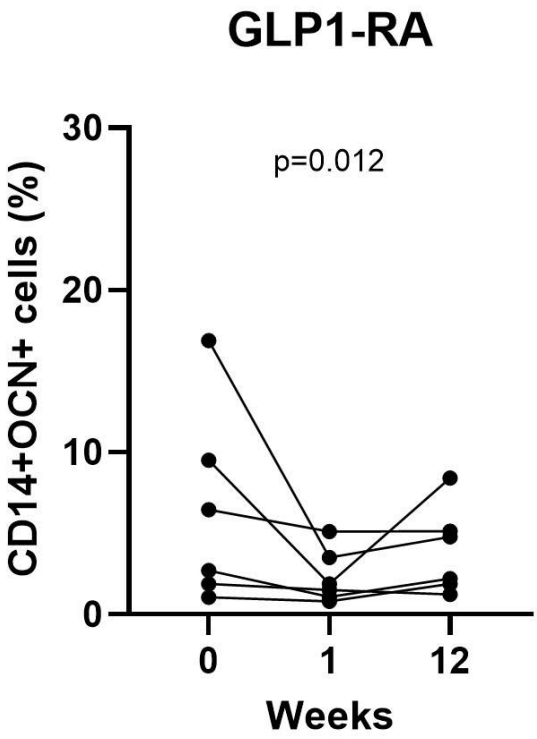

Figure 3. Levels of CD14+OCN+ cells in GLP1-RA group in three months.

2.4.2. Results Study B

Preliminary data demonstrated that treatment with oxLDL increased OCN expression by 3- to 10-fold ($p<0.05$). Following treatment with oxLDL 40µg/ml, there was a significant increase in THP-1 OCN+ cells. Preliminary results showed a power of Metformin in reducing the number of OCN+ monocytes, both under basal conditions and following stimulation by oxLDL administration, although there is still no statistical significance **(Figure 4)**.

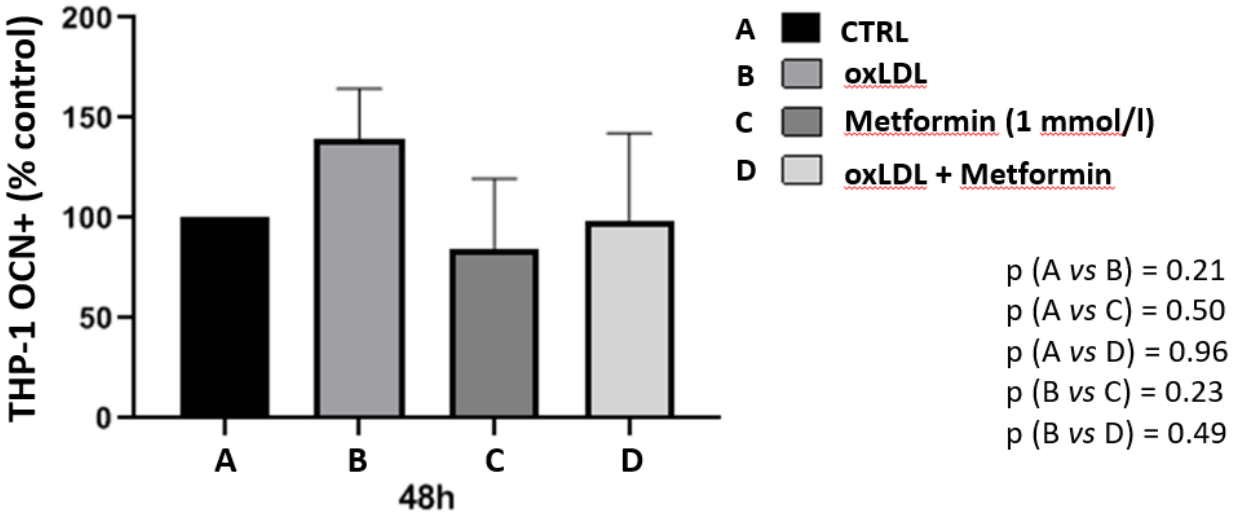

Figure 4. The amount of THP-1 OCN + cells was estimated by flow cytometry following treatment with metformin (1 mmol / l) ± oxLDL (40 µg / ml). Flow cytometric analysis was performed 48 hours after treatment.

