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PH.D. THESIS

UNRAVELING THE ROLE OF METHYLGLYOXAL ON GLUCOSE HOMEOSTASIS AND THE PRO-ANGIOGENIC POTENTIAL OF ADSCs

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ABSTRACT

Diabetes Mellitus (DM) is a metabolic disease and represents an important risk factor for vascular disease. Tight control of blood glucose levels is a major goal for the treatment of Type 2 DM (T2DM). However, metabolic defects start up before the pathology can be diagnosed. Indeed, lowering blood glucose, only partially reduces the risk of vascular complications. Methylglyoxal (MGO), a highly reactive dycarbonyl, is a major precursor of AGEs. In pathological conditions, as T2DM, hyperglycemia contributes to MGO accumulation, which induces damaging effect on vascular function, insulin sensitivity and β-cell function. Although MGO-adducts have been already observed in the pre-diabetic state of spontaneously diabetic rodents, the role of MGO in the early steps of T2DM pathogenesis has not been defined. Under physiological condition, MGO is detoxified by the glyoxalase system, of which Glyoxalase 1 (Glo1) is the rate limiting enzyme. However, in pathological conditions, such as oxidative stress, inflammation, and senescence Glo1 levels are reduced.

Adipose tissue-derived stem cells (ADSCs) have gained big interest in therapeutic applications in regenerative medicine, thanks to their low immunogenicity, self-renewal ability and differentiation into various tissue-specific cells. ADSCs have the potential to secrete paracrine factors that promote angiogenesis; however, in DM this ability is compromised.

The aim of this thesis includes the investigation of two aspects: 1. The evaluation of MGO accumulation on glucose homeostasis in mice knock-down for Glyoxalase 1 (Glo1KD) and 2. The evaluation of MGO effect on the ADSCs pro-angiogenic function and the molecular mediators involved.

The results obtained *in vivo* in the first part of the study show that the endogenous accumulation of MGO in Glo1KD mice leads to an age-dependent development of glucose intolerance. This metabolic alteration associates with the presence of insular inflammatory infiltration, the islet expression of markers of senescence, and a senescence-activated secretory profile, in the pancreas from 10-month-old Glo1KD mice, compared with their wild type (WT) littermates. *In vitro,* the exposure of INS832/13 β-cells to MGO confirms its casual role on β-cells dysfunction, which can be reverted by senolytic treatment.

In the second part of the study, we show that MGO treatment impairs the pro-angiogenic function of mADSCs and induces their senescence. The impaired mADSCs pro-angiogenic

function is mediated, at least in part, by the release of soluble factors, which associates with a reduction of p38 MAPK activation in hRECs.

These data indicate that MGO is able to induce early phenotypes typical of the progression to T2DM and to reduce the pro-angiogenic ability of mADSCs, paving the way for novel prevention approaches to T2DM, and treatment strategies for the related complications.

1. BACKGROUND

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is one of the highest metabolic diseases in the world (Chawla et al., 2016; Palazzuoli et al., 2022). According to the International Diabetes Federation (IDF), more than 537 million people worldwide currently have diabetes, with an increase of 16% since the last IDF projections in 2019. The World Health Organization has estimated that DM is the ninth leading cause of death and has predicted that diabetes-related deaths will double by 2030 (Fared et al., 2022) Conventionally, two main types of DM are distinguished: type 1 DM (T1DM), known as insulin-dependent diabetes, and type 2 DM (T2DM), known as non insulin-dependent, associated with insulin resistance and decline in insulin secretory function from β-cell (Klein et al., 2021).

1.1.1 Pathogenesis of DM

A different pathogenesis underlies the two main forms of DM Wuh et al., 2016). T1DM is mainly caused by an autoimmune reaction, which causes the destruction of pancreatic β-cells, responsible for producing insulin (Gale al., 2001). T1DM patients require daily insulin administration in order to normalize blood glucose levels. Frequent urination and thirst, weight loss, blurry vision and fatigue are the main symptoms of this type of DM. T2DM is the most frequent and common form of diabetes that occurs in adulthood. It is characterized by chronic hyperglycemia and resistance to insulin of peripheral tissues. In an early phase of T2DM, the β-cell is able to produce insulin, however the target tissues are not responsive to the action of the hormone with a consequent increase in blood sugar levels. As long as the pancreas is able to compensate for insulin resistance with an increase in insulin production, there is a condition of hyperinsulinemia. If this condition persists, there will be an alteration of glucose homeostasis and a consequent functional depletion of the β-cells, with the onset of overt diabetes and the need for exogenous insulin administration. The most significant risk factors for this condition include obesity, physical inactivity, poor diet, ethnicity, family history of DM, stressful conditions, and aging (IDF, 2019; Olokoba et al., 2012; American Diabetes Association, 2015). Another important aspect to pay attention to is the pre-diabetic condition. Pre-diabetes is a term used to describe a period before the onset of T2DM, in which blood glucose levels are higher than normal but lower than the diagnostic criteria of T2DM. The number of pre-diabetic patients

is estimated to increase to more than 470 million people worldwide (Gossain et al., 2010). Pre-diabetes occurs when the patient is diagnosed with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG). The major attribute of IGT and IFG is a decreased glucose-stimulated insulin secretion of the pancreatic β-cells (Cerasi et al., 1972). The dose–response curve for glucose-induced insulin release shifted to the right and further right for pre-diabetic and diabetic patients, respectively, as compared to normal individuals [Cerasi]. Pre-diabetes is considered as a critical phase because this condition is reversible and long-term studies have shown that if lifestyle changes were adopted, the risk of pre-diabetes to diabetes progression, can be lowered until 10 years (Tuso et al., 2014; Papaetis et al., 2014; Khan et al 2019). Therefore, to be successful in the treatment of pre-diabetes, it is necessary to intervene promptly before the condition becomes irreversible and diabetes with its complications manifests itself.

1.1.2 Vascular complications associated to diabetes

Chronic hyperglycemia, the main characteristic of DM, is considered the major cause of the alterations of the endothelial barrier in diabetes, capable of modifying its function and structure (Brownlee et al., 2001). Patients with DM are at a higher risk of early atherosclerosis (Kalofoutis et al., 2007), and treatment of hyperglycemia has been shown to reduce the progression of microvascular disease, but not macrovascular disease (Ahmad et al., 2005). This derives from the different endothelial response of large and small vessels to the action of insulin and glucose (Wang et al., 2009). In fact, an increase in glycosylated hemoglobin (HbA1c) from 5.5% to 9.5% implies a two-fold increase in the incidence of macrovascular disease, but the same increase in HbA1c implies an approximately 10-fold increase in the microvascular disease (Wang et al., 2009; Costa et al., 2013).
Chronic hyperglycemia leads to both micro- and macrovascular complications that are the principal causes of mortality and morbidity associated to diabetes.

• *Macrovascular complications*

The development of atherosclerotic plaque induces endothelial damage, followed by the formation of a lipid plaque, which subsequently develops well-defined edges and evolves into fibrous plaque (Rosenfeld et al., 2000). Most of the vessels within the plaques have an endothelium, but only a few of them have mural cells such as pericytes. This feature makes them unstable, more fragile and easily damaged, causing the development of immature vessels within the lesion, which is associated with aberrant angiogenesis (Costa et al.,

2013). The onset of haemorrhagic events within the plaque lead to its destabilization and rupture (Kolodgie et al., 2003). Subsequently, plaque rupture causes blood clots, which can lead to thrombosis, embolism, hemorrhaging, and atherosclerosis. As the condition worsens, the blood vessels become completely obstructed. These obstructions increase the risk of heart attacks, strokes, cramping of the limbs, and gangrene and, ultimately, death (Huang et al., 2017).

• *Microvascular complications*

Diabetic patients often exhibit poor wound healing capacity and impaired revascularization capacity following vascular occlusion (Martin et al., 2003). Microvascular complications include:

- *Diabetic retinopathy*

Diabetic retinopathy (DR) affects about one third of diabetic patients. It develops in two consecutive stages: non-proliferative DR (NPDR) and proliferative DR (PDR). NDPR is characterized by a loss of both pericytes and ECs, with a reduction in blood flow in the retina and the onset of ischemic phenomena. In PDR, new blood vessels are formed, with a very fragile wall because it does not have pericytes. This causes vitreous hemorrhages and the formation of scar tissue which can cause the retina detachment. Symptoms of DR include the formation of neo-vessels, bleeding and/or neo-vascular glaucoma (Costa et al., 2013), blurred vision, appearance of dark spots, perception of "floating" in the visual field, eye pain, double vision, decreased perception of low light, sudden loss of vision and complete blindness. Risk factors for DR include high blood pressure, chronic hyperglycemia, hyper- or dyslipidemia and ethnicity (Fong et al., 2004; Montano et al., 2019).

- *Diabetic nephropathy*

Diabetic nephropathy (DN) is a leading cause of kidney damage worldwide. It is characterized by abnormal angiogenesis due to the overexpression of VEGF (Xu et al., 2012). The pathogenetic mechanisms underlying this pathology involve: the generation of ROS, the accumulation of Advanced glycation end products (AGEs), the activation of intracellular signaling molecules, such as protein kinase C (PKC) (Chawla et al., 2016), and the production of various pro- inflammatory factors, such as IL-1, IL-6, IL-18, TNF-α and TGF-β1. DN is characterized by: increased vascular permeability, thickening of

glomerular BM, apoptosis of ECs, chronic inflammation (Costa et al., 2013), persistent high levels of protein in the urine (proteinuria: >300 mg/24h), progressive decline in glomerular filtration rate (GFR) and increased arterial blood pressure (Khan et al., 2015).

- *Diabetic neuropathy*

Diabetic neuropathy (DN) is a heterogeneous group of disorders including diabetic peripheral neuropathy (DPN) and diabetic autonomic neuropathy (DAN), which cause damage to peripheral and autonomic nerves, respectively, and are the result of metabolic damage (Vinik et al., 2013). DPN is associated with lower extremity pain, neural desensitization, leg and foot abscesses, resulting in potential lower extremity amputation. DAN has been shown to induce symptoms in the sympathetic and parasympathetic systems, which can result in cardiac abnormalities such as myocardial ischaemia and sudden death (Pop-Busui et al. 2006). Furthermore, both patients with DAN and patients with DPN show functional and structural damage to the peripheral nerves, caused by oxidative stress, AGE accumulation, glucose toxicity and hypoxia, which ultimately lead to neuronal apoptosis.

1.2 Endothelium and its function

Endothelium is a monolayer of metabolically active endothelial cells (ECs). Initially, it was thought to be a simple barrier that lays on the surface of blood vessel. Then, the endothelium has been defined as a dynamic organ which lines the entire vascular system (Galley et al., 2004). ECs reside in the intima of blood vessels, which is the inner lining of the vasculature (Figure 1), and they are frequently silent throughout adolescence; however, they can initiate new vessel formation in response to injury or pathological conditions (Kozanoglu et al., 2022).

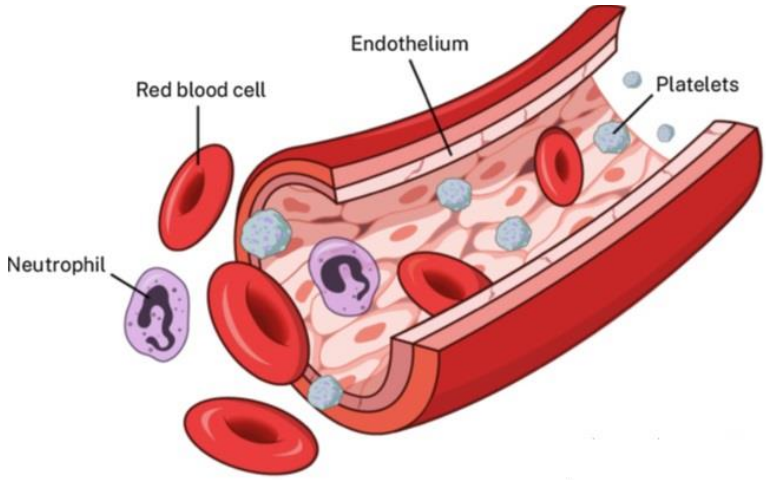


Figure 1. Schematic representation of endothelium (Kozanoglu et al., 2022).

The endothelium has important autocrine, paracrine and endocrine functions necessary to maintain vascular homeostasis under physiological conditions (Vallance et al., 2001; Bonetti et al., 2003). In detail, the endothelium is involved in: the regulation of vascular tone, injury repair, barrier integrity, metabolism, hemostasis, immune response, inflammatory response, cell adhesion, and angiogenesis (Feletou et al., 2006; Feletou et al., 2011) (Figure 2).

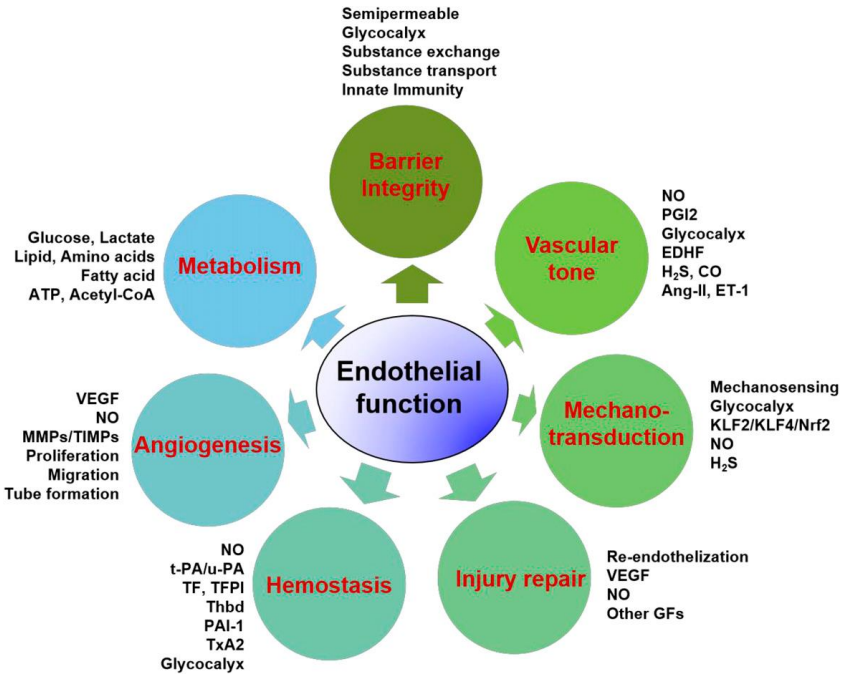


Figure 2. Endothelial cells functions. In physiological conditions, endothelium regulates vascular homeostasis through biological processes summarized in figure (Xu et al., 2021).

In order to regulate the vascular tone, the endothelium releases a multitude of factors. Among these, endothelium maintains the balance between vasodilation and vasoconstriction through the production of vasodilators such us nitric oxide (NO), prostacyclin (PGI2) and endothelium derived hyperpolarizing factor (EDHF), and vasoconstrictors such as thromboxane (TXA2) and endothelin-1 (ET-1) (Sandoo et al., 2010). ECs also produce anti-thrombotic (NO and PGI2) and pro-thrombotic molecules, such as vonWillebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1), which promote platelet aggregation and inhibit fibrinolysis, respectively. The endothelium also maintains a balance between the inhibition and promotion of migration and proliferation of VSMCs (NO, angiotensin II, ROSs), besides fibrinolysis and thrombogenesis as well as prevention and stimulation of platelets adhesion and aggregation (Moncada et al., 2006). An imbalance of these physiological events leads to endothelial dysfunction (Sena et al., 2013; Kozanoglu et al., 2022; Wang et al., 2022).

1.2.1 Angiogenesis

One of the major functions exerted by the endothelium is the angiogenesis, defined as the formation of new blood vessels out of preexisting ones (Gerritsen et al., 2008). Angiogenesis is a vital process that occurs throughout life. Indeed, during the embryonic development, it is required for the formation of a living vertebrate organism and, in the neonate, contributes to the development of certain organs, such as the retina. In adults, angiogenesis is an essential process involved in wound healing and reproduction (Gerritsen et al., 2008). Nevertheless, angiogenesis can also play a detrimental role in pathological conditions, acting as one of the major contributors of processes such us cancer, age-related macular degeneration, endometrioses, psoriasis and diabetic complications (Gerritsen et al., 2008). Angiogenesis can be described as a multistep process in which ECs move from a quiescent state to an angiogenic phenotype, following proangiogenic stimulation. In detail, ECs produce proteolytic enzymes, which degrade the basement membrane (BM). Then, ECs proliferate and migrate into the perivascular area, forming the “primary sprouts”. Subsequent lumenation of these primary sprouts leads to the formation of capillary loops, followed by the synthesis of a new temporary extracellular matrix (ECM). Then, ECs proliferate and invade the ECM and participate in the formation of immature capillary structures and the synthesis of new BM. Finally, pericytes (PCs) are recruited, thereby stabilizing the new vessels (Montemagno et al., 2020) (Figure 3).

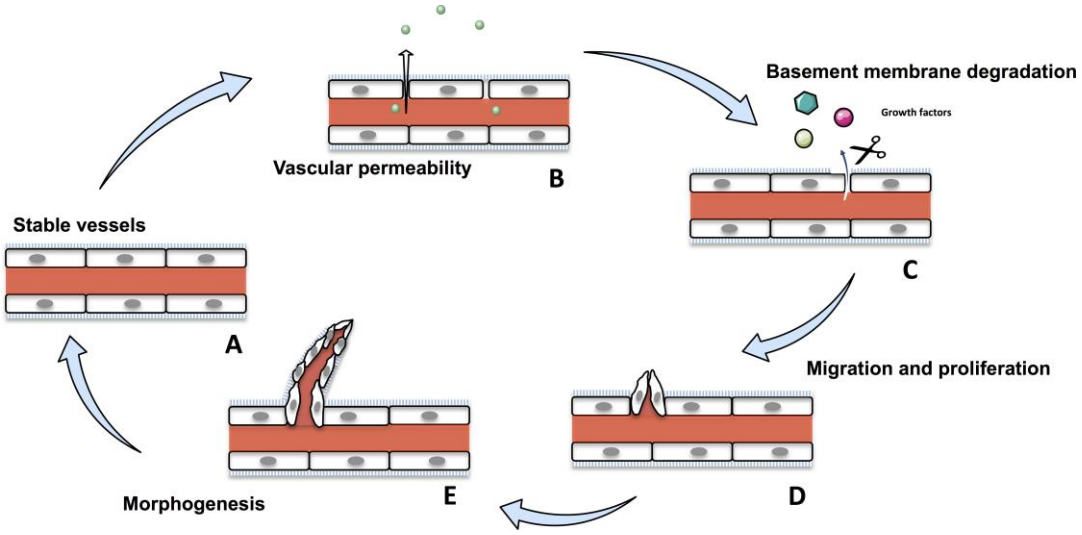


Figure 3. The angiogenic process. (A) Stable vessels undergo a vascular permeability allowing extravasation of plasma proteins (B). Degradation of the extracellular matrix releasing growth factors (C). ECs proliferate and migrate, (D) undergo morphogenesis and form new tubes followed by new stable vessels (Montemagno et al., 2020).

During this process soluble growth factors, membrane-bound proteins, cytokines, cell-matrix and cell-cell interactions act to regulate and influence angiogenesis (Rajabi et al., 2017; Koch et al., 2007). Among these, some of them have pro-angiogenic activity and others have anti-angiogenic activity, and their balance is important to ensure vascular homeostasis (Rajabi et al., 2017; Schönborn et al., 2022). Inhibitors of this process include: pigment epithelium-derived factor (PEDF), vascular endothelial cell growth inhibitor (VEGI), angiostatin, endothelin-1 (ET-1) and endostatin. Important pro-angiogenic factors are: vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), angiopoietins, platelet-derived growth factors (PDGFs), transforming growth factors (TGFs), various integrins, VE-cadherins, ephrins, NO and interleukin-8 (IL-8) (Musa et al., 2016).

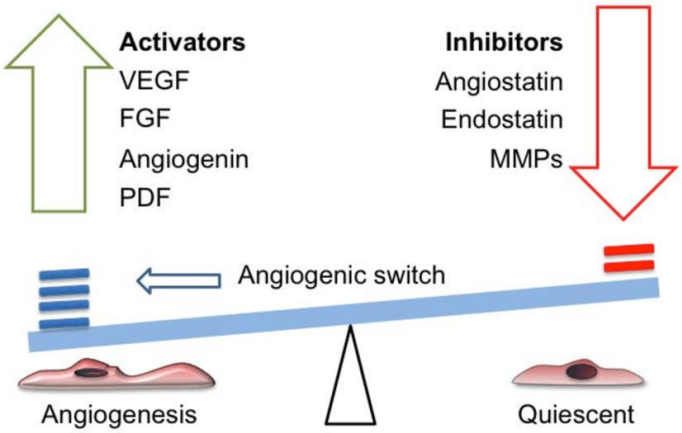


Figure 4. The angiogenic balance between the angiogenic activators and the angiogenic inhibitors regulates vascular homeostasis. Under physiological and pathological conditions, angiogenesis is associated with the up-regulation of angiogenic factors and/or the down-regulation of angiogenic inhibitors. An alteration of this balance may be associated with impaired neovascularization capacity. VEGF, vascular endothelial growth factor; FGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; MMPS, Matrix metalloproteinases (Ramcharan, 2016).

The most potent pro-angiogenic protein reported is VEGF- factor A (VEGF-A), also known as VPF, which belongs to a large family of potent angiogenic regulators that includes placental growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D (also known as FIGF) (Gunda et al., 2012; Shibuya et al., 2006; Ferrara et al., 2003). VEGF-A binds to VEGF receptor-1 (Flt1), VEGFR2 (also known as KDR or FLK1), a tyrosine kinase receptor selectively expressed on vascular ECs, and VEGF receptor-3 (FLT4). It is involved in the regulation of all stages of the angiogenic process (Ferrara et al., 2003; Dvorak et al., 1996). When VEGF-A binds to the extracellular domain of the KDR receptor, dimerization and autophosphorylation of the intracellular tyrosine kinase domain occur, activating a cascade of downstream proteins (Otrock et al., 2007). Thus, VEGF-A is responsible for the proliferation, sprouting and tube formation of ECs. VEGF-A induces endothelial expression of NO synthase, resulting in an increased production of NO, which contributes to the initial vasodilation and the increases the permeability of vascular endothelium (Dvorak et al., 1996; Dvorak et al. 2001). In addition, VEGF-A contributes to BM degradation by inducing the expression of plasminogen activators and matrix metalloproteinases (MMPs) (Gerritsen et al., 2008). Transcription of VEGF-A is regulated by hypoxia-inducible factor (HIF), specifically HIF-1α. Under hypoxic conditions, HIF-1α stabilizes and dimerizes with HIF-1β. This complex binds to and activates hypoxia responsible element of the VEGF-A promoter resulting in VEGF-A overexpression (Doxey

et al. 1995). HIF-1α is also a direct regulator of stromal-cell-derived factor-1α (SDF-1α), a chemokine involved in angiogenesis and endothelial progenitor cells (EPC) recruitment (Schönborn et al., 2022).

Angiopoietins are a family of proteins that bind Tie1 and Tie2 receptors and play an important role in vessel maturation, growth and stabilization. In angiogenesis, Tie1 receptors are needed for the integrity and survival of ECs (Otrock et al., 2007), whereas Tie2 receptors play a key role in sprouting and branching vessel (Schönborn et al., 2022).

FGF family consists of several members, including FGF-1, FGF-2, FGF-7, FGF-10, and FGF-22. FGF exerts its angiogenic effects through a complex process which involves cellular proliferation and vascular basement membranes degradation (Schönborn et al., 2022). FGFs binds FGF tyrosine kinase receptors (FGFR) present on the surface of the ECs and induce the proliferation, migration of ECs and the production of collagenase and plasminogen. Indeed, FGF increases the production of urokinase-type plasminogen activator (uPA) and MMPs, promoting ECM degradation, a fundamental step in angiogenesis (Otrock et al., 2007).

PDGFs are a family of proteins recognized as some of the forerunning factors of mitosis in mesenchymal cells such as smooth muscle, glial cells, and fibroblasts. They also exhibit chemotactic properties and are expressed by various cell types, including vascular smooth muscle cells and endothelium (Schönborn et al., 2022). PDGF is composed of the A and B chains and exists as a heterodimer (PDGF-AB) or as a homodimer (PDGF-AA or PDGF-BB), and its receptor is composed by a complex of α and β subtype. It is known that the PDGFR-β receptor plays a role in promoting angiogenesis, and that PDGF stimulates the proliferation of PCs and smooth muscle cells, both of which have been shown to express PDGF-β receptor (Doxey et al., 1995; Beer et al., 1997).

Finally, TGF-β belongs to the cytokine family, of which TGF-β1 is one of the most common members. TGF-β stimulates type II receptors, which in turn phosphorylate type I receptors and activate SMADs proteins. TGF-β1 has two roles: at low concentrations it is an important stimulator of angiogenic switch through the overexpression of angiogenic factors and proteinases, while at high concentrations it inhibits ECs proliferation, promotes the formation of new BM and facilitates differentiation and the recruitment of VSMCs (Otrock et al., 2007, Goumans et al., 2003).

1.2.2 Endothelial dysfuncion

An altered ability of endothelium to maintain vascular homeostasis following metabolic, mechanical and inflammatory stress, causes EC dysfunction (ECD) (Pober et al., 2007; Widlansky et al., 2003; Cines, et al., 1998; Corban et al., 2021). Although the term is often used to refer the loss of bioavailable NO, endothelial dysfunction is also associated with the increased vasoconstriction, impaired fibrinolytic capacity, enhanced turnover, overproduction of growth factors, increased expression of adhesion molecules and inflammatory genes, excessive generation of reactive oxygen species (ROSs), increased oxidative stress and enhanced permeability of the cell layer (Taddei et al., 2003; Hirose et al., 2010). Numerous risk factors directly contribute to ECD, including: elevated low density lipoprotein (LDL) cholesterol and oxidized LDL, reduced high-density lipoprotein (HDL) cholesterol, elevated triglycerides, hypertension, elevated C-reactive protein (CRP) and circulating lipoprotein-associated phospholipase A2 (Lp-PLA2– a specific marker of vascular inflammation), hyperglycemia, increased omega-6: omega-3 ratio (Wan et al., 2010), hyperinsulinemia, elevated homocysteine levels, increased fibrinogen and PAI-1, smoking, aging and vitamin D deficiency (Versari et al. 2009; Bhatti et al., 2010). ECD is one of the earliest signs and a key event in the onset of the vascular diseases, atherosclerotic process and insulin resistance, features of diabetes mellitus (DM) (Hirose et al., 2010; Wan et al., 2010; Kozanoglu et al., 20022) (Figure 5).

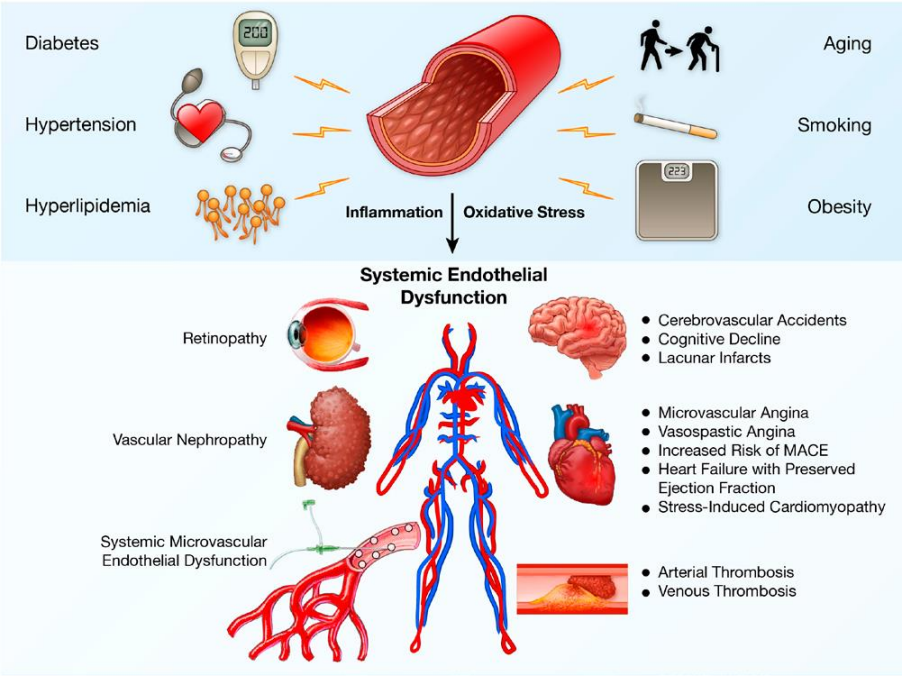


Figure 5. Endothelial dysfunction. Risk factors and widespread systemic manifestations of ECD (Corban et al., 2019).

In DM, a metabolic pathology with a multifactorial etiology, the activity of TGF-β increases matrix proteins synthesis, causing thickening and changes in the composition of the BM (Chen et al., 2001). Conversely, the thickness of the glycocalyx is reduced (Nieuwdrop et al., 2006; Nieuwdrop* et al., 2006), and this involves mononuclear cells and platelets adhesion to the endothelial surface, decreased NO availability and an increased macromolecular loss from the vascular endothelium (Van den Berg et al., 2006). Moreover, DM induces ECD via ROS generation, derived from hyperglycemia or glycation. In this context, AGEs and reactive carbonyl species (RCS) also directly affect the endothelium. The dysfunction of detoxification systems contributes to increase the levels of RCS, which can either directly or indirectly induce tissue damage. ROS generated in these pathways lead to lipid peroxidation, which in turn generate substances involved in further damaging atherosclerotic processes (Stratmann et al., 2022). In diabetic patients, poor vasodilatation and elevated blood levels of vWF, thrombomodulin, selectins, type IV collagen are indicators of ECD (Escandon et al., 2001). Furthermore, it has been demonstrated that endothelial nitric oxide synthase (eNOS) activity is significantly reduced in diabetic mouse model and ECs exposed to chronic hyperglycemia (Escandon et al., 2001; Triggle et al., 2010; Srinivasan et al., 2004).

1.2.3 Factors responsible for endothelial damage induced by hyperglycemia

ECD is an important factor involved in the onset, progression and clinical manifestations of diabetes (Cines et al., 1998). In this context, chronic hyperglycemia causes endothelial damage through the activation of various glucotoxic mechanisms (Figure 11):

- Increased flux of glucose and other sugars via polyol pathway;
- Overactivation of the hexosamine pathway;
- Activation of protein kinase C (PKC) isoforms;
- Increased formation of intracellular AGEs;
- Increased expression and activation of the AGE receptors (RAGE) (Brownlee et al., 2001).

The combined effects of these mechanisms lead to an increase in oxidative stress, apoptosis and vascular permeability (Sena et al., 2013) (Figure 6).

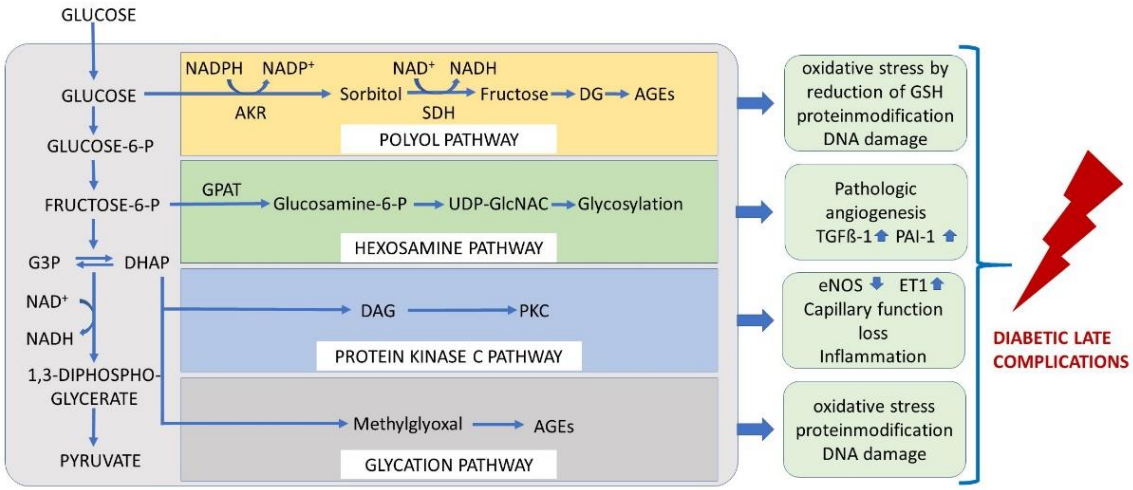


Figure 6. Molecular pathways activated by hyperglycemia. Intracellular glucose accumulation leads to an increase of glucose flux to sorbitol via the polyol pathway, to the hexosamine pathway, to the activation of PKC, and to the formation of AGEs in ECs. AKR, aldose reductase; SDH, sorbitol dehydrogenase; GFAT, glutamine, fructose-6-phosphate amidotransferase; GlcNAc, N-Acetylglucosamin; DAG, diaclyglycerol; G3P, glyceraldehyd-3-phosphate; DHAP, dihydroxyacetone phosphate (Stratmann et al., 2022).

In chronic hyperglycemia conditions, one of the major metabolic pathways responsible for ECD and, consequently, for the vascular complications associated with DM, is the formation of AGEs (Brownlee et al., 2001).

AGEs constitute a non-homogenous, chemically diverse group of compounds formed by non-enzymatic glycosylation, oxidation and/or carbonylation (de Vos et al., 2016; Twarda-clapa et al., 2022). They are physiologically formed during the aging processes, but their production is accelerated in the presence of high glucose levels (Baynes et al., 1999). The production of AGEs occurs through the Maillard reaction, a non-enzymatic process in which the carbonyl groups of reducing sugars react with the amino group of proteins, fatty acids or nitrogenous bases to form glycated molecules. In brief, the carbonyl group of the reducing sugar interacts with an amino group of proteins to form an unstable compound called Schiff base. During the second phase, further rearrangements take place to form stable, but still reversible, compounds called Amadori products (Cho et al., 2007). In the last phase, Amadori products can generate reactive carbonyl compounds that through a very slow reaction, lasting from weeks to months, generate AGEs. Some AGEs derived from this process include: pentosidine, imidazolone, glucosepane and Nε-carboxymethyl-lysine (CML), the main AGE used as a biomarker in human tissues (de Vos et al., 2016). Others faster processes involved in the AGEs formation are lipid peroxidation and glycolysis. In the lipid peroxidation, ROSs transform lipids in reactive carbonyl compounds. This oxidation then evolves into AGEs or advanced lipoxidation products

(ALE), such as malondialdehyde. In the glycolytic pathway, a small part of glucose is converted in reactive carbonyl compounds, of which the best-known and the most reactive is Methylglyoxal (MGO).

AGEs are not only formed by endogenous mechanisms, but also derive from exogenous sources, through the intake of food products rich in AGEs (Goldberg et al., 2004), the inhalation of polluted air and cigarette smoke, containing reactive glycation products which rapidly form AGE (Koschinsky et al., 1997). Indeed, it has been shown that serum AGEs are significantly elevated in smokers who smoke at least a package a day as compared to nonsmokers (Koschinsky et al., 1997). In particular, the amount of AGEs produced during cooking is determined by the temperature at which the food is cooked (Goldberg et al., 2004). Serum CML has been shown to increase after 6 weeks of a high-AGE diet and decrease with a low-AGE diet in DM patients (Cai et al., 2004; de Vos et al., 2016). The role of AGEs in the macro- and microvascular complications of DM has been demonstrated (Goldin A. et al., 2006), including impaired wound healing capacity (Peppa et al., 2009; Alqahtani et al., 2021). Indeed, they can alter the structure and function of different cell types present in the vasculature (Goldin et al., 2006). It is also known that AGEs increase the concentrations of TNF- α and CRP, responsible for inflammatory reactions, in gestational DM (Twarda-clapa et al., 2022). Moreover, AGEs form cross-links with molecules of the ECM, such as collagen and elastin, causing tissue stiffness. AGEs bind to receptor for RAGE, a member of transmembrane multifunctional immunoglobulin superfamily. There are various isoforms of RAGE, generally with extracellular C1, C2, and V domains, a transmembrane domain (TM), and a cytoplasmic domain. Ligand binding to the extracellular domain initiates a cascade of intracellular signaling events, leading to the generation of ROS and inflammatory cytokines, cell proliferation, cell apoptosis, and further upregulation of RAGE (Bongarzobe et al., 2017; Lee et al., 2013). RAGE has been implicated in several diseases, including cardiovascular diseases, neurodegenerative diseases, cancer, and diabetes (Reddy et al., 2022). AGE-RAGE interaction induces the activation of NF-kB and its target genes, which can promote the release of pro-inflammatory molecules (de Vos et al., 2016). The AGE-RAGE signaling induces the activation of MAPK, p38 and PKC, which mediate insulin resistance by downregulating insulin receptor expression, impairing IRS-1 tyrosine phosphorylation, and promoting the IRS-1 serine phosphorylation (Khalid et al., 2022). Moreover, a study has showed RAGE binds with toxic islet amyloid polypeptide (IAPP) intermediates and leads to the formation of amyloid plaque, increasing β-cells apoptosis in DM (Khalid et al., 2022). It is known

that AGEs physiologically increase with age and contribute, in addition to the development of cardiovascular diseases, also to the development of neurodegenerative diseases (Bierhaus et al., 2009; Schlotterer et al., 2009).

1.3 MGO: its formation and detoxification.

The major precursor of AGEs is MGO, a highly reactive dicarbonyl formed during glucose, protein and fatty acid metabolism. MGO readily reacts with proteins, lipids and nucleic acids to form AGEs, the accumulation of which is highly damaging to cellular function (Allaman et al., 2015). Excessive production of MGO can increase the production of ROSs causing oxidative stress (Desai et al., 2010). The major amount of endogenous MGO is formed by the non-enzymatic degradation of triosephosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) during the glycolytic process (Phillips et al., 2003). Minor sources of MGO are produced through the degradation of glycated proteins, oxidation of acetone in the catabolism of ketone bodies during diabetic ketoacidosis, catabolism of threonine, and lipid peroxidation. MGO is also present in several products consumed on a daily basis, but is also found in honey, soft drinks, coffee and other beverages, processed foods, but these exogenous sources of MGO may not be important because MGO in foods is metabolized or reacts with proteins before it is absorbed in the gastrointestinal tract (Schalkwijk et al., 2019) (Figure 7).

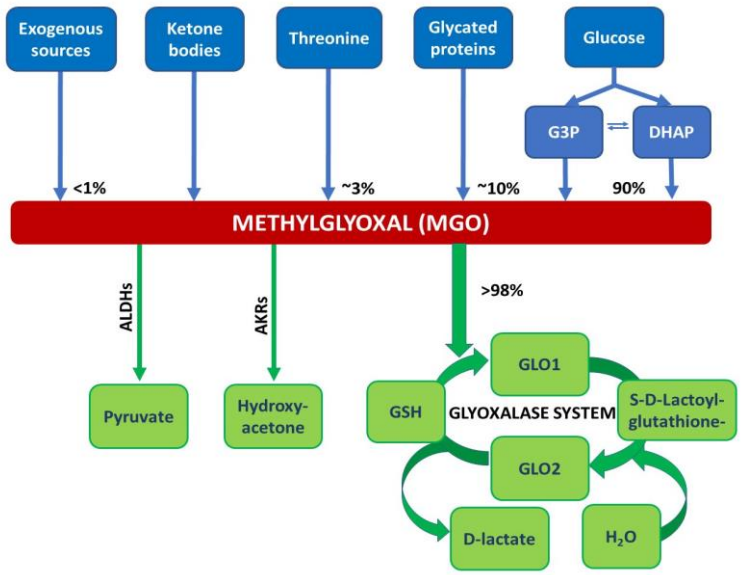


Figure 7. Sources that lead to MGO formation. MGO is mainly formed through the spontaneous degradation of the triosephosphates G3P and DHAP. Other sources of MGO are constitute by: exogenous sources, threonine, or ketone bodies and glycated proteins (Strattmann et al., 2022).

Under physiological conditions, MGO levels are about 50-150 nM in the plasma and 1-4 µM in tissues (Rabbani et al., 2014). MGO accumulation is well known to be associated with diabetes, obesity, central nervous disorders, cardiovascular disease, and diabetes (Verma et al., 2002; Eringa et al., 2004). Indeed, MGO concentrations are increased by 5 to 6-fold in patients with type 1 DM (T1DM) and by 2-3-fold in patients with Type 2 DM (T2DM) (McLellan, et al. 2014). MGO interacts mainly with arginine residues to form MGO-H1, the most common MGO-derived AGE found *in vivo*, leading to structural change, inactivation, and degradation of target proteins (Schalkwijk et al., 2015; Nigro et al., 2017). The interaction of MGO with arginine on serum albumin is responsible for the inhibition of antioxidant capacity and induces an increased synthesis and secretions of proinflammatory markers (TNF-α, IL-1β) in monocytes. Increased MGO-H1 modifications of mitochondrial proteins are associated with increased oxidative stress (Maessen et al. 2015). In physiological conditions, MGO accumulation is prevented by several detoxifying pathways. The most important of these is the glyoxalase system, while minor pathways include aldehyde dehydrogenase (ALDH) and aldose reductase (AKRs), which convert MGO to pyruvate and hydroxyacetone, respectively (Maessen et al. 2015). The glyoxalase system is a cytosolic enzyme system present in all mammalian cells. It consists of two enzymes, glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2), and a catalytic amount of reduced glutathione (GSH). Glo1, the rate limiting enzyme, catalyses the isomerisation of the hemithioacetal formed non-enzymatically from MGO and GSH to S-D-lactoylglutathione, while Glo2 catalyses the hydrolysis of S-D-lactoylglutathione to D-lactate, reforming GSH consumed in the Glo1-catalysed reaction (Thornalley, 1993; Rabbani et al., 2010) (Figure 8).

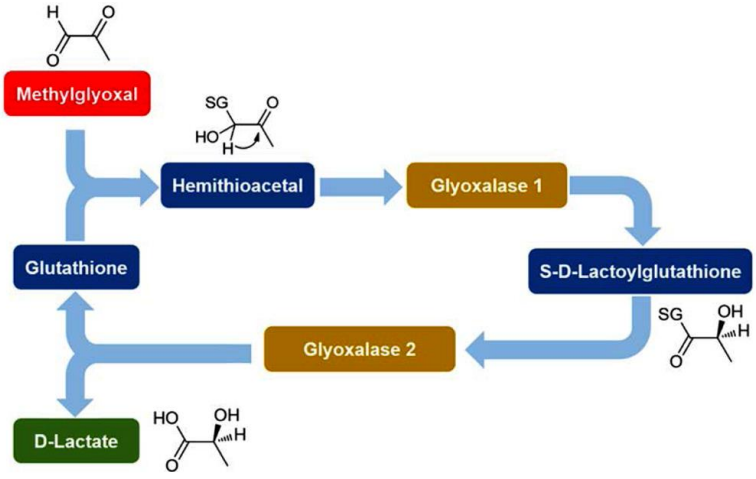


Figure 8. The glyoxalase system. Glo1 catalyzes the conversion of hemithioacetal to the thioester S-D-lactoylglutathione. Glo2 catalyzes the hydrolysis of S-D-lactoylglutathione to form D-lactate (He et al., 2020).

Glo1 gene is ubiquitously expressed in all tissues of prokaryotic and eukaryotic tissues. Glo1 protein is approximately three times more abundant in fetal tissues than in adult tissues, and its activity physiologically decreases with age (Thornalley, 2003). The activity of Glo1 depends by both gene expression regulation and post-translational modifications. Indeed, Glo1 activity is reduced by interaction with S-Nitrosoglutathione, an GSH-NO adduct, that converts Glo1 in an inactive form (Mitsumoto et al., 2000). Under hypoxic conditions, Glo1 gene expression is negatively regulated by hypoxia-inducible factor 1α (HIF1α) which binds to an antioxidant-response element (ARE) located on Glo1 promoter (Zhang et al., 2012). Hypoxia plays a pivotal role in the induction of dicarbonyl stress, by increasing MGO formation through anaerobic glycolysis besides reducing Glo1 expression (Seagroves et al, 2001). *GLO1* gene is also a hotspot for functional copy number variation (CNV) which have been reported to increase Glo1 expression by 2 to 4-fold (Redon et al., 2006; Cahan et al., 2009). In any case, further investigations are essential to understand if these mutations are useful against MGO accumulation and damage.

1.3.1 Damaging effect of MGO on vascular homeostasis

It is known that MGO accumulation has a harmful effect on vasculature, and it is involved in the development of diabetic complications causing damaging effects both on macro- and micro-vasculature (Nigro et al., 2017).