Treatment of THP-1 cells with pioglitazone, on the other hand, showed a statistically significant reduction in OCN+ cells under basal conditions ($p=0.03$), with even greater statistical strength when combined with oxLDL treatment ($p<0.001$) **(Figure 5)**. This

finding corroborates the previously cited claims in the literature demonstrating the ability of metformin and pioglitazone to act on atherosclerotic disease.

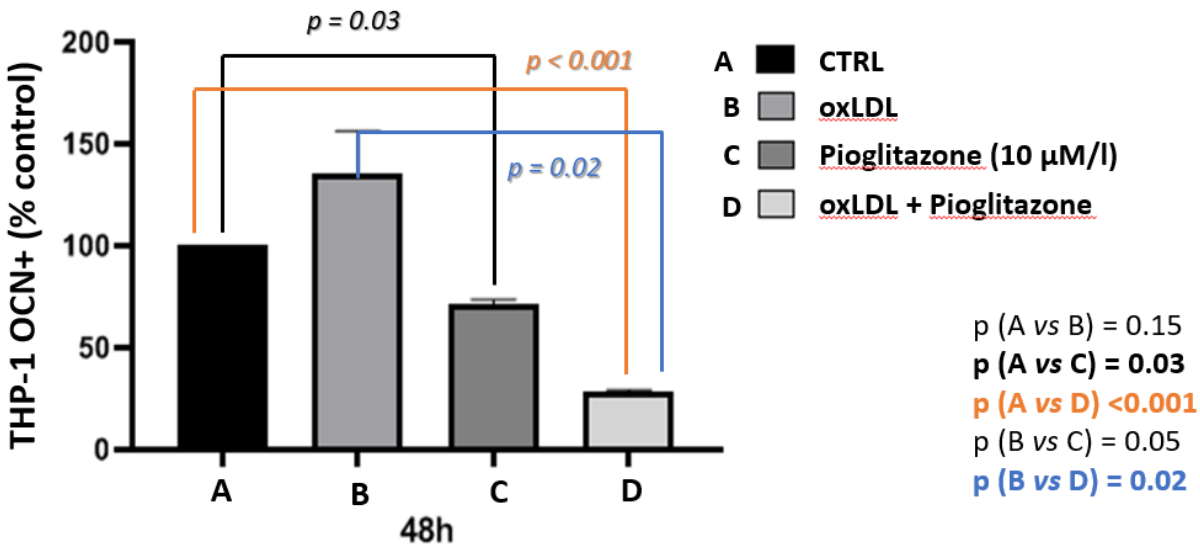

Figure 5. **The amount of THP-1 OCN + cells was estimated by flow cytometry following treatment with pioglitazone (10 µM/l) ± oxLDL (40 µg / ml).** Flow cytometric analysis was performed 48 hours after treatment.

The population enrolled to evaluate the effects of metformin and pioglitazone on osteogenic cells isolated from PMBCs had a mean age of 30.6 years, and the mean BMI fell within the Caucasian definition of normal weight (between 20 and 25 kg/m2). In both groups, in vitro treatment of PMBCs taken from peripheral blood with metformin and pioglitazone led to a significant reduction (in both cases, $p<0.001$) in the percentage of CD14+/OCN+ osteogenic cells compared to the control group. Metformin ± oxLDL led to a non-statistically significant OCN expression reduction, whereas *in vitro* treatment with pioglitazone led to a significant reduction in OCN expression on THP-1 cells both alone ($p=0.03$) and after

stimulation with oxLDL ($p<0.001$). Three-month results and further in vitro data are in development.