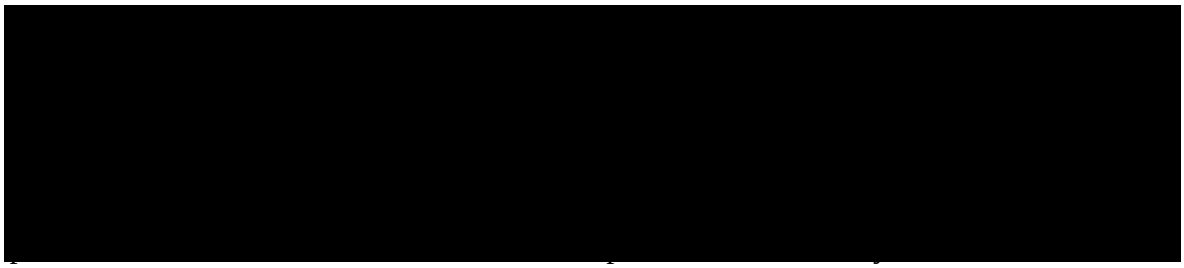
In microvascular complications, the small caliber vessels, (arterioles, capillaries and venules) (Stratton et al., 2000), which have the task of maintaining blood pressure, of guaranteeing correct supply of nutrients to the tissues (Orasanu et al., 2009) and to regulate vascular permeability and myogenic responses, are compromised (Costa et al., 2013). On the other hand, in macrovascular complications, the impaired vessels are the arteries of large and medium caliber, such as the aorta (Stratton et al., 2000). Microvascular diseases in DM are represented by retinopathy, nephropathy, and peripheral neuropathy. MGO contributes to the development and aggravation of these three diseases. On the other hand, macrovascular complications lead to atherosclerosis and thromboembolism, which are responsible of cardiovascular diseases (CVD), including cerebrovascular, coronary, and peripheral vascular disorders (Costa et al., 2013). Among the common features of micro- and macrovascular complications there are the alterations in ECs function (Fadini et al., 2019). Elevated plasma MGO concentrations have been associated with the development of CVD in T1D and with cardiovascular mortality in T2DM (Nigro et al., 2019) Vulesevic

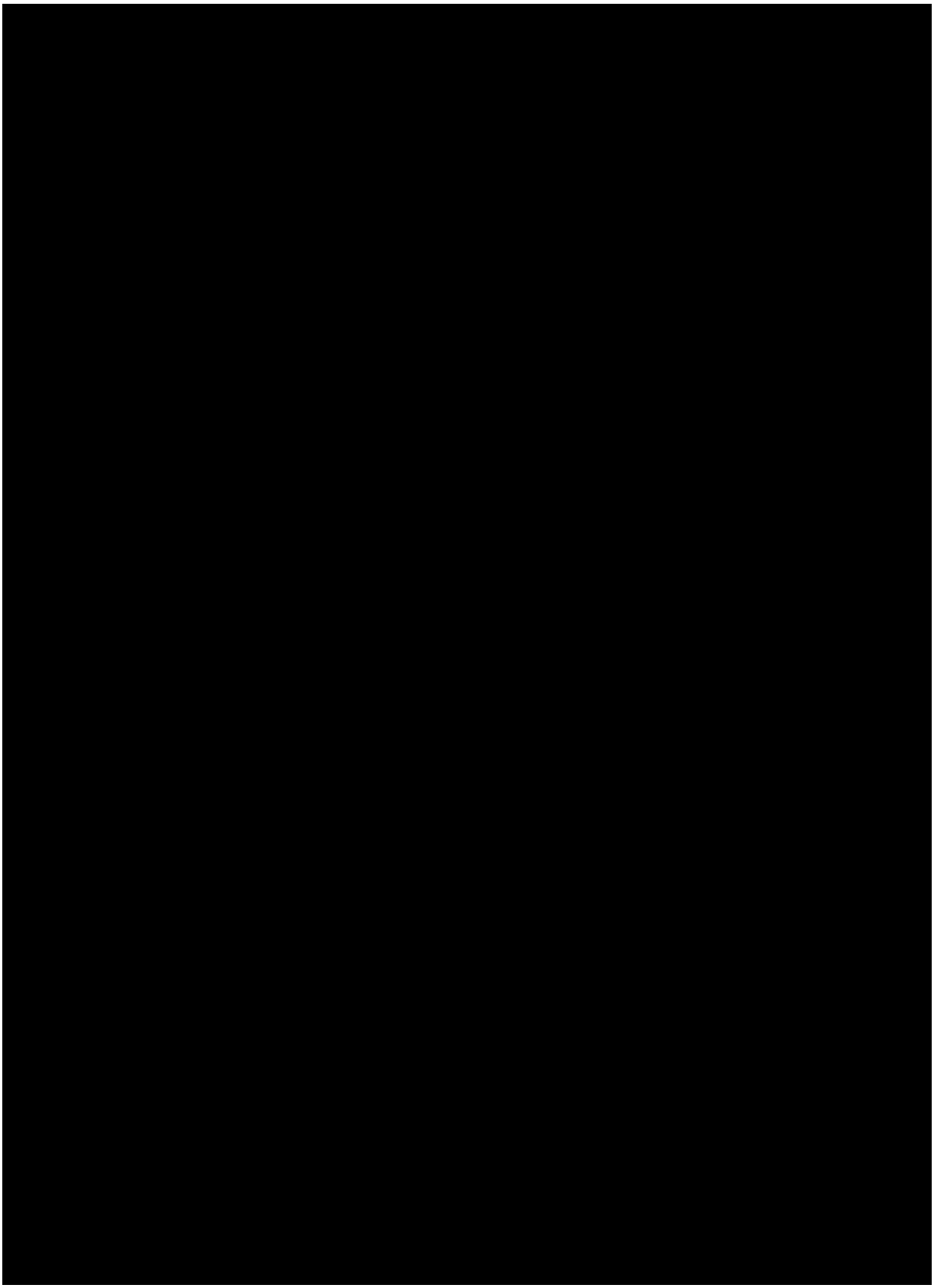
B et al. have shown how MGO is involved in the development of cardiomyopathy, which increases inflammation and ECs loss in diabetic mice (Vulesevic et al., 2016). Similar to this, MGO treatment of Goto-Kakizaki rats exacerbate ECD in part increasing oxidative stress, AGEs formation and inflammation (Sena et al., 2012). In addition, high MGO levels induce vascular dysfunction in arterial walls of spontaneously hypertensive rats (Mukohda, et al., 2012) and activates NF-κB via RAGE, increasing renin-angiotensin levels and blood pressure in Sprague-Dawley rats (Dhar et al. 2014, Nigro et al., 2017). MGO is also involved in the formation of atherosclerotic plaque. A study showed that increasing plasma MGO to levels observed in diabetic mice increase vascular adhesion and atheroneogenesis in normoglycemic apoE-/- mice to a similar extent as that observed in diabetic mice (Tikellis et al., 2014). Moreover, MGO modifies LDL increasing their atherogenicity, molecular density and binding to proteoglycans in the arterial wall (Rabbani et al., 2011). High levels of MGO-H1 residues in humans have been found in carotid atherosclerotic plaques associated with a rupture-prone phenotype (Hanssen et al., 2014), and have been proposed as markers of early stages of atherosclerosis in childhood diabetes (Heier et al., 2015; Nigro et al., 2019). MGO is known to alter the angiogenic process. Indeed, high MGO concentrations induce the formation of aberrant capillaries in zebrafish by upregulation of VEGFR2, ECs exposed to MGO show impaired viability, migration and tube formation due to RAGE-mediated and autophagy induced VEGFR2 degradation (Nigro et al., 2019; Hongtao et al., 2012). Exposure of cultured cells and kidneys from diabetic mice to high concentrations of MGO sensitizes microvascular ECs to inflammatory effects of TNF-α (Yao et al. 2007). Elevated serum levels of MGO-H1 are associated with the development of DR (Fosmark et al., 2006). Moreover, in diabetic patients it has been found a correlation between MGO-derived AGEs and early disease progression (Beisswenger et al., 2013), confirmed by higher GO and MGO levels found in T2D patients with DN compared to those without DN (Wang et al., 2019). *In vivo* studies shown that exogenous administration of MGO to rats induces diabetes-like microvascular changes including impaired vasodilation (Berlanga et al., 2005; Sena et al., 2012), loss of ECs and PCs, thickening of basement membrane, early neuronal dysfunction and increased oxidative stress (Schalkwijk et al., 2019; Nigro et al., 2019).

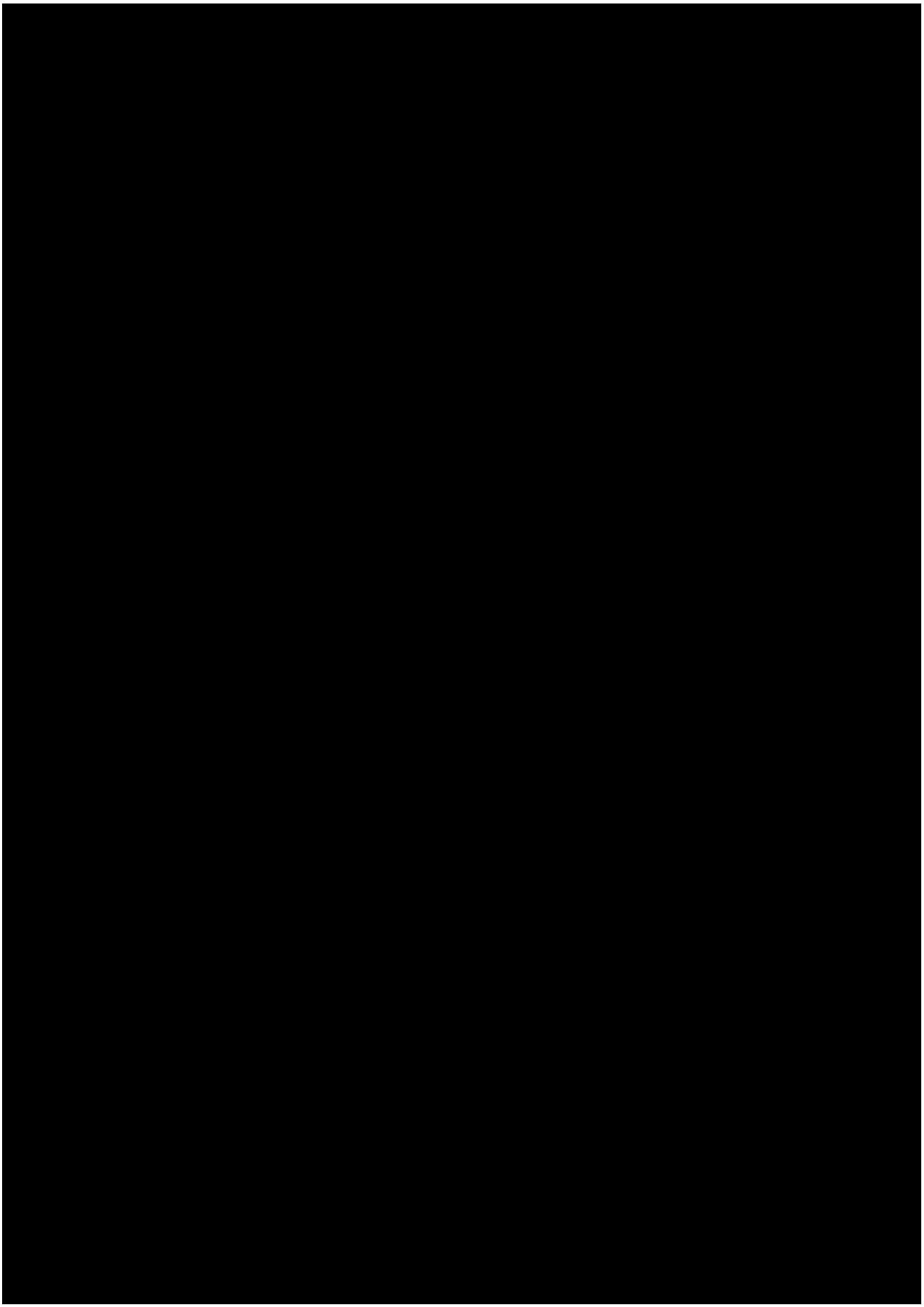
Our group has previously demonstrated that MGO inhibits the activation of insulin dependent IRS1/Akt/eNOS pathway in ECs, thus reducing the NO production in response to insulin, production both *in vivo* and *in vitro*. Furthermore, MGO increases ERK1/2 activation and ET-1 release, fostering endothelial vasoconstriction (Nigro et al., 2014).

This effect is, at least in part, mediated by the down-regulation of miR-190a and miR-214, and the following increased levels of the kinase Kirsten rat sarcoma viral oncogene homolog (KRAS) and the Akt phosphatase PH Domain And Leucine Rich Repeat Protein Phosphatase 2 (PHLPP2), respectively (Schalkwijk et al., 2019). Inhibitors of AGEs formation, such as aminoguanidine, pyridoxamine and benfotiamine, have been shown to reduce AGEs formation directly or indirectly, and prevent the development of nephropathy, retinopathy, and neuropathy in diabetic rats (Hammes et al. 2003; Alderson et al., 2003). In turn, oxidative stress induced by MGO is associated with the impairment of endothelium-dependent vasodilation. Indeed, a study has indicated that acetylcholine-induced vasodilation in rat aortic tissue is impaired by MGO, and this effect is attenuated by aminoguanidine and N-acetyl-cisteine (Dhar et al. 2010). The evidence that these inhibitors can reduce MGO-mediated effects may emphasize the important role of MGO in microvascular complications. Knockdown of GLO1 in human aortic ECs increases MGO and alters the expression of genes associated with endothelial inflammation and apoptosis (Schalkwijk et al., 2019). In line with this, it has been demonstrated that increased Glo1 alleviates dicarbonyl stress, delays the development of obesity and prevents the development of diabetic microvascular complications (Rabbani et al., 2018). Moreover, Glo1 transgenic mice prevented an increase in renal MGO-H1 content in streptozotocin-induced diabetes, with the concomitant prevention of albuminuria and mesangial expansion (Rabbani et al., 2014). Glo1 overexpression is also able to prevent the impairment of angiogenesis in hyperglycemia (Ahmed et al., 2008), of NO-mediated vasodilatation in DM (Brouwers et al., 2010) and renal ischaemia-reperfusion injury in rats (Kumagai et al., 2009). Finally, overexpression of Glo1 improves diabetes-induced impairment of vasodilatation and diabetes-induced expression of the adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1).

All these studies show that counteracting MGO accumulation may be crucial for preserving vascular homeostasis.







1.3.2 Effect of MGO on glucose homeostasis

Insulin is a peptide hormone produced by β-cells of islets of Langerhans, which regulates glucose homeostasis. In blood, insulin exists as an active monomer, consisting of an α-chain of 21 amino acids and a β-chain of 30 amino acids linked by two disulfide bonds. In presence of insulin, the insulin receptor (IR) activates its intracellular tyrosine kinase activity, phosphorylating insulin receptor proteins (IRS proteins) and subsequently activating several protein kinases. Insulin resistance and β-cell loss are the hallmarks of T2DM (Schalkwijk et al., 2019). Metabolic insulin resistance is characterized by an impaired responsiveness to insulin action on glucose uptake in multiple target organs. MGO alters the β-chain of human insulin, specifically the N-terminus arginine residue and it is associated with changes in insulin structure and stability (Oliveira et al., 2011). Thus, the formation of the MGO-insulin adducts impairs autocrine control of insulin secretion by β-cells, decreases insulin clearance, and reduces insulin-mediated glucose uptake in target cells (Schalkwijk et al., 2020). Studies from our group and others have demonstrated that a chronic administration of MGO by i.p. injection, minipumps infusion or supplementation of drinking water leads to insulin resistance (Dhar et al., 2011; Guo et al., 2009), causes pancreatic cell dysfunction, and induces T2D (Dhar et al., 2011). In addition, MGO impairs the insulin signaling and insulin action on glucose-induced insulin secretion in the pancreatic β-cell line INS-1E (Fiory et al., 2018). MGO modulates the secretory activity of β-cells depending on the levels of glucose, as well as other metabolic factors. MGO decreases insulin secretion in the presence of acetylcholine but promotes insulin secretion in the presence of potassium and epinephrine (Elmhiri et al., 2014). Although intracellular MGO may injure the pancreatic β-cells, the underlying mechanisms responsible for β-cell injury remain to be elucidated. Acute exposure to MGO induces the inhibition of insulin-stimulated phosphorylation of protein kinase B (PKB) at Ser473 and Thr308 and extracellular-regulated kinase 1/2 (ERK1-p44 and ERK2-p42) with impaired insulin signaling in muscle and pancreatic β-cells (Chavey et al., 2016). Moreover, MGO impairs insulin secretion of pancreatic β-cells through increased ROS production, which induces upregulation of JNK/P38 and UCP2, resulting in reduced MMP and ATP production (Jinshuang et al., 2016). Moreover, MGO induces oxidative damage in pancreatic β-cell line INS-1 cells through Ire1α-JNK and mitochondrial apoptotic pathway (Chongxiao et al., 2017). Non-cytotoxic MGO concentrations impair both insulin action and secretion by inhibiting insulin-induced activation of IRS1/ phosphatidylinositol 3 kinase (PI3K)/protein

kinase B (PKB), which in turn forms AGE adducts on IRS, and inhibits insulin secretion by glucose (Nigro et al., 2019). MG induced the activation of TAK1/p38/mTORC1 signaling in adipocytes causing multiple serine phosphorylation on IRS-1, which potentially contribute to insulin resistance (Sung-Ping et al., 2022). However, an acute exposure to MGO induces a dual effect on rat pancreatic islet function, increasing insulin secretion at basal glucose concentrations, but exerting the opposite effect on hyperglycemia (Elmhiri et al., 2014). Thus, MGO can act as an agonist of islet cells transient receptor potential ankyrin 1 (TRPA1) channel in acute stimulation, promoting Ca2+ influx and insulin secretion (Cao et al., 2012). Therefore, MGO may have different effects in the acute and chronic exposure of pancreatic cells, likely inducing a transient increase in insulin secretion, but reducing long-term β-cell function by inhibiting insulin signaling and synthesis mechanisms (Matafome et al., 2017). Moreover, phosphocreatine has protective and therapeutic effects against MGO-induced pancreatic β-cell injury by reducing apoptosis, intracellular calcium, and oxidative stress, as well as improving mitochondrial functions and activation of dual signalling pathways: the AKT/IRS-1/GSK-3β and STAT3/Cyp-D, both *in vitro* and *in vivo* (Wang* et al., 2022).

In conclusion, MGO negatively impacts both on insulin production and action and the development of new strategies for preserving glucose homeostasis will be useful for preventing the progression of T2DM.

1.3.3 Effect of MGO on senescence

Age-related damage to intracellular and extracellular macromolecules is known to be progressive and irreversible due to the impairment of glycolytic and oxidative defence mechanisms. MGO and MGO-derived AGEs increase in aging tissues contributing to macular degeneration in the retina, osteoarthritis in human cartilage, impaired endothelium-dependent vasorelaxation in vascular tissue, endoplasmic reticulum (ER) stress and apoptosis in skin fibroblasts and skin aging (Kalapos et al., 2010; Johnson t al. 2005). The first link between AGEs and aging was demonstrated by Morcos et al. in C. Elegans, showing that the age-related decrease in Glo1 activity increases mitochondrial ROS production, thereby limiting lifespan. Consistently, the overexpression of Glo1 was shown to prevent the accumulation of MGO-H1 residues in mitochondrial proteins, reducing ROS production and increasing the lifespan of C. elegans (Morcos et al., 2008).

In a study performed in mice has been demonstrated that aging-related Glo1 reduction delays wound healing (Fleming et al. 2013). MGO-induced AGEs, such as CEL and CML, accumulate in tissue proteins with age and are implicated in the aging of tissue proteins (Kalapos et al., 2010). Therefore, MGO and its derived AGEs are all associated with the aging process, and they have been correlated with many aging-related diseases such as cardiovascular complications of diabetes, neurodegenerative diseases, cancer and connective tissue disorders (Christensen et al., 2020). MGO changes several biochemical processes that determine aging through: mitochondrial dysfunction, inflammaging, genomic instability, loss of proteostasis and cellular senescence (Nigro et al., 2019) (Figure 11).

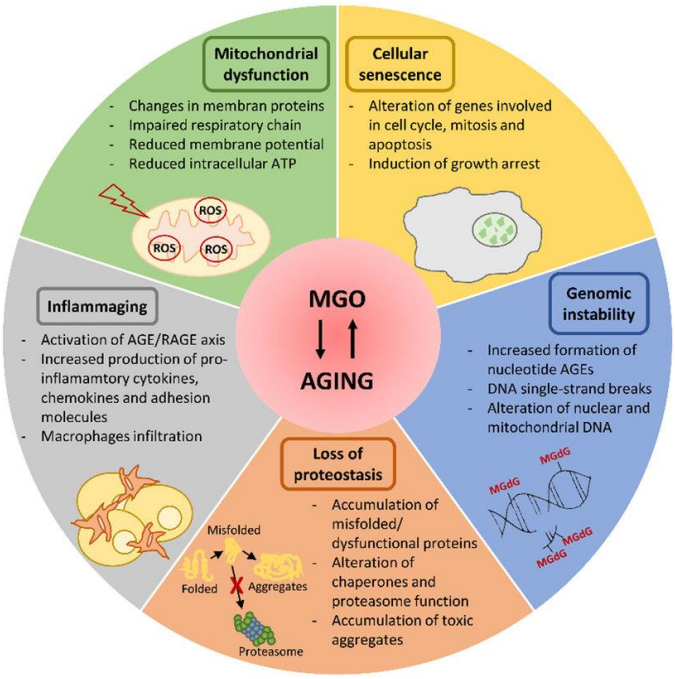


Figure 11. Cellular processes compromised by MGO and aging (Nigro et al., 2019).

In detail, many studies have showed that increased levels of MGO in diabetes and aging induce cellular senescence. Santos et al. demonstrated that a combination of GO and MGO, in addition to increased ROS and AGEs formation, can increase p21 expression and arrest human vascular ECs in the G2-phase of the cell cycle, which can be abrogated by antioxidant and dicarbonyl scavenger treatment (Santos et al., 2017). Similarly, high MGO concentrations upregulate p53 gene expression inducing growth arrest and apoptosis in HUVECs. Carnosine has been proposed as a strategy to ameliorate the harmful effect of MGO on HUVECs because it counteracts gene expression changes induced by MGO in

HUVECs (Braun et al., 2019). Sejersen and Rattan showed that the treatment of human skin fibroblasts with MGO (400 μM) or glyoxal (1 mM) has induced a senescent phenotype within 3 days, as indicated by an G2 cell cycle arrest, increased senescence-associated β-galactosidase (β-Gal) activity, increased CML levels and altered antioxidant enzyme (Kalapos et al., 2010). Moreover, they demonstrated that hMSC-TERT exposed to 0.75 mM and 1 mM of Glo induce cellular senescence in three days as observed by increased levels of a cell cycle inhibitor p16, senescent morphology and β-Gal activity (Larsen et al., 2012). Aguayo-Mazzuccaro et al. showed that the cytokines MCP1, TNF-α and IL-1β, which are part of senescence-associated secretory phenotype (SASP), were secreted by senescent β-cell (Aguayo-Mazzucato et al., 2019). It has been reported that MGO accelerated cellular senescence in human dermal fibroblasts and regulated longevity of Caenorhabditis elegans regulating TORC2/SGK-1/DAF-16 signaling pathway (Shinet al., 2020). GLO induces senescence in human keratinocyte in early-stage with the activation of PKB/FOXO3a/p27KIP1 pathway, while in late-stage through the p16INK4/pRb pathway (Halkoum et al., 2022).

1.4 Stem cells

Stem cells are unspecialized cells able to differentiate into any cell of an organism and have the ability of self-renewal. Stem cells exist in both embryonic and adult cells (Ramalho-Santos et al., 2007). There are several steps of specialization. Totipotent stem cells are able to divide and differentiate into cells of the whole organism. Indeed, totipotency represents the highest differentiation potential and cells can form both embryonic and extraembryonic structures. An example of a totipotent cell is the zygote, which is formed following the fertilization of the egg. On the other hand, pluripotent stem cells (PSCs) can differentiate into cells belonging to all germ layers but not to extra-embryonic structures (e.g. the placenta) (Zakrzewski et al., 2019; Miana et al., 2018). Examples of pluripotent cells are embryonic stem cells (ESCs), derived from the inner cell mass of preimplantation embryos, and induced pluripotent stem cells (iPSCs), which are produced in the laboratory through a complex genetic reprogramming of somatic cells (Zakrzewski et al., 2019), thus avoiding the ethical issue associated with the use of human embryos (Chagastelles et al., 2011). Multipotent stem cells have a more limited differentiation capacity than PSCs and can, therefore, differentiate only into a small number of specialized cells. Adult stem cells are multipotent cells and include: hematopoietic stem cells and mesenchymal stem cells

(MSCs). Hematopoietic stem cells have the ability to differentiate into different types of blood cells and are usually isolated from the bone marrow or cord blood. On the other hand, MSCs are of stromal origin, have the ability to differentiate into a large variety of cells and can be isolated from any tissue of the organism (Meirelles et al., 2006; Meirelles et al., 2009; Chagastelles et al., 2011) such as, from bone marrow, adipose tissue, menstrual blood, pulp of deciduous teeth, umbilical cord tissue and blood (Aboushady et al., 2018; Macrin et al., 2017; Teng et al., 2017). Several studies have shown that MSCs derived from differentiated tissues show heterogeneity in their biological and functional characteristics due to their different proliferative, differentiative, pro-angiogenic and immunomodulatory capabilities and they are promising candidate for therapeutic use in regenerative medicine. Finally, unipotent stem cells have the narrowest differentiation capacity and a special property of dividing repeatedly (Han et al., 2017; Kumar et al., 2019; Zakrzewski et al., 2019).

1.4.1 Stem cells derived from adipose tissue

Among the MSCs, adipose tissue derived stem cells (ADSC) have been identified as one of the most promising stem cells populations identified because they are ubiquitous, have high proliferative capacity (Kunze et al., 2020; Liu et al., 2021), can maintain their phenotype in culture for a long time and they are relatively easy to harvest in larger quantities with low donor-site morbidity (Zizhen et al., 2019). In humans, ADSCs can be isolated from adipose tissue wastes resulting from plastic surgery, liposuction aspirates and from reconstructive surgery, or from the whole adipose tissue pieces (Lindroos et al., 2011). It is known that ADSCs present in the subcutaneous white adipose tissue have a much higher yield and differentiation potential, compared to those present in the visceral white adipose tissue (Vachkova et al., 2016) and that the anatomical area of adipose tissues collected, induce different properties to the ADSCs. For example, ADSCs collected from abdominal regions are significantly less apoptotic than ADSCs collected from other areas, and those isolated from the thigh have a better yield of brown adipose tissue. However, these cells have different characteristics and are more likely to encounter to muscle differentiation. Finally, it has been observed that age, gender, and body mass index (BMI) of the donor also influence the differentiation capacity of ADSCs. In fact, advanced age, a high BMI and DM have been found to reduce the proliferative and differentiation potential of ADSCs. (Zuk et al., 2001; Wankhade et al., 2016) Currently, the most widely used

method for the isolation of ADSCs is that proposed by Zuk et al. and requires the enzymatic degradation of the collected white adipose tissue with collagenase. Subsequently, from the digested tissue it is possible to isolate the stromal vascular fraction (SVF), which consists of a heterogeneous cell population including, in addition to ADSCs, also pre-adipocytes, ECs, fibroblasts, PCs, vascular smooth muscle cells, white blood cells and red blood cells (Bacakova et al., 2018; Zizhen et al., 2019)

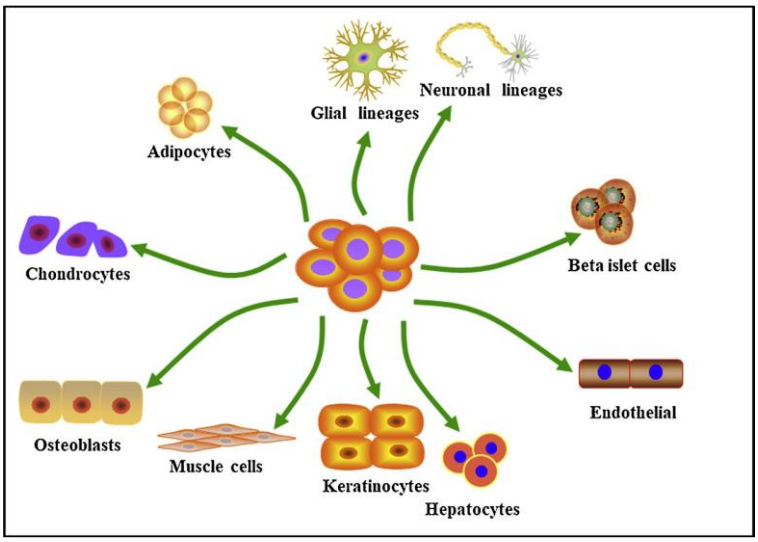


Figure 12. The differentiation capability of ADSCs (Zizhen et al., 2019).

The International Society for Cell Therapy (ISCT) and the International Federation for Adipose Tissue Therapy and Science (IFATS) have established three minimum criteria for identifying cells as ADSCs: i. the ability to grow in adhesion; ii. the expression of the surface markers CD73, CD90, CD105 and the absence of markers CD45, CD14, CD19, CD11b and HLA-DR; iii. the ability to differentiate into pre-adipocytes, chondrocytes, and osteoblasts. Subsequently, the ISCT indicated additional surface markers including CD13, CD29 and CD44 as constitutively expressed on the surface of 80% of ADSCs, while CD31, CD45 and CD235a as primary negative markers which should be expressed on less than 2% of ADSCs (Zizhen et al., 2019; Lindroos et al., 2011). It has been shown that ADSCs, despite its mesodermal origin, have the ability to differentiate even in cells of ectodermal and endodermal origin (Radtke et al., 2009; Timper et al., 2006). In fact, under certain conditions they can also differentiate into vascular smooth muscle cells, keratinocytes, hepatocytes, and β-cells (Visvader et al., 2016; Varghese et al., 2017; Ding et al., 2013).

1.4.2 Applications of ADSCs in regenerative medicine

Regenerative medicine is a multidisciplinary field of research that uses biomaterials, growth factors, and stem cells to repair, replace, regenerate tissues and organs damaged by injury or disease (Margiana et al., 2022). Stem cells are ideal candidates for use in regenerative medicine because of their ability to self-renew and to differentiate into multiple cell lineages (Fathi et al., 2016; Mohammadian et al., 2016). Among stem cells, ADSCs are currently at the centre of considerable interest from the scientific community in the field of regenerative medicine due to their availability, plasticity and versatility. Initial studies have suggested that ADSCs simply act by differentiating into different cell lines, thereby compensating for the function of defective cells or the absence of excised cells (Noel et al., 2008; Lin et al., 2008; Hong et al., 2006). ADSCs can also perform their supporting function through the paracrine release of growth factors, necessary to accelerate and direct the repair of tissues by host cells (Rehman et al., 2004; Miranville et al., 2004). Numerous pre-clinical and clinical studies have demonstrated the pivotal role of ADSCs in the reconstruction and repair of target organs such as bone, myocardium, liver, nervous system, and skin (Zhang et al., 2020). More in detail, it has been shown that ADSCs: 1. can form new bone and, therefore, repair large cranial defects or defects involving the maxilla and mandible (Sandor et al., 2003; Zhang et al., 2009; Zizhen et al., 2019); 2. they are a very promising alternative for the treatment of acute liver failure and chronic liver disease (Liang et al., 2019; Berardis et al., 2014); 3. they can promote neurogenesis and therefore could provide a valid therapeutic option for the regeneration of nerves and nervous tissue (Erba et al., 2010; Abdanipour et al., 2011; Wankhade et al., 2016); 4. they can be used as treatment for myocardial infarction (Nagata et al., 2016; Zhang et al., 2020). Moreover, Bacau et al (Bacau et al., 2004) showed a study on rats in which cells differentiated from ADSCs were injected into the injured tibialis anterior muscle and after 60 days the treated group had increased contractility compared to the untreated control group. Meanwhile, another study showed that in model of Duchenne muscular dystrophy, ADSCs transplanted in mice induce the production of dystrophin in mice (Rodriguez et al., 2005). In addition, studies on murine models observed cell aggregates similar to the islets of Langerhans formed from ADSCs in the pancreas, which were able to secrete insulin (Chandra et al., 2009; Chandra et al., 2016; Miana et al., 2018; Krawczenko et al., 2022).

The potential of ADSCs to self-renew and regenerate tissues has great implications in wound healing and restoration of damaged skin. Wound healing is a complex biological

process in which the skin has the ability to regenerate itself following damage. However, in some circumstances such as deep burns, chronic wounds and acute skin loss, and in some pathological conditions, such as DM, this process is compromised (Groeber et al., 2011; Guo et al., 2010). Supportive approaches are therefore required for this process to be preserved. In addition, chronic and deep wounds can undergo infections that could further delay or avoid the healing process, thus causing the amputation of a limb or, even, the death of the patient. The use of stem cells represents a promising approach to overcome the limitations of conventional treatments (donor site morbidity and invasive surgical procedures) (Tanaka et al., 2016) and accelerate the healing of both acute and chronic wounds (You et al., 2014). ADSCs exert their actions in wound healing, in different ways: 1. by differentiating into different cell types; 2. through their secretory profiles, able to modulate some biological processes, including cell proliferation and differentiation, angiogenesis and inflammation; 3. through the reduction of apoptosis and oxidative stress; 4. through antibacterial properties. It has been shown that, when applied to wounds, ADSCs are able to differentiate into fibroblasts, keratinocytes, epithelial cells and also into ECs (Shingyochi et al., 2015, Ebrahimian et al., 2009). However, the main mechanism by which ADSCs improve wound healing is the autocrine and paracrine secretion of factors that promote differentiation and proliferation of both the stem cells themselves and neighboring cells (Hassan W. U. et al., 2014). Indeed, when exposed to an outbreak of inflammation or ischemic injury, ADSCs secrete growth factors and cytokines that are useful in promoting tissue healing and regeneration (Ebrahimian et al., 2009). In addition, ADSCs have antiapoptotic and antioxidant properties, thanks to which the wound healing process is supported (Cerqueira et al., 2013; Shingyochi et al., 2015). Furthermore, the remarkable resistance to the cytotoxic effects of different bacterial strains can make ADSCs a good option for treating infected wounds (Fiedler et al., 2013; Hassanshahi et al., 2018).

Zhao et al. observed accelerated wound healing, re-epithelialization, enhanced collagen production, angiogenesis, cell proliferation, inhibited apoptosis, and reduced inflammation using human ADSCs to treat diabetic cutaneous wounds in mouse model (Krawczenko et al., 2022). ADSCs therapy is also promising in the treatment of many vascular disorders, thanks to the stabilizing effects these cells have on microvasculature (Kelly-Goss et al., 2014). In particular, it has been shown that an intravitreal injection of ADSC can stabilize retinal microvascularization, allowing the repair and regeneration of damaged capillary beds in DR (Cronk et al., 2015; Wankhade et al., 2016).

1.4.3 Pro-angiogenic potential of ADSCs

It is known that ADSCs can enhance the angiogenic process thanks to their differentiation capacity, cellular interaction, and paracrine effects (Watt et al., 2013). In particular, the proangiogenic effects of ADSCs are attributable to the secretion of several factors including bFGF, VEGF, TGF-β, PDGF, Angpt1, PIGF, IL-6, IL-8, IGF-1 and MCP-1, able to activate and recruit endogenous cells involved in the angiogenic process (Kwon et al., 2014; Krawczenko et al., 2022). In detail, IL-6 is a cellular factor that shows a powerful pro-angiogenic and anti-apoptotic activity; MCP-1 is a cytokine with a chemoattractive action critical for angiogenesis (Boomsma et al., 2012); VEGF, as a pro-angiogenic factor expressed by ADSCs, promotes their differentiation (Beckermann et al., 2008), regulates ECs migration and differentiation and promotes the recruitment of ECs for angiogenesis (Shimamura et al., 2013). Other factors secreted by ADSCs, capable of promoting local angiogenesis, are the hepatocyte growth factor (HGF) and the factor derived from stromal cells-1 (SDF-1) (Pasquet et al., 2010). HGF exerts its pro-angiogenic activity through the phosphorylation of its specific receptor, c-Met, expressed by ECs and smooth muscle cells (Kaga et al., 2012). On the other hand, SDF-1 is an inducible and constitutively expressed chemokine, that can promote ECs proliferation and capillary formation (Zhou et al., 2012). It has also been shown that ADSCs are involved in postnatal angiogenesis and in the vascularization in vivo by differentiating into ECs (Lin et al., 2012; Tao et al., 2016). Several studies investigated the ways in which ECs or the secretome influence the angiogenic ability of ADSCs, especially in culture conditions. Studies have shown that Activin A is induced in ADSCs in response to exposure to ECs and inhibits vascular network formation. Similarly, inhibition of activin A secretion in ADSCs increased network density in parallel studies (Krawczenko et al., 2022). ADSCs in co-culture with EPCs promote angiogenesis increasing the migration and invasion of EPCs and simultaneously upregulating the expression of angiogenesis markers in EPCs (Gan et al., 2022, Rautainen et al., 2021). A study conducted by Pu et al. described that ADSCs and the conditioned medium (CM) of ADSCs enhance neovascularization and skin survival after an ischemia injury (Krawczenko et al, 2022). Interestingly, CM obtained from senescent ADSCs attenuates the angiogenic potential of these cells (Ratushnyy et al., 2020). However, in some pathological conditions such as in the case of chronic hyperglycemia, hyperinsulinemia and metabolic disorders typical of DM, the functionality of the ADSCs is compromised. A fundamental role in this alteration seems to be played by

the accumulation of ROS. It is known that in such pathological conditions ROS are involved in the physiological modulation of cell differentiation, proliferation, and migration (Bonnard et al., 2008). Excessive caloric intake has been observed to lead to the accumulation of ROS in adipose tissue (source of ADSC) of T2DM mice and to promote cellular senescence by increasing β-Gal activity, p53 expression and proinflammatory cytokine production (Minamino et al., 2009). It has also been reported that ADSCs from diabetic models or those treated with high glucose show greater stemness, evidenced by the overexpression of the pluripotent genes Sox-2, Oct-4 and Nanog, although they show reduced proliferation (Cheng et al., 2016). In addition to hyperglycemia, also hyperinsulinemia, characteristic of DM, can cause ROS accumulation. In fact, Scioli and colleagues have shown that the increased apoptosis of ADSCs induced by high insulin levels, through the generation of ROS, compromises the correct homeostasis of stem cells (Scioli et al., 2014). The angiogenic capacity of ADSCs is also compromised in the case of DM. Indeed, ADSCs isolated from patients with coronary heart disease and DM show reduced angiogenic activity due to an imbalance of pro and antiangiogenic growth factors secreted by ADSCs (Dzhoyashvili et al., 2014). Surprisingly, bFGF has been shown to reverse the effect induced by DM on ADSCs, improving their morphology, increasing their proliferative activity, and reducing their senescence and apoptosis by decreasing oxidative stress (Nawrocka et al., 2017). Finally, T2DM-related metabolic dysfunction was observed to influence the immunomodulatory properties of ADSCs as well. Indeed, compared to ADSCs isolated from lean subjects, ADSCs deriving from obese or T2DM patients show an increase in the expression and secretion of inflammatory cytokines, activation of the NLRP3 inflammasome, greater migratory, invasion and phagocytosis capacities, but less efficacy in suppressing T and B cell proliferation, activating M2 macrophages and increasing TGF-β1 secretion (Serena et al., 2016; Qi et al., 2019). However, *in vitro* preconditioning of ADSCs with the iron chelator deferoxamine (DFX), a hypoxia mimetic agent with antioxidant properties, increase the production and secretion of pro-angiogenic, neuroprotective, and anti-inflammatory molecules important for the clinical management of DN (Oses et al., 2017).

However, the beneficial effects of ADSCs on angiogenesis may be related not only with their paracrine activity but also with their differentiation potential towards pericyte-like cells, which play an important role in stabilization of the vessel wall and preventing vascular leakage. These observations performed *in vitro* under both normal culture conditions and in high glucose concentration medium, to mimic the altered

microenvironment of a diabetic eye, indicated that pericyte-like differentiation of human ADSCs may be a therapeutic option to replace irreversibly loss of PCs in patients with DR (Mannino 2020; Mannino et al., 2021). Therefore, improving the functionality of the ADSCs is a fundamental requirement in order to optimize the therapeutic strategies that involve the use of autologous ADSCs for the treatment of vascular diseases.

2. AIM OF THE STUDY

Tight control of blood glucose levels is the primary treatment goal for DM and its complications. However, clinical studies have shown that factors other than poor glycemic control may induce vascular complications in DM. Among the biochemical mechanisms by which hyperglycemia affects cellular function, the increase of reactive dicarbonyls, including MGO, has been identified as an important player, which lead to cellular dysfunction and the progression of tissue damage. In physiological conditions, MGO is detoxified by Glo1 and Glo2. Decreased Glo1 activity and transcription have been described to occur physiologically with aging, and in other pathological conditions, such as oxidative stress, inflammation, and senescence. MGO exerts a damaging effect on the angiogenic function, on peripheral insulin sensitivity and β-cell function. However, the specific effect of MGO accumulation on the early steps of T2DM pathogenesis and tissue repair has not been fully clarified yet. In recent years, numerous studies have focused on the potential use of ADSCs in the field of regenerative medicine thanks to their low immunogenicity and their ability to self-renew. Indeed, ADSCs have a crucial role in the repair of damaged tissues, like ischemic wound in diabetic patients, and they play a pivotal role in angiogenesis, through the activation and recruitment of endogenous cells involved in this process and the stabilization of new formed vessels. However, in pathological conditions, including diabetes, ADSCs function is compromised.

The present work includes a first part aiming to investigate the causal effect of MGO on glucose homeostasis perturbation *in vivo* in a mouse model knock-down for Glo1 (Glo1KD mice). The second part aims to test the effect of MGO on the pro-angiogenic function of ADSCs, in order to identify molecular targets useful to optimize the use of autologous ADSCs for the treatment of vascular defects in diabetic patients.

3. MATERIALS and METHODS

Reagents

Media, sera and antibiotics for cell culture were provided by Innoprot (Derio, Bizkaia, Spain) and Gibco (Waltham, Massachusetts, USA). MGO (40% in water), was from Sigma-Aldrich (St Louis, MO, USA). Insulin was from Eli Lilly (Florence, Italy). Protein electrophoresis and western blot reagents were from Bio-Rad (Richmond, VA, USA) and electrochemiluminescence reagents from Pierce (Rockford, IL, USA). The antibodies used are anti-MGO (Abcam, Cambridge, UK) and anti-14.3.3 (Santa Cruz Biotechnology, Dallas, Texas, USA), anti-p53 (Cell Signaling Technology, Danvers, MA, USA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HMGB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-histone H3 (Abcam, Cambridge, UK), and anti-vinculin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Technology, Danvers, MA, USA). CD29-APC, CD44-PE, CD45-FITC, CD31-APC antibodies were provided by Miltenyi Biotec (Auburn, CA, USA). All other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Mice

Glo1 knockdown (Glo1KD) mice were were generated and characterized as previously described (Bierhaus et al., 2012; Queisser et al. 2010; Giacco et al. 2014). Mice were housed in a temperature-controlled (22°C) room with a 12-h light/ dark cycle in accordance with the *Guide for the Care and Use of Laboratory Animals*. All the experiments were conducted in age-matched male mice with the approval of the local ethics committee of the MIUR.

Measurement of MGO

The MGO content in the pancreas tissue and plasma was determined by HPLC after derivatization with 1,2-diaminobenzene (Rabbani et al. 2014).

mADSCs isolation

ADSCs were isolated from subcutaneous adipose tissue biopsies taken from C57bl6 mice available in our laboratory. Under a laminar flow hood, the biopsies were washed with culture medium consisting of: DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12), 10% FBS, Pennicillin 100 (U/ml), Streptomycin (50 μg/ml) and

Amphotericin B (1.5 μg/ml) and minced mechanically with the use of sterile scissors. The finely shredded tissues were processed with the "Adipose tissue Dissociation kit" (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. It consists in a combination of mechanical dissociation with enzymatic degradation of the extracellular matrix, which maintains the structural integrity of tissues. In particular, the tissues were digested into the gentleMACS C Tube with an enzymatic mixture at 37° C for 40 minutes. The digested tissues were then filtered using 100 μm filters and centrifuged at 1500 rpm for 5 minutes. After the centrifuge, the samples were stratified in: *i.* an oily layer; *ii.* a fatty portion, consisting of adipocytes; *iii.* a liquid portion, consisting of the medium in which the biopsy samples were digested and iv. a pellet, consisting of the SVF containing the ADSCs. After removing the supernatant, the pellet was resuspended in the complete DMEM/F12 culture medium and plated in a T25 flask.

Cell culture

INS-1 832/13 rat insulinoma cells (cod. SCC207, Sigma-Aldrich, St Luis, MO, USA) were plated in T75 flasks and grown in RPMI 1640 with sodium bicarbonate, supplemented with 10% (vol/vol) fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA USA), 10 mm HEPES (pH 7.4), 2 mm L-glutamine, 1 mm sodium pyruvate, and 50 μm β-mercaptoethanol (Sigma-Aldrich, St Luis, MO, USA).

hRECs were provided by Innoprot (Derio, Bizkaia, Spain) and cultured in T75 flasks, previously coated with fibronectin (Innoprot, Derio, Bizkaia, Spain), using ECM (Endothelial Cell Medium) consisting of 500ml of basal medium, 25 ml of fetal bovine serum (FBS), 5 ml of EC growth supplement (ECGS) and 5 ml of a penicillin/streptomycin solution (P/S solution).

mADSCs were cultured in T25 flask and grown in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12), with 10% FBS, Pennicillin 100 (U/ml), Streptomycin (50 μg/ml).

Cell cultures were maintained at 37 °C in a humidified 5% (v/v) CO2 incubator. Where indicated cells were treated with MGO (Sigma-Aldrich, St Luis, MO, USA) for 16h or 48h, treated or not with dasatinib (31 nM) and quercetin (6 μM) in combination (Sigma-Aldrich, St Luis, MO, USA), or with 10 μM SB203580 for 30 minutes (MedChemExpress, Shanghai, China).

RNA isolation, Reverse transcription, and quantitative Real-Time PCR (qPCR)

RNA was isolated from mouse tissues (skeletal muscle, perigonadal adipose tissue, liver, brain, pancreas, and pancreatic islets) or INS-1 832/13 cells using miRNeasy mini kit (QIAGEN, Hilden, Germany) and from mADSCs using TRIzol reagent, according to the manufacturer's protocol. After quantification with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), 1 μg of total RNA was reverse transcribed using SuperScript III (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA obtained was used as a template for Real Time-PCR, performed in triplicate by using iQ SYBR Green Supermix on iCycler Real Time detection system (Bio-Rad). *Cyclophilin A* and *β-Actin* were used as reference genes. Gene expression data from cells are shown as relative quantification using the $2^{-\Delta\Delta Ct}$ method. Gene expression data from mice are shown as the absolute quantification normalized on reference gene expression (ratio of gene of interest copy number to reference gene copy number; Schmittgen & Livak, 2008). Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design specific primers, which were then purchased from Sigma-Aldrich (St Louis, MO, USA). Primers used for real-time Real-time-PCR are as follow:

β-Actin mouse	Forward: 5'-ACCGTGAAAAGATGACCCAG-3'
	Reverse: 5'-GTACGACCAGAGGCATACAG-3'
Adipoq	Forward: 5'- CTGACGACACCAAAAGGGC-3'
	Reverse: 5'- TCCAACCTGCACAAGTTCCC-3'
Adgre1 mouse	Forward: 5'-ACAGTACGATGTGGGGCTTT-3'
	Reverse: 5'-GTGTGGTCATCCCCCATCTG-3'
Ap2	Forward: 5'-TCTCACCTGGAAGACAGCTCC-3'
	Reverse: 5'-GCTGATGATCATGTTGGGCTTGG-3'
Cdkn1a mouse	Forward: 5'- GCAGACCAGCCTGACAGATTT-3'
	Reverse: 5'- CTGACCCACAGCAGAAGAGG-3'
Cdkn1a rat	Forward: 5'-TCGTACCCCGATACAGGTGA-3'
	Reverse: 5'- TGTCTAGGAAGCCCTCCCG-3'
Cd68 mouse	Forward: 5'- GCTAGGACCGCTTATAGCCC-3'
	Reverse: 5'- GGATGGCAGGAGAGTAACGG-3'
Cyclophilin A mouse/rat	Forward: 5'-GCAGACAAAGTTCCAAAGACAG- 3'
	Reverse: 5'-CACCCTGGCACATGAATCC- 3'
Glyoxalase 1 mouse	Forward: 5'-CCCTCGTGGATTTGGTCACA-30
	Reverse: 50-AGCCGTCAGGGTCTTGAATG-30
Glut4	Forward: 5'-CAATGTCTTGGCCGTGTTGG-3'
	Reverse: 5'-GCCCTGATGTTAGCCCTGAG-3'
Fgfr1 mouse	Forward: 5'-CCTGAACAAGATGCACTCCCA-3'
	Reverse: 50-TCTGGGGATGTCCAGTAGGG-3'
Igf1r mouse	Forward: 5'-ATTCTGATGTCTGGTCCTTCG-3'
	Reverse: 50-AGCATATCAGGGCAGTTGTC-3'
Pparγ2	Forward: 5'-CAGTGGAGACCGCCCAGGCT-3'
	Reverse: 5'-TGGAGCAGGGGGTGAAGGCT-3'

Trp53 mouse	Forward: 5'-CCTCTCCCCCGCAAAAGAAA-3' Reverse: 5'-GACTCCTCTGTAGCATGGGC-3'
Trp53 rat	Forward: 5'- GTGATATGTACCAGCCACAGG-3' Reverse: 5'- CGAACAGACGACGGCATACT-3'

Glucose tolerance test (GTT)

Mice were fasting overnight. Blood glucose was collected from the tail vein at 0, 15, 30, 45, 60, 90, 120 min after the administration of a 20% w/vol D-glucose solution (2g/kg body weight) by an intraperitoneal injection. Blood glucose was assessed using a glucometer (One Touch® Ultra, Lifescan, Johnson & Johnson, Milpitas, Italy).

Insulin tolerance test (ITT)

Mice were fasted for 4 h before an intraperitoneal injection of insulin (0.75 U/kg body weight Humulin R, Eli Lilly Italia S.p.A., Italy). Whole blood samples were collected at the following time points: 0, 15, 30, 45, 60, 90, 120 minutes, after insulin injection, and serum insulin levels measured by an ELISA assay (EMD Millipore Corporation, Billerica, MA, USA).