2.5. Discussion

There are numerous factors that can direct the differentiation of the monocyte line. The study identifies, in the treatment with metformin, pioglitazone or GLP-1 RA, possible factors capable of preventing differentiation towards pro-calcifying phenotypes. The results showed both in the treatment group with GLP-1 RA + metformin and in the treatment group with metformin alone, global reductions in the percentage of OCN+ cells at V1 compared to V0, therefore already in the acute phase, that is, after only one week of treatment: the results obtained by measuring the OCN+ cells in the various treatment groups (metformin ± GLP-1 RA) are indicative of a superiority of GLP-1 RA compared to metformin in the reduction of OCN+ cells. After one week of treatment, reductions were recorded in the group on metformin monotherapy compared to the first week, although not significant. This data was partially confirmed in the 3-month observation (V3), therefore showing a reduction or in any case a maintenance of the levels of OCN+ cells in the group in add on to GLP1RA and a greater dispersion of the data therefore an inconsistent increase in the levels of OCN+ cells in the group in monotherapy with metformin. The reinforcement of the lifestyle changes adopted early on in joining the clinical trial, therefore better adherence to diet and lifestyle, could justify the initial improvement in the group in treatment with metformin in monotherapy, despite the group not seeing changes in the therapeutic indications with the study. The group treated with GLP-1 RA could also have received the same beneficial effect given by enrollment, but in fact, to confirm the presence of an uncommon factor between the two groups, therefore associated with the different therapy between the two groups, there is the statistical significance in the difference between the V1/V0 ratios of the OCN+ cells of the group in add-on therapy with GLP-1 RA and the group in monotherapy with metformin, indicating the presence of a discriminating factor present in the treatment group with GLP-1 RA + metformin compared to simple metformin. Certainly, a greater number of

measurements could further improve the statistical robustness of the study. Numerous evidences in the literature have highlighted various pleiotropic effects of GLP-1 RA on various cellular elements. These studies allow us to hypothesize numerous other routes of action of GLP-1 RA that can integrate their effects on the vascular wall. One study highlighted a possible ability of GLP-1 RAs to direct macrophage polarization towards the M2 phenotype, typical of alternative activation, and therefore with anti-inflammatory and restorative action[159]. This phenotypic change could contribute to the protective effects of GLP-1 RAs in the context of cardiovascular disease, and provide explanations for its anti-atherogenic capacity. Furthermore, a further study has demonstrated the expression of GLP-1 receptors at the level of human monocytes, which is reduced when monocytes differentiate into macrophages or foam cells[160], the latter being characteristic cells of the atherosclerotic lesion. Recent in vitro and in vivo evidence has also shown an improvement in bone metabolism following administration of GLP-1 RAs, but if there are studies that suggest an association between the use of GLP-1 RAs and the risk of fracture[161], others refute this thesis[162]. GLP-1 may also affect the fat-bone axis, promoting osteogenic differentiation and inhibiting adipogenic differentiation from bone mesenchymal precursor cells (BMSCs, Bone Marrow-Derived Stem Cells), characterized by the expression of the GLP-1 receptor[163]. This double differentiation possibility for BMSCs allows them to balance the presence of the two cell populations, constituting a key node in bone homeostasis[164]. Osteocalcin is the most expressed protein in the bone matrix with the exception of proteins from the collagen family, and is synthesized by osteoblasts, the same cells involved in atherogenic mechanisms and calcium deposition at the vascular level[92] . Consequently, the reduction in osteocalcin detected by the study also suggests a reduction in the calcium deposition phenomenon. Consequently, GLP-1 RAs could act through different biochemical pathways and by acting on numerous cells to lead to the beneficial effects described. Study B, on the

other hand, demonstrated that both metformin and pioglitazone have the in vitro ability to reduce OCN expression, with a greater reduction given by metformin compared to pioglitazone. Several lines of evidence have indicated a role for PPARγ as a key regulator in bone cell differentiation[145]. Specifically, pioglitazone may act by blocking the activity and reducing the blood concentration of β-catenin, which is necessary for stem cells to differentiate towards the osteoblastic lineage, and consequently blocking osteoblastogenesis. This behavior is supported by evidence from studies on PPARγ-negative embryonic stem cells, which passively acquire an osteoblastic phenotype, which is instead avoided in the presence of PPARγ agonists[146,147]. Furthermore, a role for PPARγ in immune regulation has been detected, in particular monocyte-macrophage activity[148]. PPARγ transduces a signal on the monocyte capable of inhibiting the production of inflammatory cytokines, but it has also been shown to be capable of opposing vascular and valvular calcification and the complication of plaques, although the molecular mechanism remains unclear[149–151]. Furthermore, an induction of OCN expression in human monocyte cell cultures mediated by the addition of oxidized low-density lipoprotein (OxLDL), a known marker of monocyte atherogenic activation, at a concentration of 40 µg/ml at 12, 24, 48 and 72 hours has been demonstrated. The percentage of OCN+ monocytes increased, with temporal correlation, up to 3-10 times. Furthermore, the OCN content of the lysed cells was significantly increased at 48 hours of treatment with OxLDL, also indicating a possibility of autologous production of OCN by the stimulated monocytes[80].

The results of study B represent both a new starting point for study A and for further work. The in vitro data for metformin indicate a positive effect of the drug on the percentage of OCN+ cells. Study A uses metformin as a control group compared to GLP-1 RA. A confirmation also in vivo through specific studies on the effects of metformin on vascular calcification in the long term, also including patients without a diagnosis of T2D, would

allow to provide new therapeutic indications, aimed at reducing a cardiovascular risk factor currently without treatment such as arterial calcification, to a drug that is inexpensive and generally well tolerated in patients. Indeed, preventing vascular calcification by extensive use of GLP-1 RA would be a strategy that is not very applicable, due to the high costs for the health service and the difficulties in the drug production chain. Metformin would overcome this limit. A further prospect for the future is the possibility of expanding study A by implementing an in vivo comparison with a group of patients undergoing add-on therapy with pioglitazone, to study its efficacy by virtue of the data highlighted with study B. The data from study B relating to Pioglitazone, in fact, also indicate for this drug a power of reduction of OCN+ cells, with an important statistical significance. Pioglitazone is a drug of ever decreasing use in clinical practice, but if its ability to prevent vascular calcification equal to or superior to metformin were to be demonstrated in vivo, it would be possible to bring back into vogue another drug reasonably prescribable on a large scale. At present, the study has some limitations, such as the small number of patients undergoing GLP1-RA therapy, currently penalized by the inconsistent availability of the drug class, the observational nature of the study, which did not allow either randomization of patients or the use of placebo, the short follow-up which did not allow for correction of the results obtained for possible confounding factors such as improvement in glycemic control. The study also has some important strengths. Metformin continues to be the cornerstone of pharmacological therapy for T2D, has a well-defined safety profile, is generally well tolerated, and is a drug well known to both patients and non-specialist clinicians. It is easy to prescribe and has a very limited number of contraindications. GLP-1 RAs have also gained great importance in clinical practice, and through new strategies aimed at facilitating their prescription, and an ever-increasing interest also from general practitioners and specialists in other disciplines, they are becoming a new cornerstone in the internal medicine management

of diabetic patients. As a perspective for the future, the study may expand a line of research on an important cardiovascular risk factor using drugs that are fundamental in T2D.

3. Conclusions

The results of this study, to be confirmed in a larger sample, show that both metformin and GLP-1 RAs can impact the circulating levels of CD14+/OCN+ MCC, with a greater efficacy and significance of GLP1-RAs, in agreement with the greater cardiovascular benefits of this class of drugs. The in vitro data, in addition to confirming in the plate the activity of metformin in preventing the trans-differentiation of monocytes towards a calcifying phenotype, suggest possible benefits on this pathway also of pioglitazone, to be confirmed in a clinical study already started. Overall, the results of this experimental thesis work suggest a new mechanism of cardiovascular action of these classes of drugs, now widely used in people with T2D.

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