Pancreatic islets isolation

Islets of Langerhans were isolated by collagenase digestion (Li et al, 2009). Mice were killed by cervical dislocation, and an incision around the upper abdomen was made to expose the liver and the intestine. Once located the ampulla, it was clamped on the duodenum wall and the pancreas was perfused by injecting 3 ml of Collagenase P (Roche Applied Sciences, Penzberg, Germany) solution (1,000 U ml^{-1}) in Krebs (NaCl 120 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgCl_2$, $6H_2O$ 1.2 mM, $NaHCO_3$ 24 mM, Hepes 5 mM, penicillin/streptomycin 50 U/ml, pH 7.35). Digestion was completed in a shaking water bath (37°C) for 8–10 min. The digested pancreas was treated with DNase I (QIAGEN, Hilden, Germany). The islets were hand-picked under a stereomicroscope using a syringe with a 25-gauge needle and then pelleted for RNA extraction, homogenization, or incubated overnight in RPMI for *ex vivo* glucose-stimulated insulin secretion (GSIS).

Glucose-stimulated insulin secretion (GSIS)

Overnight-fasted mice were injected with a 30% w/v D-glucose solution (3 g/kg body weight), and whole blood glucose was collected from mandibular vein at 0, 3, and 30 min after glucose injection. Blood samples were allowed to clot for 30 min and then centrifuged

at 4,500 rpm for 5 min at room temperature for serum collection. One mouse each group a time was in parallel analyzed to minimize potential confounders. Ex vivo GSIS was performed as previously described (Lombardi et al, 2012). Briefly, batches of 20 size matched islets were preincubated at 37°C for 30 min in glucose-free Krebs–Ringer bicarbonate HEPES buffer (KRBH) and then incubated for 1 h in a thermomixer at 37°C; incubation was with 1 ml KRBH medium containing 2.8 mmol/l glucose or 16.7 mmol/l glucose. Islets were then pelleted, supernatant fractions were collected to measure insulin secretion, and the genomic DNA was extracted from pellets to normalize insulin secretion. DNA extraction was performed using Wizard Genomic DNA purification kit (Promega, Milan, Italy), according to the manufacturer's protocol. In vitro GSIS was analyzed in INS-1 832/13 as previously described (Fiory et al, 2011). Briefly, at the end of treatments, cells were maintained for 1 h in KRBH with 0.25% (w/v) BSA and then incubated in KRBH with 2.8 mM (basal condition) or 16.7 mM glucose (stimulated condition). Incubation was stopped by putting the plates on ice, the supernatants were collected for insulin measurement, and cells solubilized for protein extraction as previously reported (Mirra et al, 2017) for insulin normalization. Insulin levels in serum, islets, or cell supernatants were measured using an ELISA assay (EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer's protocol.

Histological analysis

Sections of formalin-fixed, paraffin-embedded (FFPE) pancreas were cut into 4 µm sections, deparaffinized, rehydrated through graded alcohols and stained with hematoxylin and eosin for the evaluation of islets morphology. Seven mice each phenotype were analyzed, and 3 sections per mouse were evaluated to determine number, size, morphology, and infiltration of islets, by the use of Olympus DP21 software (Olympus, Center Valley, PA, USA). Immunohistochemical staining was carried out on whole sections from FFPE tissues to evaluate the expression of insulin, glucagon, and F4/80 markers. A manual procedure was perfomed for insulin and glucagon. Primary antibodies were Rabbit anti-glucagon (clone EP3070- 1:6,500) and Rabbit anti-insulin (clone EPR17359- 1:8,000) from Abcam (Cambridge, UK). Secondary reagent was Dako EnVision^{+} System-HRP Labelled Polymer Anti-Rabbit (Dako; Agilent Technologies, Santa Clara, CA, USA). Peroxidase reactivity was visualized using 3,3'-diaminobenzidine (DAB). F4/80 immunohistochemical staining was carried out using the BOND III slide stainer (Leica Biosystems, Wetzlar, Germany) in accordance with the manufacturer's instructions.

Staining was achieved using NBP2-12506 F4/80 antibody (Clone SP115, Novus Biologicals, Centennial, CO, USA) and visualized using the Bond™ Polymer Refine Detection kit (Leica Biosystems, Wetzlar, Germany) using 3,3'-diaminobenzidine tetrahydrochloride chromogen as substrate. Using a semi-quantitative scoring system microscopically, an observer (P.I.) performed a blind evaluation of the intensity, extent, and subcellular distribution of the markers by Olympus BX43 light microscope and Olympus DP21 microscope camera and software (Olympus, Center Valley, PA, USA). For each sample, ten fields and at least > 500 cells were analyzed. There are no standardized criteria for insulin, glucagon, and F4-80 markers staining evaluation.

For insulin and glucagon, the staining intensity was graded as follows: 0, negative; 1, weak; 2, moderate; and 3, intense. The intensity score was multiplied with percentage of positive cells in order to generate an immunoreactive score (IS). For insulin, high expression is defined as an IS ≥ 150, for glucagon ≥ 93, which represent the average value of the IS. F4/80 stain showed for all positive samples the same staining intensity. For this reason, we evaluated the percentage of immunostained cells and we scored it as follows: high when positivity was ≥ 1%.

Multiplex immunoassay

Whole pancreata or isolated islets from WT and Glo1KD mice were treated with Bio-Plex Cell Lysis kit (Bio-Rad, Hercules, CA, Stati Uniti) and homogenized by TissueLyser LT (QIAGEN, Hilden, Germany) accordingly to the manufacturer's instructions. At the end of treatments, INS-1 832/13 were cultured in serum-free RPMI medium with 0.25% (w/v) BSA for 16 h. Mouse pancreatic lysates and cell-conditioned medium were used to measure, respectively, the tissue and secreted concentration of interleukin-1 beta (IL-1b), tumor necrosis factor-alpha (TNF-a) and monocyte chemoattractant protein- 1 (MCP-1) by the use of a custom-blended ELISA multiplex assay (Bio-Plex ProTM, Bio-Rad) according to manufacturer's protocol. Islets lysates and serum samples were used to measure insulin and glucagon content by the use of an immunoassay in multiplex (Bio-Plex Pro mouse diabetes immunoassay, Bio-Rad) according to manufacturer's protocol. Data were acquired using a Bio-Plex 200 system equipped with Bio-Plex Manager software v5.0 (Bio-Rad). The standard curves optimization and the calculation of analyte concentrations were performed by using the Bio-Plex Manager software.

Characterization of mADSCs

a) *Immunophenotype*

The identification of the mADSCs population was evaluated by searching for cellular markers defined as clusters of differentiation (CD). A flow cytometric analysis was carried out in order to evaluate the expression of the surface antigens CD29, CD44, CD45 and CD31.

Once confluence was reached, the cells were collected and the pellet was washed with 1X PBS. After centrifuging at 300g for 10 minutes and removing the supernatant, the pellet was resuspended in 300 µl of cold buffer (PBS 1X pH 7.2, BSA 0.5%, EDTA 2 mM). Then, the cells were aliquoted, and a part was resuspended in a mix containing the CD29-APC and CD45-FITC antibodies in 100 µl of 1X PBS; the other part was resuspended in a mix containing CD44-PE and CD31-APC antibodies in 100µl of 1X PBS. The samples were placed for 10 minutes at 4 °C in the dark. Then, the cells were washed with 1 mL of 1X PBS and centrifuged at 300 g for 10 minutes at 4 °C. Subsequently, the samples were resuspended in 300 µl of PBS 1X, read with the BD LSRFortessa FACS (BD Biosciences, San Jose, CA, USA) and analyzed using the BD FACS Diva software.

b) *Adipogenic differentiation*

$2.5x10^5$ mADSCs were plated in MW6 and cultured in DMEM/F12 10% FBS until 80% confluence (day -2). After two days (day 0), they were induced to differentiate in DMEM/F12 (1: 1) 3% FBS with the addition of a differentiation mix consisting of 850 nM insulin, 0.5mM IBMX (3-isobutyl-1-methylxanthines), 10 µM dexamethasone and 10 µM rosiglitazone. After three days (day 3), the medium was replaced to adipocyte medium consisting of 850 nM Insulin, 1 µM dexamethasone and 1 µM rosiglitazone, changed every three days until complete differentiation (21 days).

c) *Oil Red-O staining*

After 21 days of differentiation protocol, the cells were stained with Oil Red-O. The latter is a dye capable of binding the neutral lipids contained in the fatty vesicles of mature adipocytes and highlighting them in red. After removing the culture medium, the adipocytes were washed twice with 1X PBS. Subsequently, cells were fixed with 4% formaldehyde and incubated at room temperature (RT) for 5 minutes. After two washes with 1X PBS, the plates were washed quickly with 60% isopropyl alcohol and then

incubated for 30 minutes at RT with Oil Red-O. Cells were then washed twice with 1X PBS and observed under a light microscope. Subsequently, samples were eluted in 60% Isopropanol and the color intensity was quantized using the BECKMAN model spectrophotometer (DU-730), evaluating the absorbance at the wavelength of 490 nm.

Western blot analysis

For whole cell protein extraction, cells were solubilized in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l Na2P2O7, 2 mmol/l Na3VO4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100) for 2 h at 4°C. Cell lysates were clarified by centrifugation at 16,000 g for 20 min. Nuclear proteins were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit purchased from Thermo Fisher (Waltham, Massachusetts, USA), according to the manufacturer's instructions. Cell lysates were then separated by SDS-PAGE and transferred into 0.45-lm Immobilon-P PVDF membranes (Merck, Darmstadt, Germany). Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by electrochemiluminescence according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL, USA) and densitometric analysis was performed using ImageJ software.

Detection of Senescence-associated β-galactosidase

SA-β-gal activity has been evaluated *in vitro* by a fluorescence-based assay using flow cytometry, as described in (Debacq-Chainiaux et al, 2009). Briefly, at the end of treatments, INS-1 832/13 cells were exposed to 100 nM bafilomycin A1 for 1h in fresh cell culture medium to induce lysosomal alkalinization. Subsequently, cells were incubated in 33 μM C12FDG (5-dodecanoylaminofluorescein di-b-D-galactopyranoside) for 2h and then washed twice with PBS, harvested by trypsinization, and resuspended in ice-cold PBS. Cell suspension was run in a BD FACS and the acquisition and analysis performed by BD FACSDiva Software (BD Bioscience). Once selected the analysis region which excludes dead cells and cellular debris, the percentage of positive cells were estimated by dividing the number of events within the bright fluorescence compartment (set at fluorescence intensity > 10^4) by the total number of events. The same procedure was also used to evaluate SA-β-gal activity in mADSCs.

Cell viability

mADSCs were seeded in equal numbers (10^4 cells/ well) in MW-96 and grown in the standard culture medium previously described. Subsequently, they were treated with MGO at increasing concentrations (25 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM) for 16 hours. The viability of the mADSCs was then assessed by using the "Cell Viability Assay" kit (Biotium, Fremont, CA, USA) according to the instructions provided by the manufacturer. The colorimetric assay consists in the cleavage of the tetrazolium salt MTT (bromide of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (of yellow color), to formazan (of blue/violet color), by the active mitochondrial succinate dehydrogenase enzyme only in living cells. This reaction is evaluated and measured by reading the sample with a spectrophotometer (Infinite 200, Tecan) at a wavelength of 595 nm.

Cell migration assay

The migration of hRECs was evaluated through a 2D co-cultures system using a MW24 "Transwell® Permeable Supports" inserts (BD Falcon, Franklin Lakes, NJ) containing 8 μm pores polycarbonate membrane. mADSCs were seeded in MW-24 and treated or not with 100 μM of MGO for 16h. Subsequently, $5x10^4$ hRECs were seeded on the upper side of 8.0 μm transwell inserts placed in MW24 where mADSCs had been cultured. As negative control, hRECs were seeded on the upper side of the insert in the absence of mADSCs.

For the hRECs migration assay with CM, mADSCs were seeded in MW24 and treated with 100 μM of MGO for 16h. Subsequently, Optimem was added on mADSCs for 24h to obtain the CM. After 24h, the CM from mADSCs treated or not with MGO was collected, centrifuged, diluted 1:2 and placed in MW24 for the hRECs migration assay. Subsequently, $5x10^4$ hRECs were seeded on the upper side of 8.0 μm transwell inserts placed in the MW24 where CM was previously placed. Basal media (BM) was used as negative control of hRECs migration. For both cell migration assays after a 24-hour incubation, the upper surface of the insert was cleaned to remove the excess of cells that had not crossed the porous membrane. The inserts were transferred to a clean MW-24 and both the upper and lower surfaces of the insert were washed 3 times with 1X PBS. Inserts were incubated 30 minutes at room temperature with 11% glutaraldehyde to fix cells migrated on the bottom of inserts, and then washed 3 times with 1X PBS. Cells were then stained *with Crystal Violet* for 15 minutes. The excess of dye was removed by washing the inserts in distilled

H_2O. The inserts were air dried and then the photos were acquired under an optical microscope with a 4x and 10X magnification and counted by imageJ.

Statistic procedures

Data are expressed as means ± SD and means ± SEM, as indicated in figure legends. Data distribution was tested for normality by Shapiro-Wilk test. Comparison between groups were performed using Student's *t*-test or Mann–Whitney U test for comparison of non-parametric data. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (version 7.0; San Diego, CA, USA) or R studio software (version 3.5.3; R studio, Boston, MA, USA). Outlier values were calculated by performing Grubb's test and excluded from the analysis.

4. RESULTS AND DISCUSSION

SECTION 1

4.1.1 Characterization of Glo1KD mice

In order to evaluate the effect of MGO accumulation on glucose homeostasis *in vivo* we used a mouse knock-down for Glo1 gene (Glo1KD) mice and their wild type littermates (WT mice). Compared to the models already described in the literature, the advantage of using the Glo1KD mice is the opportunity to evaluate whether and which alterations result from the MGO increase by itself, excluding the presence of confounding factors deriving from hyperglycemia and obesity. To validate this experimental model, at first, we tested the Glo1 expression in different tissues by Real Time-PCR. The results show that, Glo1KD mice have a reduced Glo1 tissue expression compared to WT mice. In detail, mRNA levels of Glo1KD mice are reduced by 90%, 40%, and 62% in skeletal muscle (Figure 13A), white adipose tissue (WAT) (Figure 13B), and liver (Figure 13C), respectively, compared with WT mice at 10 months of age.

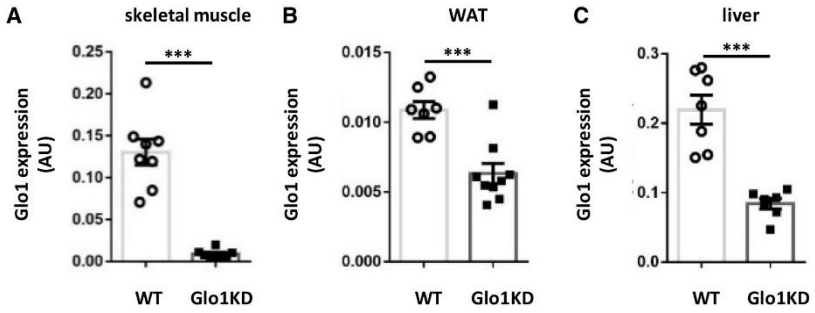


Figure 13. mRNA tissue expression of Glo1. Data distribution of the absolute quantification of Glo1 gene expression measured by RT-PCR in skeletal muscle **(A)**, WAT **(B)**, and liver **(C)** of 10-month-old Glo1KD and WT mice. *Cyclophilin A* was used as a housekeeping gene. Gene expression is shown as the ratio of Glo1 to *Cyclophilin A* copy number (arbitrary units, AU). Bars in the scatter plots show the mean ± SEM. Statistical analysis was performed by Mann–Whitney test in A and by Student t-test in B and C (***P ≤ 0.05).

Then, to test if the partial deletion of Glo1 expression was able to induce MGO accumulation in this model, endogenous MGO levels were measured by HPLC. The results show a 50% increase of MGO serum levels in Glo1KD mice compared to WT mice at 10 months of age, but not earlier at 5 months of age (Table 1). These data suggest that when

Glo1 is decreased, it fails to keep down MGO levels with age. We also monitored the metabolic characteristics of Glo1KD mice during lifespan. As shown in Table 1, we did not find significant variation in body weight, food intake, fed and fasting glycemia, serum insulin, and glucagon levels in age-matched WT and Glo1KD mice, neither at 5 nor at 10 months of age, indicating that Glo1KD mice are normoglycemic and, thus, do not display a diabetic phenotype.

Table 1. Metabolic parameters in WT and Glo1KD mice.

Variable	WT mice	Glo1KD mice	WT mice	Glo1KD mice
	5 months		10 months	
Body weight (g)	25.4±0.8	24.6±0.8	32.6±1.5 ###	35.9±2.0 ###
Food intake (g/day)	2.88±0.06	3±0.12	3±0.12	2.8±0.24
Fasting glycaemia (mg/dl)	105.9±5.3	98.1±3.5	114.6±8.4	110.4±7.4
Fed glycaemia (mg/dl)	129.7±6.4	134.8±6.4	149.3±8.5	153.1±6.1
Serum Insulin (ng/ml)	0.75±0.14	0.88±0.14	0.68±0.12	0.89±0.05
Serum Glucagon (pg/ml)	285.4±20	275.1±42.7	285.4±35.8	290.1±32.2
Plasma MGO (nM)	197.6±6.4	185.4±13.7	182.6±15.6	267.2±5.7 ***,###

Body weight, food intake, fasting and fed glycemia, serum insulin, glucagon, and plasma MGO were measured in Glo1KD mice and their WT littermates at 5 and 10 months of age. Data are reported as mean ± SEM values. Statistical analysis was performed by Student's t-test for data with a normal distribution, or with Mann–Whitney test for data with a not normal distribution (***P < 0.001, age-matched Glo1KD vs. WT mice; ###P < 0.001, 10 months vs. 5 months).

4.1.2 Glo1KD mice develop an impairment of glucose tolerance with aging

Subsequently, we measured glucose levels during an intraperitoneal Glucose Tolerance Tests (ipGTT) in WT and Glo1KD mice at 5 and 10 months of age. As shown in figure 14, glucose tolerance is impaired with age, as demonstrated by higher glycemic levels observed in Glo1KD mice at 10 months (Fig 14B) but not at 5 months of age, compared to WT mice (Fig 14A). Differently, we tested insulin sensitivity with an insulin tolerance test (ITT), and it appears not to be significantly compromised in Glo1KD mice, compared to WT mice, either at 5 (Fig 14C) or at 10 months of age (Fig 14D). This is shown by both the glycemic trend (line graphs) and the inverse area under the curve (iAUC) calculation (bar graphs). These results indicate that the impairment of glucose tolerance developed by Glo1KD mice with age was not associated with peripheral insulin resistance.

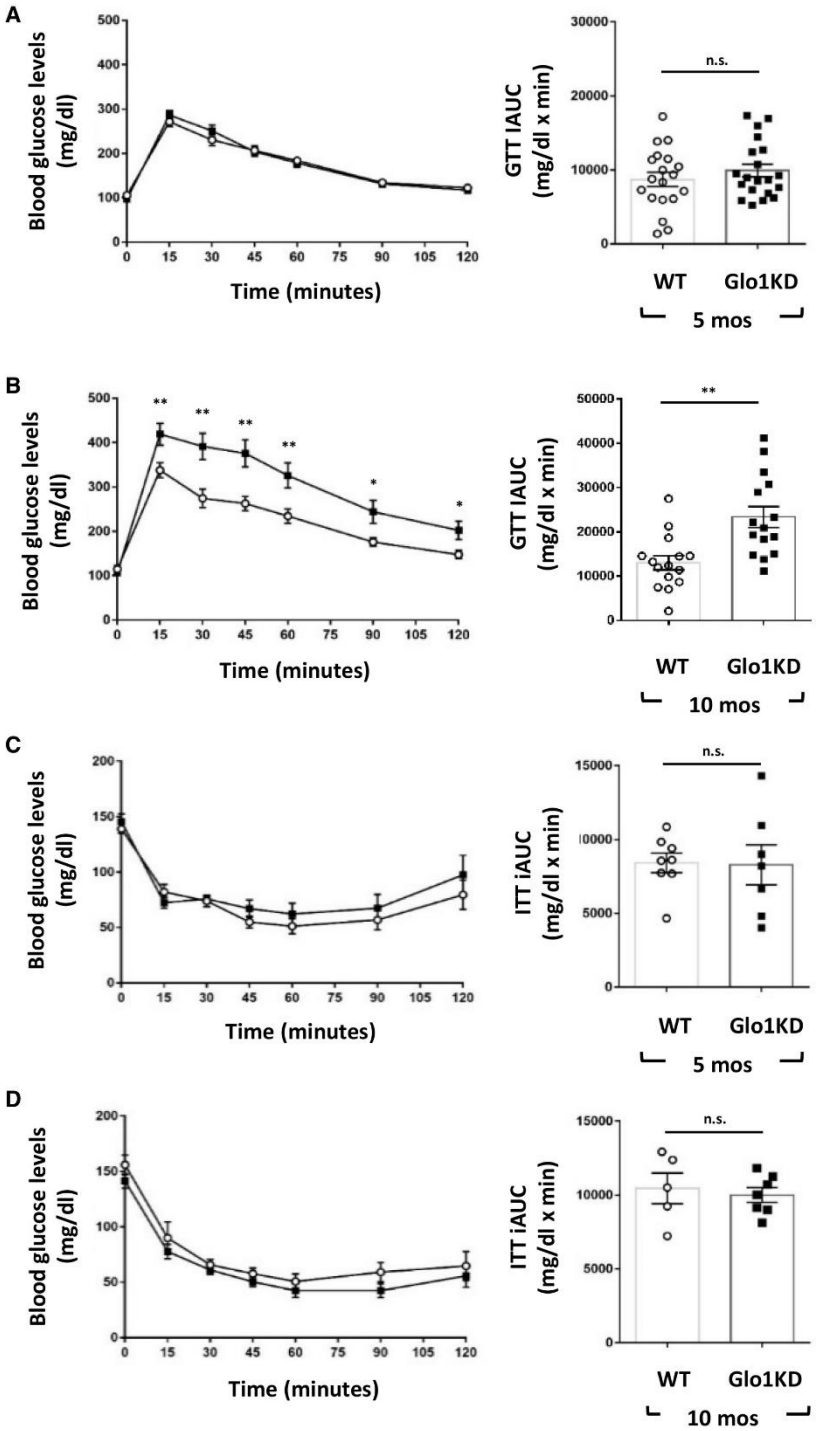


Figure 14. Evaluation of glucose tolerance and insulin sensitivity. Glucose tolerance **(A, B)** was evaluated in 5- and 10-month-old mice by ipGTT. Line graphs show the trend of blood glucose levels of WT mice and Glo1KD mice within 120 minutes after intraperitoneal administration of a glucose bolus (2 g/kg) at 5 and 10 months. Bars graphs show the GTT IAUC of Glo1KD and WT mice. Insulin sensitivity **(C, D)** was evaluated in 5- and 10-month-old mice by ITT. Line graphs show the trend of blood glucose levels within 120 minutes after intraperitoneal administration of an insulin bolus (0.75 U/kg). Blood glucose was measured using a portable blood glucometer at the indicated time points (X axes). Bar graphs show the ITT iAUC of Glo1KD and WT mice. Data are presented as mean ± SEM. Statistical analysis was performed by Student's t-test (*P ≤ 0.05, **P ≤ 0.01 for scatter plots in B) and by Mann–Whitney test (**P ≤ 0.01 for line graph in B).

Then, we measured insulin secretion in the fasted state and after 3- and 30-minutes following intraperitoneal glucose administration (3 g/kg). As shown in figure 3, insulin secretion was similar in Glo1KD mice and WT mice at 5 months of age (Fig 15A), while it was impaired in Glo1KD mice at 10 months (Fig 15B). In detail, WT mice at 10 months of age showed a 2.7-fold increase in insulinemia in the early phase of secretion, which was significantly reduced to 1.3-fold increase in Glo1KD mice. The age-dependent impairment of glucose-stimulated insulin secretion in Glo1KD mice was also evident by analyzing the incremental area under the curve (IAUC, bar graphs in Fig 15). Although ipGTT and GSIS are impaired in 10-month-old Glo1KD mice, but not in 5-month-old mice, neither fast nor fed blood glucose levels are significantly different in Glo1KD compared with WT mice. Therefore, we speculate that Glo1KD mice recapitulates with age a pre-diabetes-like phenotype. Indeed, they do not feature overt diabetes, as shown by normal levels of fed and fast glycemia. This phenotype suggests a potential role for senescence-related mechanisms triggered by MGO.

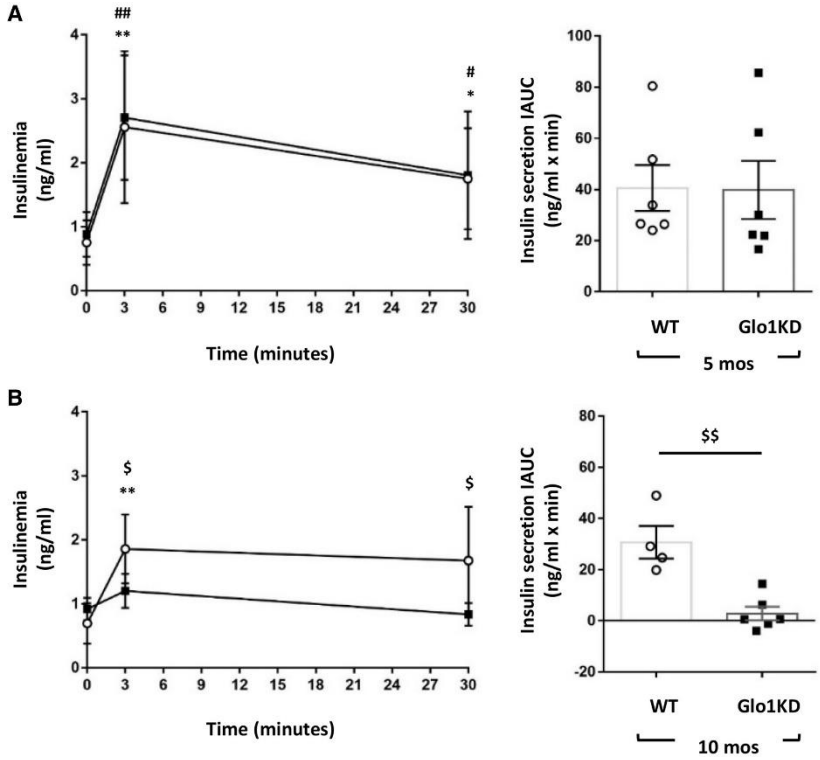


Figure 15. Evaluation of glucose-stimulated insulin secretion (GSIS). Line graphs **(A, B)** show the serum insulin levels at 0, 3, and 30 min after the administration of an intraperitoneal glucose bolus (3 g/kg) in Glo1KD and WT mice at 5 months and 10 months of age. Bar graphs represent the IAUC monitored during insulin secretion test in Glo1KD and WT mice. Data are presented as mean ± SEM. Statistical analysis was performed by Student's t-test (*P ≤ 0.05, **P ≤ 0.01: 3 and 30 min vs. time 0 of WT mice; #P ≤ 0.05, ##P ≤ 0.01: 3 and 30 min vs. time 0 of Glo1KD mice; $P ≤ 0.05: Glo1KD vs. WT mice).

4.1.2 Pancreas from Glo1KD mice reveals macrophage infiltration and impaired GSIS of isolated islets

Once demonstrated the impaired insulin secretion in 10-month-old mice, we and our collaborators performed hematoxylin and eosin staining and immunohistochemistry analysis of pancreatic sections to measure both hormones storage and islets morphology. Data in figure 16 show no differences in the number (Figure 16D), the size (Figure 16E), the diameter (Figure 16F and G), the insulin (Figure 16H) and glucagon staining (Figure 16 I) of the islet from 10-month-old WT and Glo1KD mice. Similarly, neither insulin nor glucagon content, measured by an ELISA assay, is different between the two genotypes (Figure 16 K and L). The immune system plays an important role in the regulation of metabolism (Osborn et al., 2012). Among immune cells, macrophages have been detected in the islets of patients with T2DM and in animal models in association with increased levels of cytokines and chemokines (Ehses et al., 2007; Donath et al., 2013). The contribution of other immune cells besides macrophages to islet cell infiltration in T2DM is less clear (Marchetti, 2016). Therefore, we tested the insular inflammatory infiltration, which is visible in pancreatic sections of Glo1KD mice, as shown in panel 16B, with an incidence mean value of 6% at different severity degree, but not in sections of WT mice (Figures 16B and C). More in detail, the insular macrophage marker F4/80 was present at a median value of 5% in pancreatic sections from Glo1KD mice, whereas no F4/80-positive cells we found in sections from WT mice (Figure 16 J and A, right panel). Tissue levels of MGO were increased 2.5-fold in the pancreas of 10-month-old Glo1KD mice compared with WT mice (Fig. 16 M).

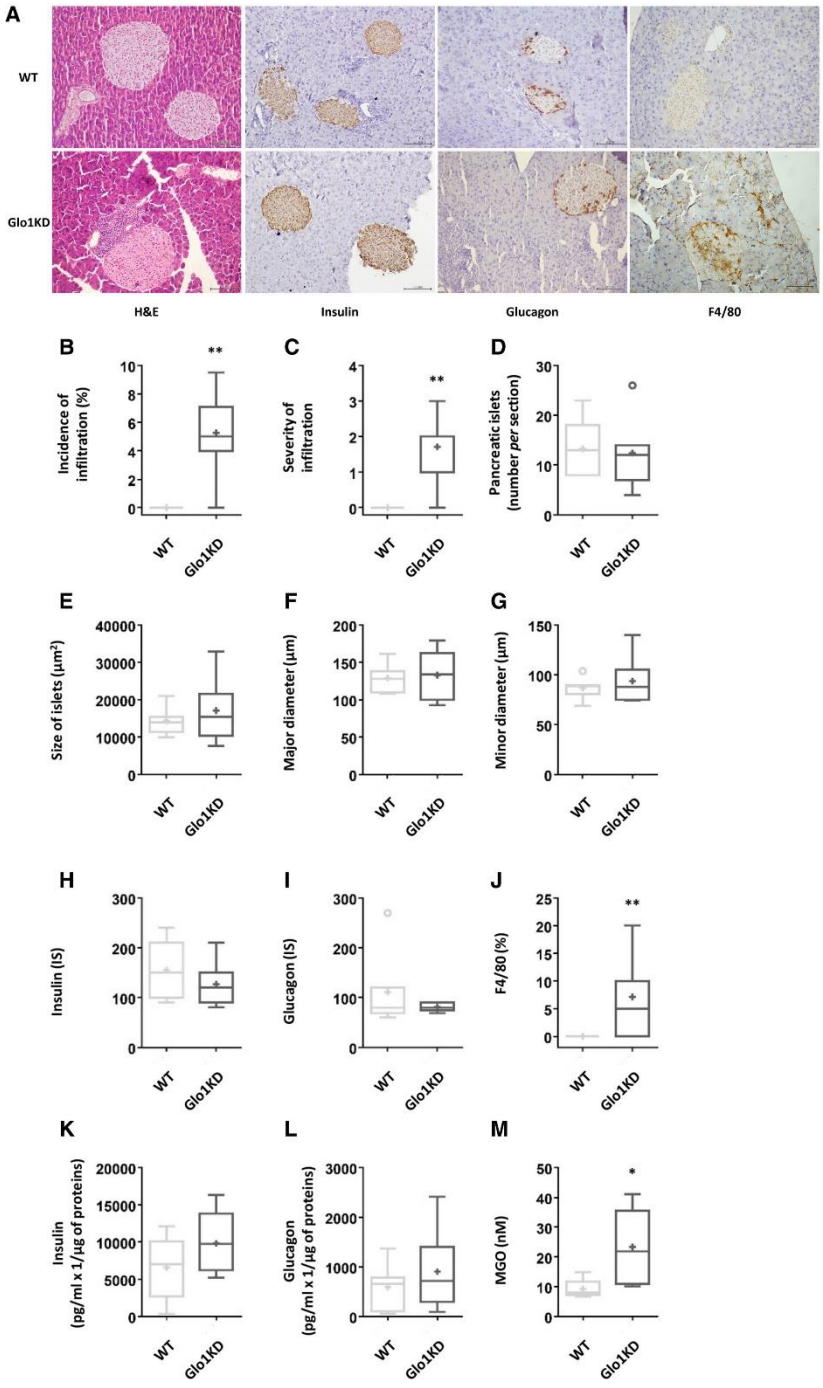


Figure 16. Immunohistochemistry evaluations and hormones measurement in pancreatic islets of 10-month-old WT and Glo1KD mice. Representative pancreatic sections (20× magnification) from 10-month-old WT and Glo1KD mice **(A)** were stained with hematoxylin and eosin, anti-insulin, anti-glucagon, and anti-F4/80 antibodies. Insular inflammatory infiltration was indicated as incidence (% of infiltrated islets) **(B)** and severity score **(C)**. Severity of infiltration has been graded as follows: absent (0), low (1), moderate (2), and high (3). Morphometric analysis of islets was indicated as the number of islets per sections **(D)** and the size of islets, calculated as the area **(E)**, the major diameter **(F)**, and the minor diameter **(G)** of the islets per mouse. Insulin and glucagon IS, was calculated as the intensity score multiplied with percentage of positive cells **(H, I)** and the percentage of islets area stained with F4/80 **(J)**. Quantification of insulin **(K)** and glucagon **(L)** content in islet extracts by immunoenzymatic assay. MGO content **(M)** in pancreatic lysates was measured by LC-MS/MS. In (B–M), box plots show the minimum and maximum values

(ends of the whiskers), interquartile range (length of the box), median (line through the box), and mean (+) values. Statistical analysis was performed by Mann–Whitney test (*P ≤ 0.05, **P ≤ 0.01).

Besides the histological staining, we also tested mRNA levels of F4/80 and CD68, used as macrophage markers. The data obtained show that pancreatic expression levels of both F4/80 encoding genes, adhesion G protein-coupled receptor E1 (Adgre1), and CD68 do not differ between the two genotypes at 5 months of age (Figure 17A and B), while pancreatic Glo1expression was reduced by about 50% in Glo1KD mice compared to WT mice (Fig.17C), as expected. Glo1KD mice at 10 months of age show an increased expression of both pancreatic Adgre1 and Cd68 compared to age-matched WT mice, consistently with immunohistochemistry analysis (Figure 17A and B). The decreased Glo1 expression found in Glo1KD mice at 5 months of age is maintained at 10 months of age in these mice compared with age-matched WT mice (Fig.17C).

We also confirmed the increased Adgre1 and Cd68 expression in the isolated islets from 10-month-old WT and Glo1KD mice, while no difference we revealed in the islets from 5-month-old mice (Fig 17D and E). These data indicate that, similarly to the impairment of insulin secretion, this inflammatory phenotype is fostered by Glo1 knockdown during aging. Cytokine and chemokine levels were previously identified as molecular signature of islet inflammation in T2D (Marchetti, 2016). In addition to the cellular components, we also tested pancreatic inflammation by the local presence of inflammatory soluble factors (cytokine and chemokine), known to be involved in the cross talk between β-cells and macrophages and to be associated with β-cell dysfunction (Donath et al., 2013; Butcher et al, 2014). TNF-α and IL-1β are known to be primary cytokines regulating the function of several other cytokines and chemokines, including MCP-1, thereby contributing to the recruitment of immune cells. Furthermore, both β-cells and macrophages are potential sources of pro-inflammatory cytokines within the islets of patients with T2D (Maedler et al., 2002; Morris, 2015). It is known that IL-1β is produced by β-cells exposed to high glucose, contributing to islet glucotoxicity and impaired β-cell function (Maedler et al, 2002; Mahdi et al., 2012). Furthermore, IL-1β sustains the autocrine and paracrine activation of both β-cells and macrophages, exacerbating the chronic inflammatory responses in the islets (Donath et al., 2013). Butcher et al. demonstrated that TNF-α and MCP-1 expression is higher in leucocyte-infiltrated islets of T2D subjects and negatively correlates with first-phase glucose-stimulated insulin secretion of the isolated islets (Butcher et al., 2014). In order to test cytokine levels in pancreatic tissue, we performed

multiplex immunoassays which have revealed a 40% increase in both MCP-1 and TNF-α concentration in the pancreatic lysate of 10-month-old Glo1KD mice, compared with WT mice. IL-1 β pancreatic levels were not found to be significantly higher in Glo1KD mice than WT mice (Table 2).

Moreover, we observed a 2.5-fold increase in insulin secretion in response to glucose stimulation in the islets from WT mice, which was decreased to 1.2-fold increase in the islets from 10-month-old Glo1KD mice (Fig 17F and G). Therefore, we observed a pro-inflammatory microenvironment which associates with an impaired GSIS *ex vivo* of the isolated islets. These results confirmed an association between the local increase in these cytokines and the impaired insulin secretion ability by β-cells in response to glucose.

In accordance with the study by Butcher et al. in the islets from T2D patients (Butcher et al, 2014), we provide evidence that the accumulation of MGO is sufficient to increase TNF-α and MCP-1, suggesting a causal role of the pro-inflammatory microenvironment in the β-cell dysfunction.

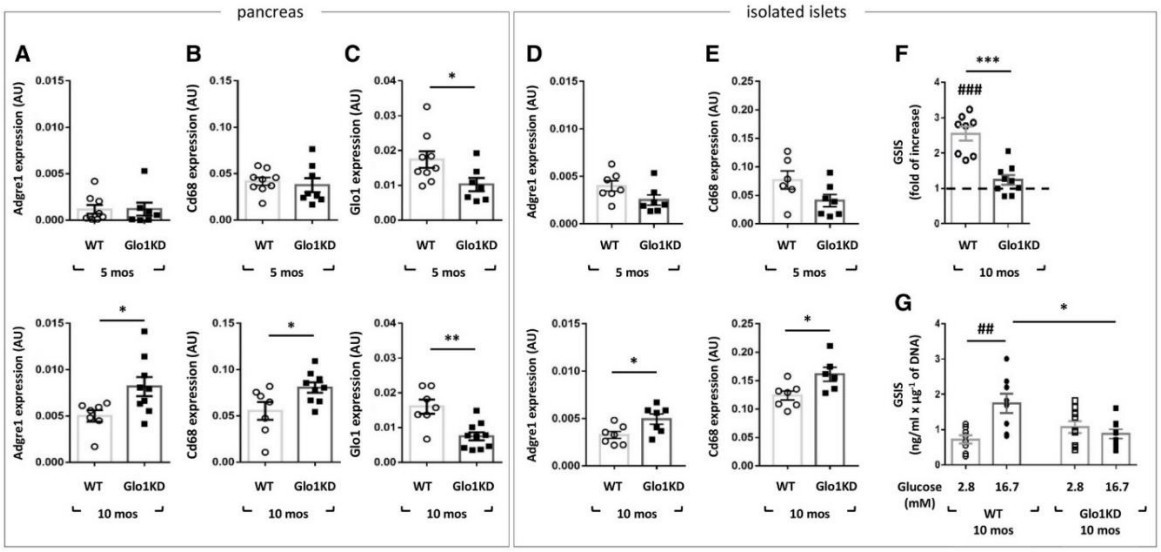


Figure 17. Macrophage markers gene expression in pancreatic tissue, isolated islets, and *ex vivo* GSIS. Data distribution of the absolute quantification of Adgre1 **(A)**, Cd68 **(B)**, and Glo1 **(C)** gene expression were measured by qPCR in pancreatic tissue from WT and Glo1KD mice at 5 and 10 months of age (lower panels). Data distribution of the absolute quantification of Adgre1 **(D)** and Cd68 **(E)** gene expression measured by qPCR in the isolated islets from WT and Glo1KD mice at 5 and 10 months of age. *Cyclophilin A* was used as housekeeping gene. Gene expression is shown as the ratio of Adgre1, Cd68, or Glo1 to *Cyclophilin A* copy number (AU). *Ex vivo* GSIS showed as fold of increase **(F)** and as insulin concentration released into the secretion media **(G)** in response to the basal condition at 2.8 mM glucose and to the stimulated condition at 16.7 mM glucose of pancreatic islets from WT and Glo1KD mice at 10 months of age. Insulin concentration was normalized on DNA islets content. Bars in the scatter plots show the mean ± SEM. Statistical analysis was performed by Mann–Whitney test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001: WT vs. Glo1KD mice; ##P ≤ 0.01, ###P ≤ 0.001: 16.7 mM glucose vs. 2.8 mM glucose).

Table 2. Cytokine levels in pancreatic tissue.

	WT mice	Glo1KD mice
IL-1β (pg/ml x 1/mg of proteins)	80.39 ± 3.68	90.47 ± 6.08
MCP-1 (pg/ml x 1/mg of proteins)	845.48 ± 55.64	1212.01 ± 137.88*
TNF-α (pg/ml x 1/mg of proteins)	678.22 ± 46.54	955.57 ± 94.84*

Mean ± SEM values of IL-1b, MCP-1, and TNF-a in pancreatic lysate from 10-month-old WT and Glo1KD mice are reported in table. Statistical analysis was performed by Student's t-test for data with a normal distribution, or with Mann–Whitney test for data with a not normal distribution (*P < 0.05, 10-month-old Glo1KD vs. WT mice).

4.1.4 Glo1KD isolated islets express senescence markers

The phenotype observed in Glo1KD mice recalls typical traits described by Aguayo-Mazzucato et al. in senescent islets characterized by the differential expression of senescence markers and induced by aging and metabolic stress (Aguayo-Mazzucato et al., 2017, 2019). These findings were followed by the description of a SASP actively produced by senescent β-cells, which plays not only an autocrine but also a paracrine effect on surrounding cells. It is worth noting that the cytokines analyzed in our models: TNF-α, MCP-1, and IL-1β are part of the SASP secreted by senescent β-cells (Aguayo-Mazzucato et al., 2019). We hypothesized that MGO accumulation induced by the knockdown of Glo1 acts as the metabolic hit leading to the senescence of pancreatic islets, which foster the onset of a pro-inflammatory condition, increasing macrophage infiltration and thereby resulting in impaired GSIS. Indeed, aged islets have been described to have impaired GSIS, but still maintain their insulin-producing capability and β-cell number. To prove this hypothesis, we tested the expression of the senescence markers transformation-related protein 53 (Trp53) and cyclin dependent kinase inhibitor 1A (Cdkn1a) (Rufini et al., 2013), besides two novel markers of age-related β-cell functional decline, insulin-like growth factor 1 receptor (Igf1r) and fibroblast growth factor receptor 1 (Fgfr1) (Aguayo-Mazzucato et al., 2017). In detail, it has been described that the β-cell expression of Igf1r associates with senescence and dysfunctional insulin secretion (Aguayo-Mazzucato et al., 2017). In mesenchymal stem cells, the FGFR1 activation prevents cellular senescence acting upstream MDM2 and its mediated degradation of p53 (Coutu et al., 2011). Our data demonstrate that mRNA levels of Trp53, Cdkn1a, and Igf1r does not show significantly difference in pancreatic islets isolated from both WT and Glo1KD mice at 5-month-old (Fig 18A, C and E). Conversely, Trp53, Cdkn1a, and Igf1r expression was increased by 48%, 88%, and 39%, respectively, in the pancreatic islets isolated from 10-month-old

Glo1KD mice, compared with WT islets (Fig 18B, D and F). Fgfr1expression was reduced by 37% in the islets from both 5- and 10-month-old Glo1KD mice, compared with the islets from age-matched WT mice (Fig 18G and H). As the reduced expression of Fgfr1 has been associated with β-cell age but not with impaired insulin secretion, it is conceivable that Fgfr1 modulation may precede the functional alteration found later at 10 months of age.

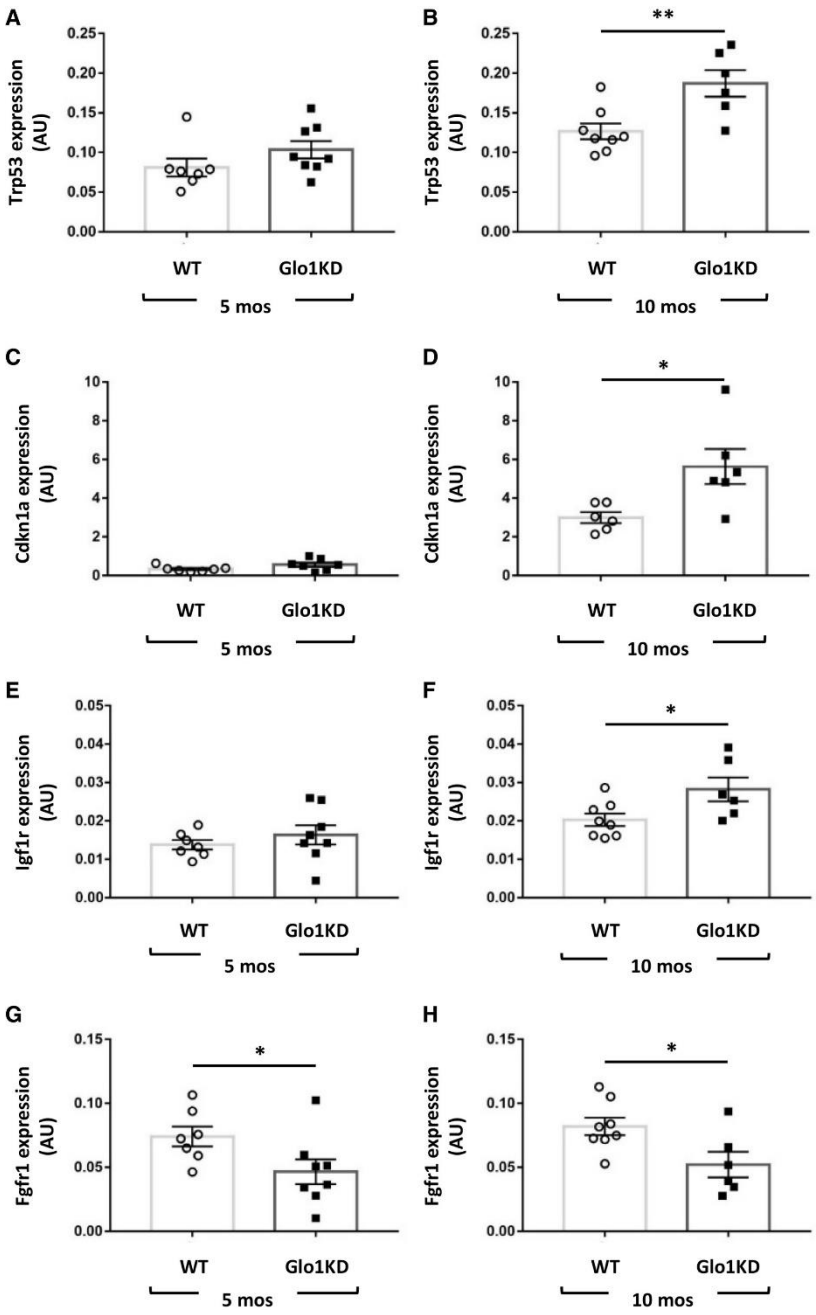


Figure 18. Trp53, Cdkn1a, Igf1r, and Fgfr1 gene expression in isolated islets. Trp53 **(A, B)**, Cdkn1a **(C, D)**, Igf1r **(E, F)**, and Fgfr1 **(G, H)** gene expression measured by qPCR in pancreatic islets of WT and Glo1KD mice at 5 months and 10 months of age. β-Actin was used as housekeeping gene. Gene expression is shown as the ratio of Trp53, Cdkn1a, Igf1r, or Fgfr1 to β-

Actin copy number (AU). Bars in the scatter plots show the mean ± SEM. Statistical analysis was performed by Student's t-test (*P ≤ 0.05, **P ≤ 0.01).

4.1.5 MGO impairs GSIS in vitro in a β-cell line through a senescence-mediated mechanism

To confirm that the β-cell dysfunction found in the Glo1KD model was an effect mediated by the MGO increase and a consequent induction of senescence, we exposed INS-1 832/13 cells to MGO *in vitro* and analyzed some functional and senescence markers. Data obtained by treated INS-1 832/13 cells with MGO for 48 hours show an increase of both the mRNA (Fig 19A and B) and protein levels (Fig 19C) of two canonical inducers of cellular senescence: p53 and p21. In detail, we obtained an increase of 35% of Trp53 expression which resulted in a 2-fold increase of p53 protein levels, and an increase of 30% of Cdkn1a expression which resulted in a 2.5-fold increase of p21 protein levels in INS-1 832/13 cells exposed to MGO, compared to not-treated cells. We treated INS-1 832/13 cells, previously exposed to MGO, with dasatinib (31 nM) and quercetin (6 μM), used in combination as senolytic agents, for further 24 hours (Aguayo- Mazzucato et al., 2019). The senolytic treatment reduced both the mRNA and protein expression of p53 and p21 at levels comparable to controls (Fig 19A–C). Several cells entering senescence lose nuclear high mobility group box 1 (HMGB1), depending on p53 activity (Davalos et al, 2013). INS-1 832/13 cells exposed to MGO show the nuclear exclusion of HMGB1, while the nuclear protein levels were rescued by the senolytics treatment (Fig 19D). The β-Gal activity was tested as further marker of senescence in our experimental model. Under MGO treatment, we observed that the percentage of β-Gal$^+$ cells is increased from 1.9% to 5.4% and it is reverted by senolytics (Fig 19E). Although the increase in β-Gal$^+$ INS-1 cells may look to be modest in response to MGO, previous evidence from our group and others demonstrated how a similar increase (threefold or less) in the percentage of senescent cells is sufficient to impair cell function and interfere with tissue homeostasis (Chen et al., 2002; Xu et al., 2018; Spinelli et al., 2022). Moreover, treated INS-1 832/13 cells show an increased secretion of the senescence-associated secretory profile (SASP) cytokines: IL-1β, MCP-1, and TNF-α, by 40%, 35%, and 30%, respectively (Fig 19F). This increase is blunted in MGO-exposed cells by dasatinib and quercetin combined treatment (Fig 19F). Therefore, the experiments performed *in vitro* in INS-1 832/13 cells confirm that MGO is able to induce the β-cell secretion of all the three cytokines analyzed, TNF-α, MCP-1, and IL-1β.

In light of these *in vitro* data, it is conceivable that in the analysis performed *ex vivo* from the whole pancreas of mice, the IL-1β increase could be hidden by the contribution of other tissue components. The observed effects associate with the modulation of GSIS. Indeed, while not-treated INS-1 832/13 cells showed a 7-fold increase in insulin secretion in response to glucose, MGO-treated cells showed a 4-fold increase, which was significantly lower than the insulin secretion observed in not-treated control cells (Fig 19G). Interestingly, treatment with dasatinib and quercetin rescue the GSIS in INS-1 832/13 cells exposed to MGO (Fig 19G). These data provide the proof of concept that the β-cell dysfunction induced by MGO is mediated by a senescence-associated phenotype.

Our results demonstrate for the first time that the MGO harmful effect on insulin secretion is mediated, at least in part, by cellular senescence.

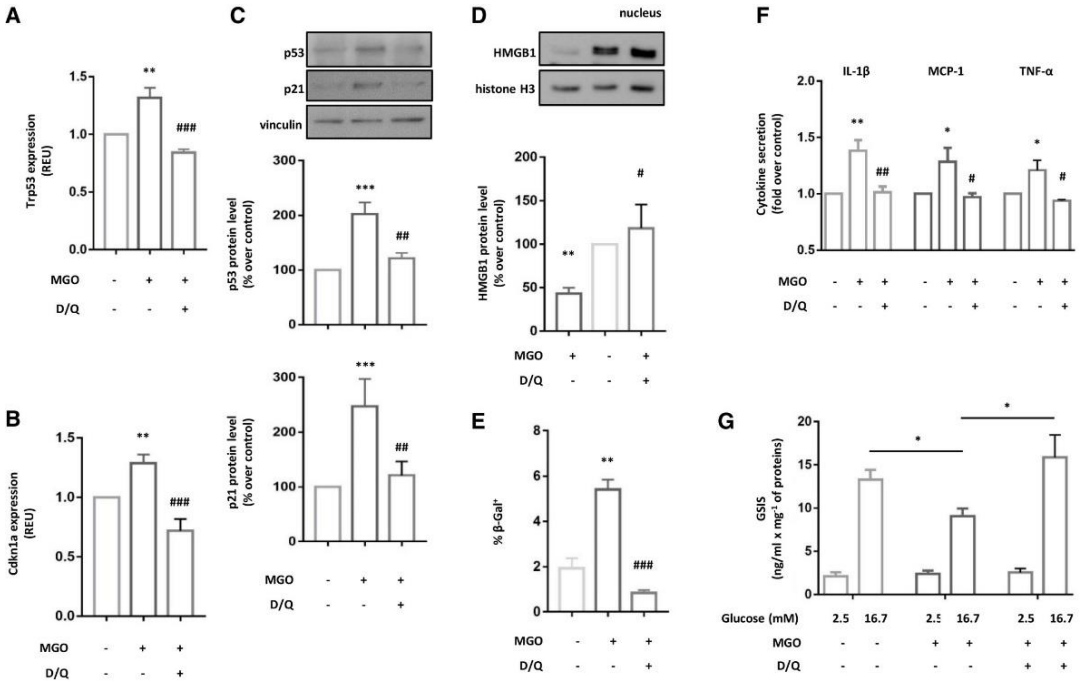
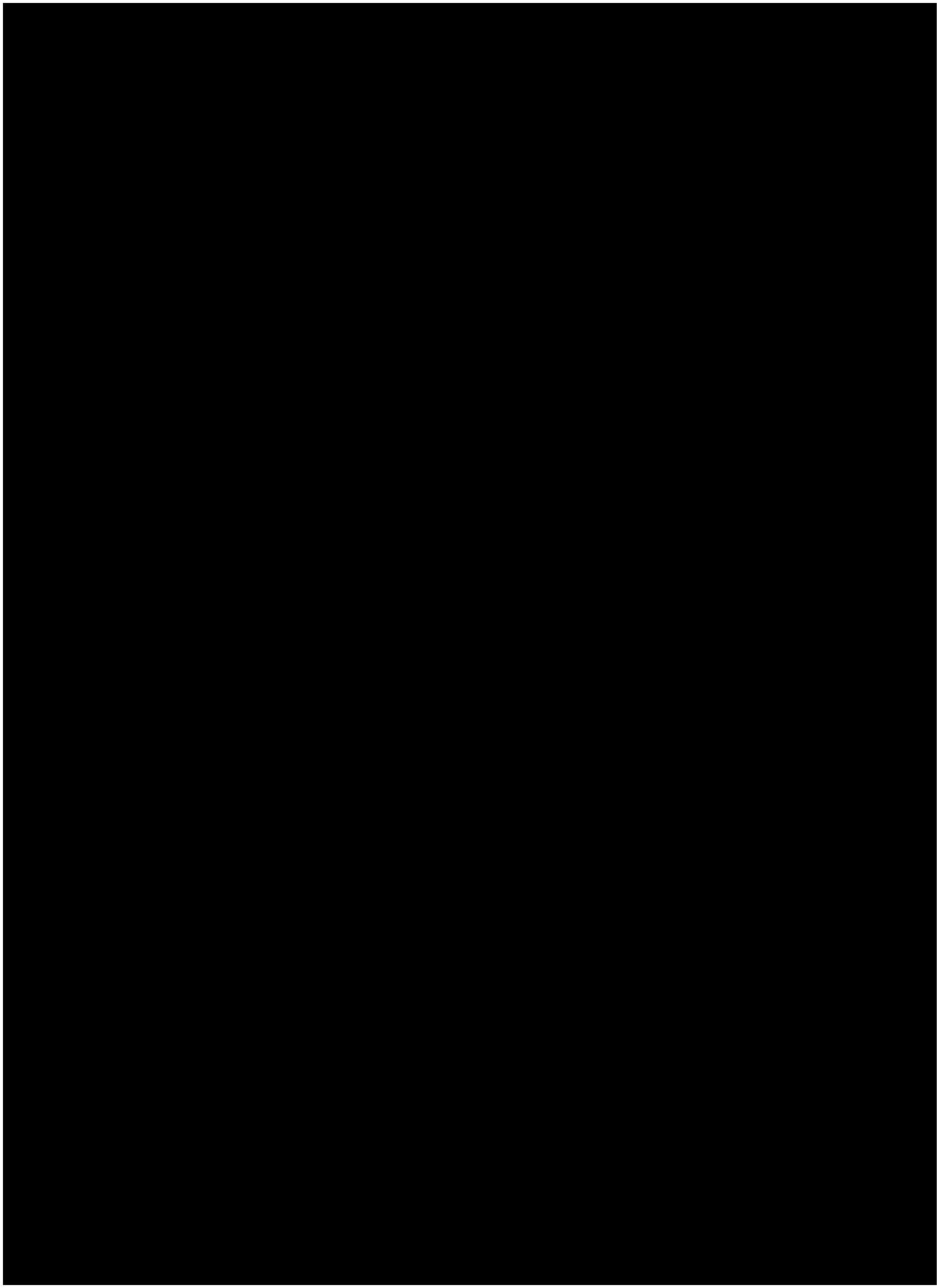
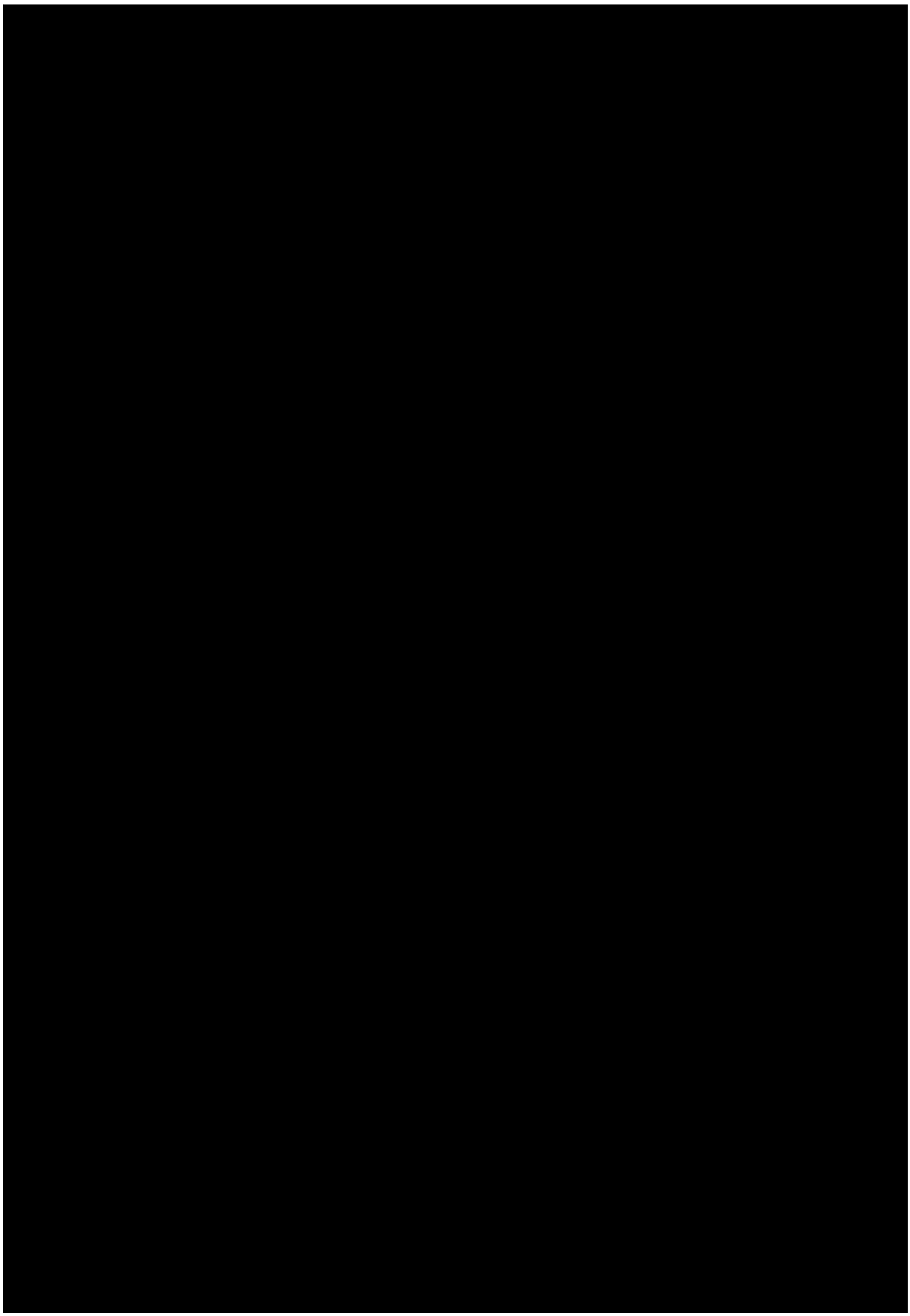


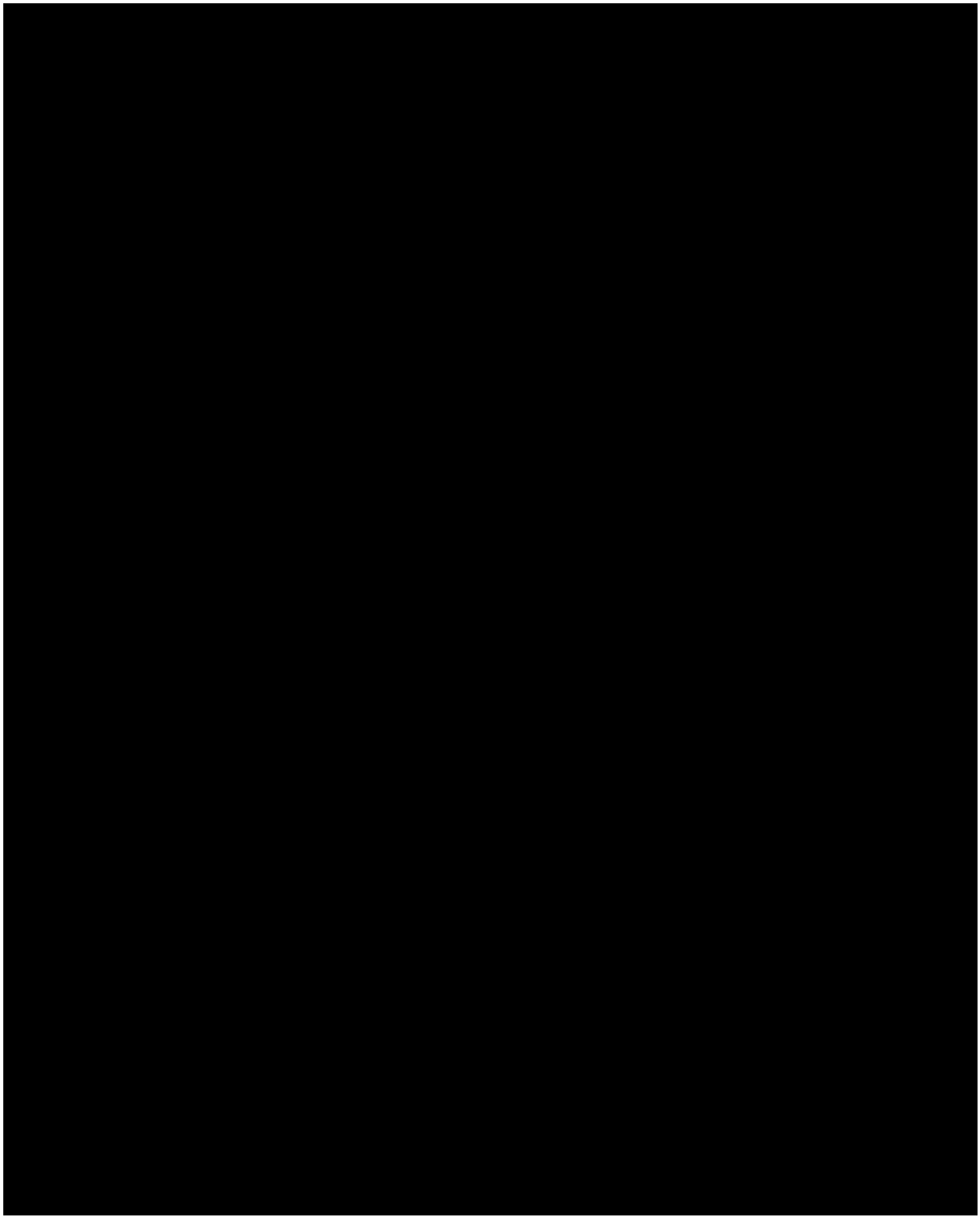
Figure 19. Senolytics effect on MGO-treated INS-1 832/13 β-cell line. mRNA levels of Trp53 and Cdkn1a **(A, B)** in INS-1 832/13 cells treated with MGO 0.05 mM for 48 h or with MGO plus 24 h of dasatinib 31 nM and quercetin 6 μM (D/Q). Gene expression is measured by qPCR as relative quantification to *Cyclophilin A*, used as housekeeping gene. Whole cell protein lysates of INS-1 832/13 β-cells **(C)** were analyzed by Western Blot with anti-p53 and anti-p21 antibodies; anti-vinculin antibody was used as loading control. Nuclear protein lysates **(D)** of INS-1 832/13 β-cells analyzed by Western Blot with anti-HMGB1 antibody; anti-histone H3 antibody was used as loading control. Proportion of β-Gal$^+$ β-cells **(E)** analyzed by a fluorescence-based assay using flow cytometry. Cytokine levels **(F)** were measured in the β-cell culture medium by a multiplex immunoassay and are shown as fold increase over not-treated control cells. GSIS **(G)** measured in the β-cell culture medium by an ELISA assay. Insulin concentration is reported at basal condition (2.5 mM glucose) and in response to glucose stimulation (16.7 mM). Data in bar graphs are

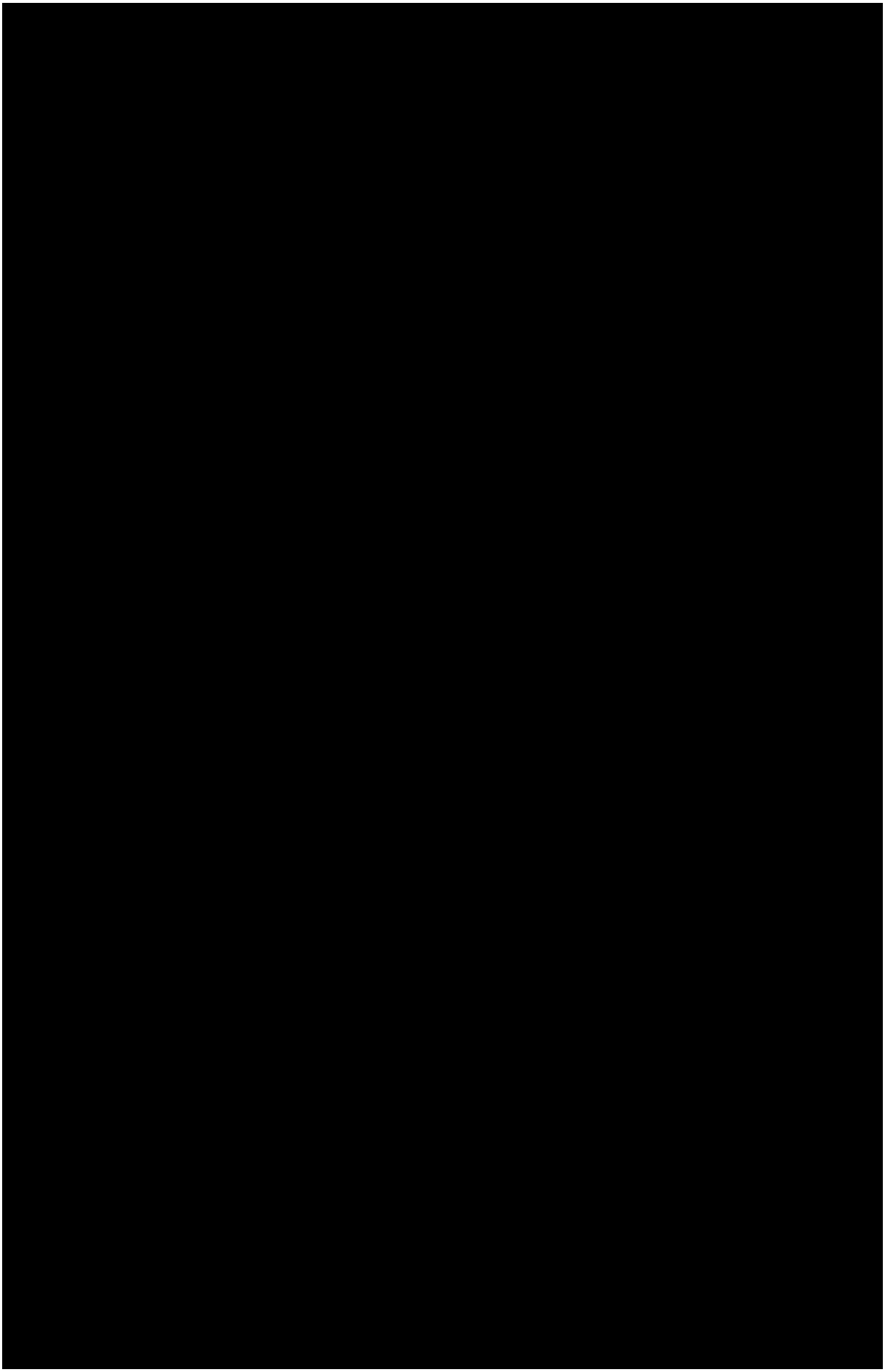
presented as mean ± SEM. In C and D, blots show representative image of replicate experiments; protein levels were quantified by the densitometric analysis of independent experiments and showed in the bar graphs as the percentage over not-treated control cells. Statistical analysis was performed by Mann–Whitney test in A–D and F and by Student's t-test in E and G (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001: MGO vs. CTR; #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001: D/Q vs. MGO).

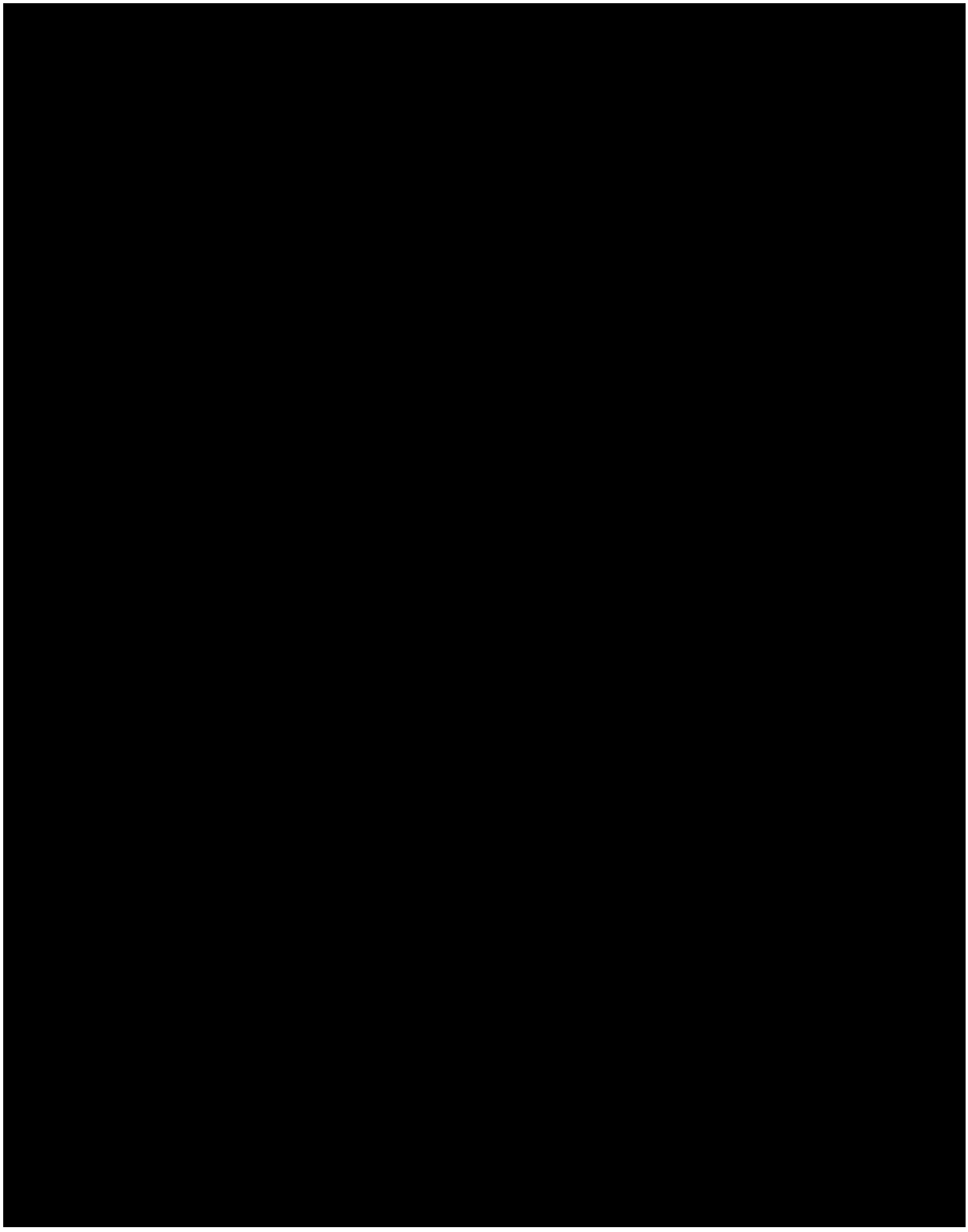
SECTION 2

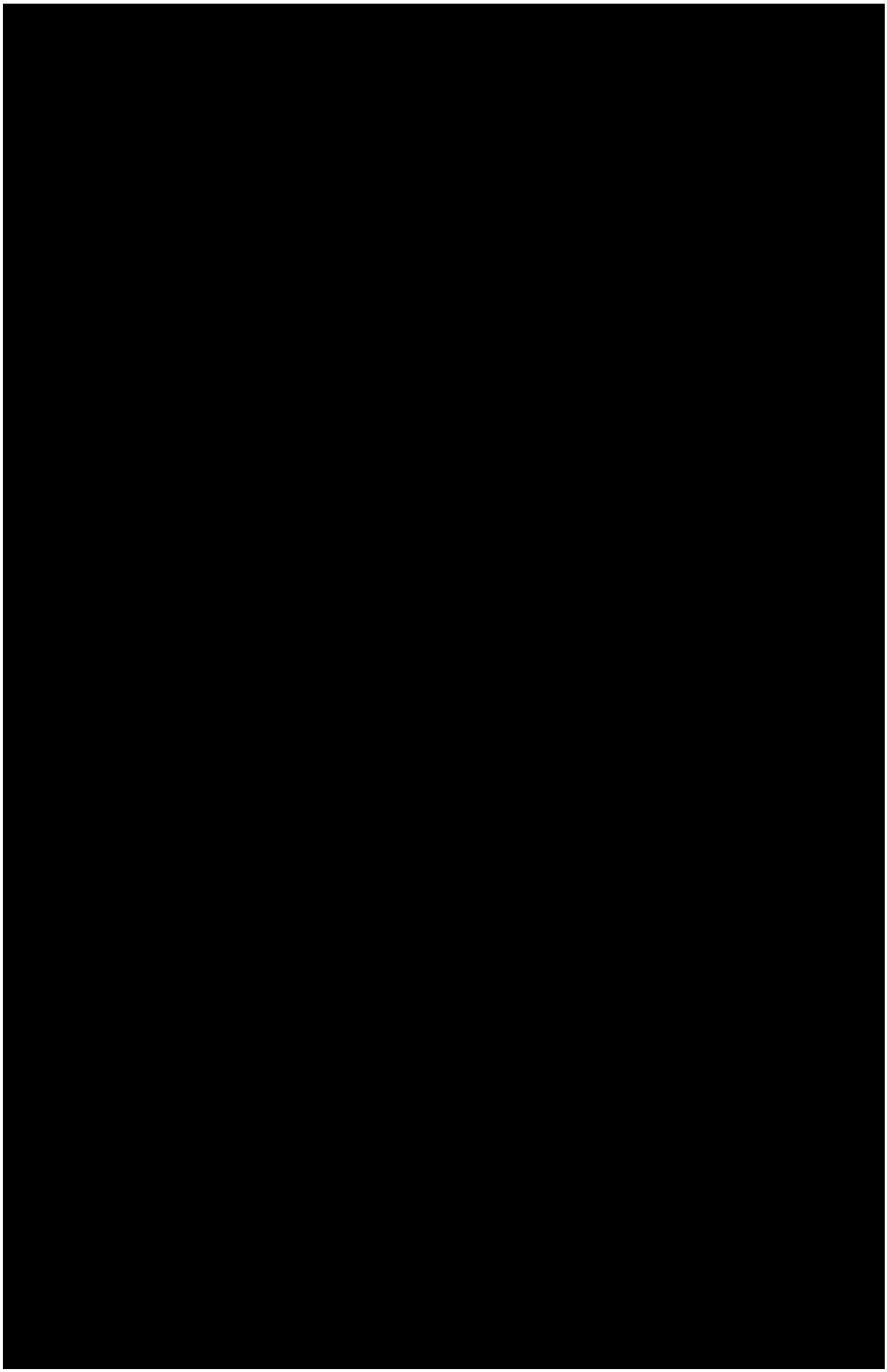


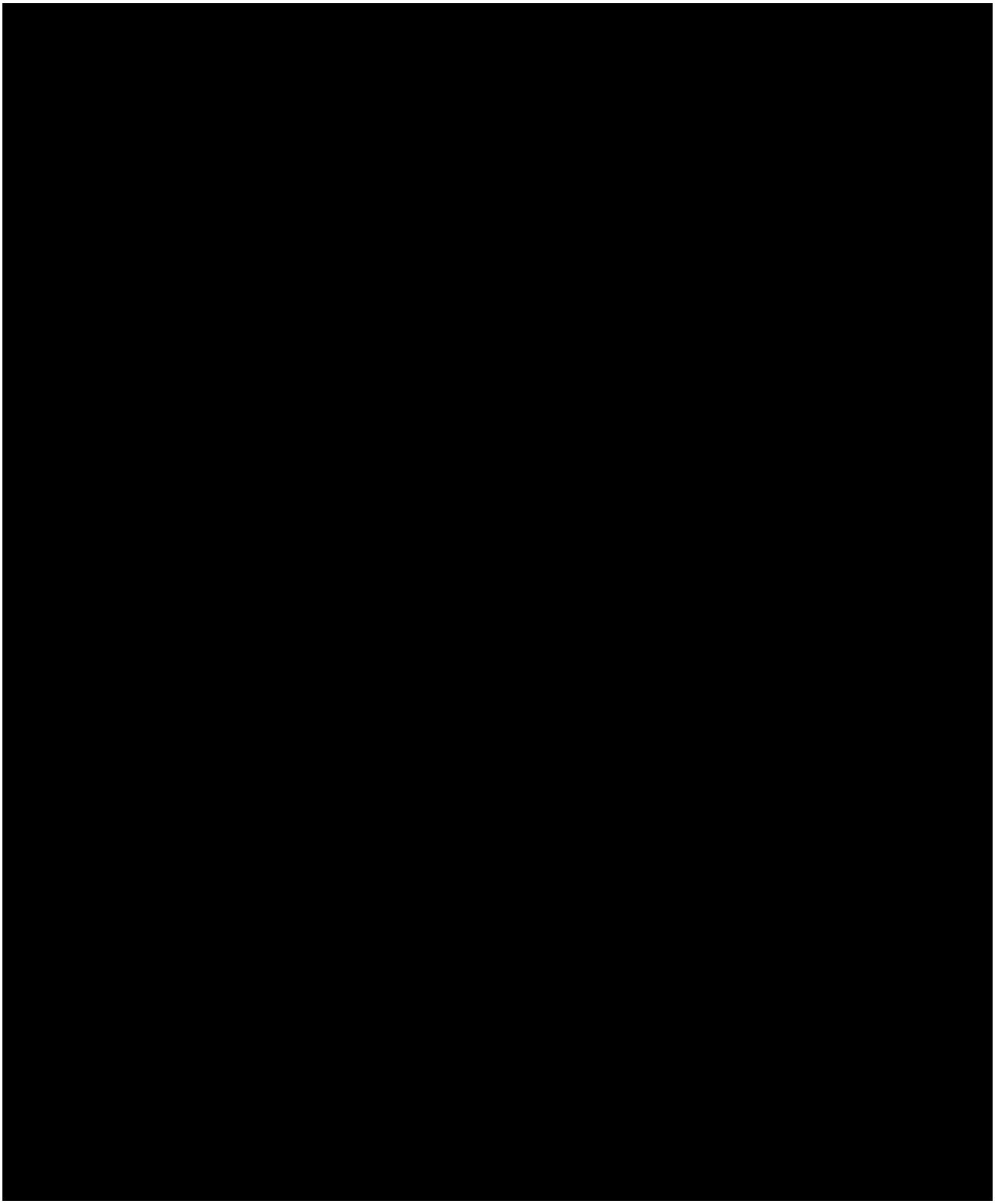


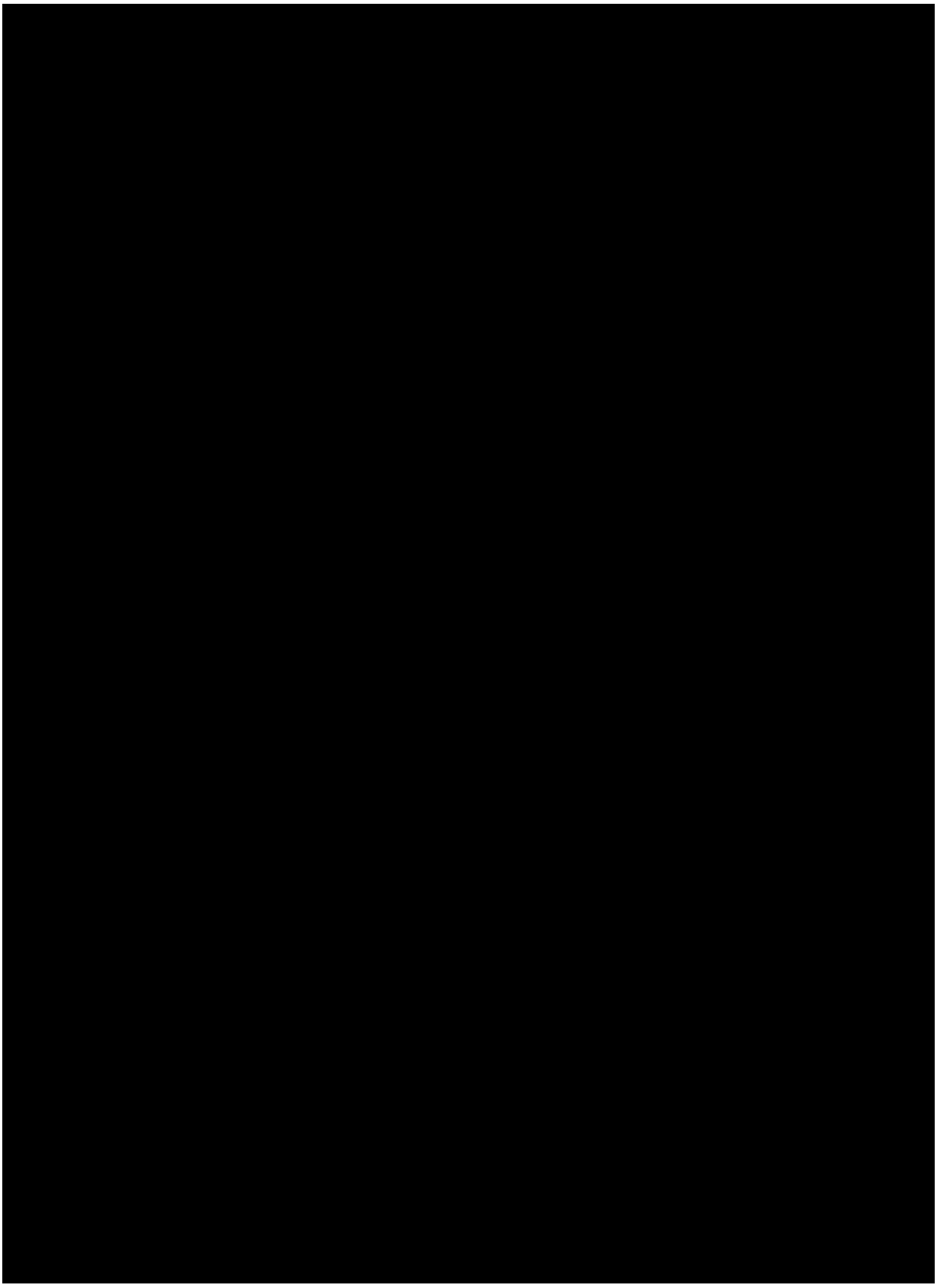


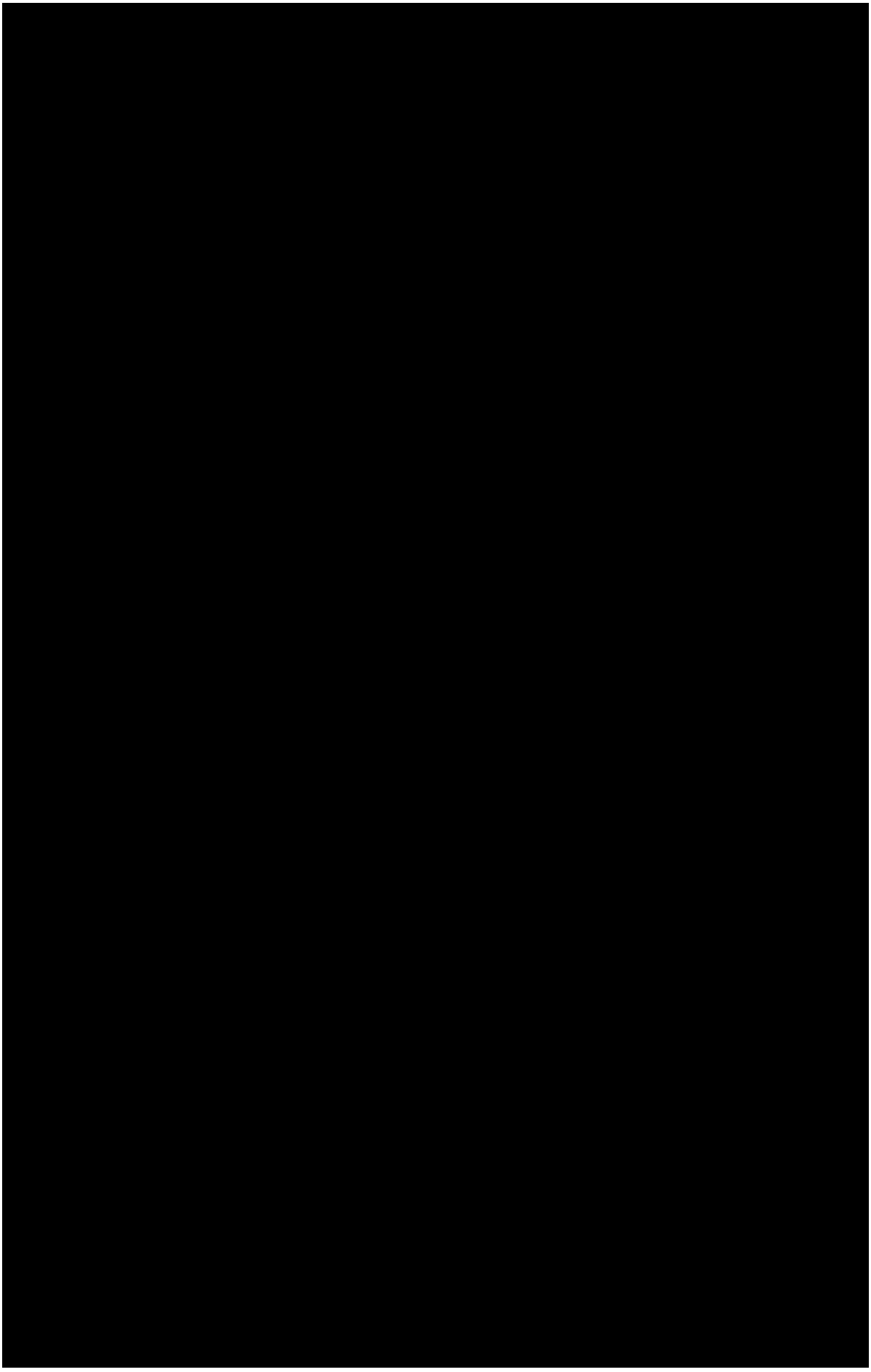


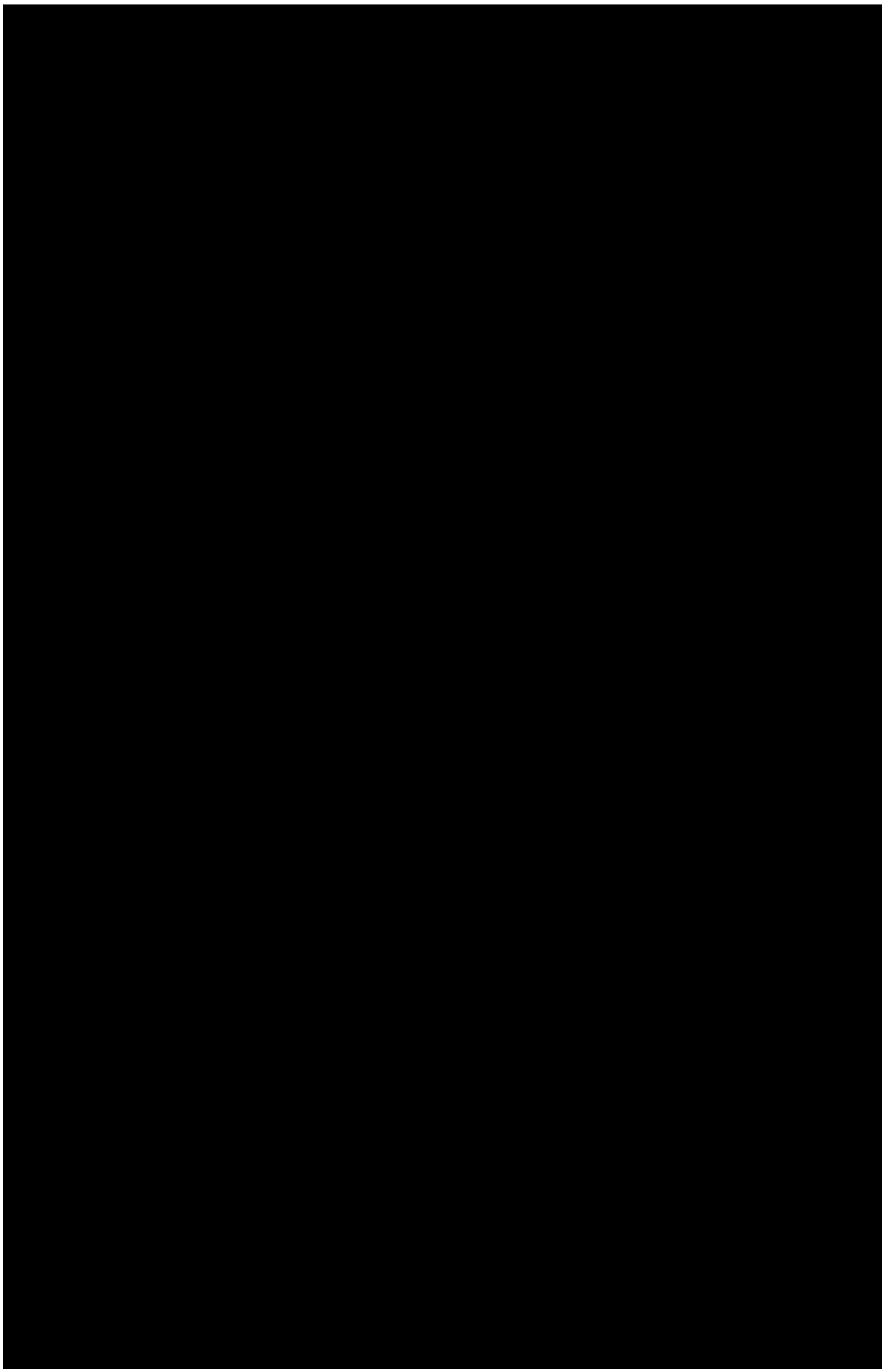












5. Conclusion

Although chronic hyperglycemia seems to be the leading cause of MGO accumulation, it has also been demonstrated that reducing HbA1c to normal levels is not sufficient to prevent the onset of vascular events in diabetic patients (Lind et al. 2010), suggesting that hyperglycemia itself is not the only driver of tissue dysfunction and complications progression in diabetes. Moreover, even if normalized on glucose levels, intracellular MGO is higher in diabetic patients (Fleming et al. 2012). Therefore, despite the progress made in the recent years in this field, further research is needed to pinpoint the role played by MGO in the development and progression of DM. To this aim, a promising contribution is provided by the use of experimental models accumulating MGO. In the first part of the study, we used a unique model in which the increase in MGO levels is due to the partial deletion of Glo1 to demonstrate that MGO accumulation induces a pancreatic damage similar to that observed in T2DM. While *Glo1*KD mice are not hyperglycemic, they develop an age-dependent glucose intolerance due to a senescence-associated pro-inflammatory phenotype of islets resulting in GSIS impairment. Our results demonstrate for the first time that the MGO harmful effect on insulin secretion is mediated, at least in part, by cellular senescence. Further studies involving the use of senolytic agents *in vivo* will be useful to draw the clinical implications of the phenotype described here for the Glo1KD mouse.

Besides its contribution to the onset of the early phenotypes of T2DM progression, MGO plays a major role in endothelial damage and development of vascular disease. In recent years, ADSCs have proved to be useful for the treatment of patients with vascular diseases (Wankhade et al., 2016; Ntege et al. 2020). However, in some pathological conditions, including T2DM, ADSCs functionality is impaired (Bonnard et al., 2008). This represents the main limitation for their use in regenerative therapy which involves the use of autologous ADSCs.

In the second part of this thesis, we have demonstrated that MGO impairs the mADSCs pro-angiogenic function and induces senescence in these cells. The harmful effect played by MGO on the pro-angiogenic capacity of ADSCs is mediated, at least in part, by the modulation of soluble factors released by mADSCs in the extracellular environment. The analysis of the mADSCs secretome will be useful for the identification of the soluble factors responsible for the impaired mADSCs functionality, whose release can be modified by MGO accumulation. Moreover, we have identified a reduced activation of p38 MAPK,

which is a mediator of angiogenesis, in hRECs exposed to CM from mADSCs treated with MGO. Together with the mADSCs secretome analysis, further investigations (e.g. the use of receptors inducers or antagonists of specific cytokines/growth factors) on hRECs angiogenesis ability will be needed to confirm the involvement of p38 MAPK and/or other pathways in the impaired pro-angiogenic function of mADSCs induced by MGO.

In conclusion, these data provide new evidence for an active role of MGO in both the ethology of T2DM and the worsening of complications, highlighting novel targets for prevention approaches to T2DM progression and useful hints for optimizing therapeutic strategies involving the use of autologous ADSCs for the treatment of complications.

6. References

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LIST OF PUBLICATIONS

1. I. Prevenzano, A. Leone, M. Longo, **A. Nicolò**, S. Cabaro, F. Collina, I. Panarese, G. Botti, P. Formisano, R. Napoli, F. Beguinot, C. Miele, C. Nigro. “Glyoxalase 1 knockdown induces age-related β-cell dysfunction and glucose intolerance in mice”. EMBO Reports (2022), 5-23(7):e52990 http://doi.org/10.15252/embr.202152990
2. A. Leone, C. Nigro, **A. Nicolò**, I. Prevenzano, P. Formisano, F. Beguinot, C. Miele. “The Dual-Role of Methylglyoxal in Tumor Progression –Novel Therapeutic Approaches”. Front. Oncol. (2021), 11:645686 https://doi.org/10.3389/ fonc.2021.645686