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"ROLE OF GASOTRANSMITTERS AND NON CANONICAL MEDIATORS IN INFLAMMATORY-BASED VASCULAR DISEASES (IBVD)"

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Ai miei genitori faro in oceano aperto,

A mio fratello ancora inamovibile,

“Il tempo sfugge, ma il segno del tempo rimane”.

Index

Abstract

Background and purpose

"The novo biosynthesis" is the most relevant pathway of SLs synthesis, where serine-palmitoyltransferase (SPT) is the rate limiting enzyme of the entire pathway. Its activity is negatively regulated by Nogo-B, a resident protein. Within vasculature, the physiological SPT/Nogo-B ratio modulates the SLs production rate [3]. Inflammatory-based vascular diseases (IBVD) are characterized by chronic inflammatory vascular process. Although they have different etiology, they have a as a common feature the endothelial dysfunction, defined as reduction of NO biosynthesis. Despite SLs homeostasis has been altered in diabetic condition [5], their involvement in IBVD is not well-clarified. Among anti-diabetic drugs metformin is one of the most used worldwide. Increasing clinical data support the beneficial role metformin on the endothelial function in diabetic conditions. Based on these findings, the aims of this study were: 1) to investigate a possible involvement of SLs in endothelial dysfunction associated to type 1 diabetes 2) to investigate the possible protective action of metformin on hyperglycemia-induced endothelium dysfunction through SLs signaling.

Experimental approach

Non-obese diabetic mice (NOD) were divided in two groups according to glycemia levels, normoglycemic or NODI and diabetic or NODIII mice and treated with Myriocin (0.3 mg/Kg), a pharmacological inhibitor of SPT, or vehicle. After 24 h, aortas were harvested and used for testing vascular reactivity. Molecular analysis and mass spectrometry-based metabolomics was performed for the evaluation of SLs content. In a separate set of experiments, NODIII mice were treated with Metformin (300 mg/Kg/Daily) and mesenteric arteries harvested to evaluate vascular function. *Ex vivo* and *in vitro* studies on microsomal fractions from mouse lung and human umbilical vein endothelial cells (HUVEC) have also been performed to confirm the data obtained *in vivo* and *ex vivo*.

Key results and conclusions

*D*e *novo* biosynthesis is altered during development of endothelial dysfunction-induced by type 1 diabetes, demonstrated by a significant increase of both SPT expression and activity, in *ex vivo* and *in vitro*. Determination of SLs by metabolomic approach revealed an accrual of both ceramides in diabetic aortas suggesting that in diabetic condition the over-expression of SPT leads to ceramide accumulation within the vasculature altering its function and reactivity. Metformin administration is largely associated to beneficial effect in endothelial function [9] but the exact mechanisms of action is still unknown. Metformin treatment of NODIII mice induced an improvement of vascular reactivity in mesenteric arteries (MA) due to a recover of eNOS/NO signaling. Incubation of both aorta and MA from healthy mouse with ceramide recapitulated the impairment observed in diabetic mice confirming the detrimental action of ceramide accrual induced by diabetes.

The take home messages of this project are as follows: 1) *de novo* biosynthesis of SLs is altered in T1D due to an increase of SLs metabolism and accumulation of ceramide in vasculature. 2) Metformin treatment in T1D restored endothelial function, improved significantly NO availability and reduced SPT activity leading to physiological extent independently to its anti-glycemic activity. According to this study, the effect of metformin on SLs homeostasis could be accreditable as a new mechanism of action leads to protect against endothelial dysfunction associated to type 1 diabetes mellitus.

1. Introduction

For many years, the endothelium was considered an inert lining of blood vessels, with the only function of a barrier between tissues and bloodstream. In 1628 Willam Harvey described for the first time the existence of a vessels' network that physically separated blood from tissues. In 1800 Von Reckingausen stated that the vascular wall was lined with a layer of cells, and a century later Heidenhahn defined the endothelium as an active and secreting cellular system rather than a static barrier. From electron and physiological microscopy studies last decades evidenced that the endothelium is a disseminated, heterogeneous and dynamic organ with secreting, synthesizing, metabolic, and immunological vital functions.

The endothelium is a continuous monolayer of cells that covers an area of about 350 m^2 in adult humans[10]. It regulates the transport of nutrients between the blood and tissues[11] by complex adhesion structures between endothelial cells [12].

Contiguous cells display intercellular junctions, that are i) tight junctions ii) adheres junctions and iii) gap junctions. These junctions are tighter in arterioles compared to capillaries and are quite loose in venules. This not homogeneity in the type of junctions in the vascular tree reflects the specific role of blood vessels based on their localizations. In addition, endothelium may be continuous or discontinuous. Continuous endothelium, in turn, could be fenestrated or non-fenestrated. Non-fenestrated continuous endothelium is found in arteries, veins, and capillaries of the brain, skin, heart, and lung. Fenestrated continuous endothelium occurs in vessels where it is required filtration or trans-endothelial transport. Fenestrated ≈70 nm in diameter are present for example, in the lamina propria of the human small intestine on the side that faces the absorptive epithelial layer of the mucosa [13]. Finally, discontinuous endothelium is found in certain sinusoidal vascular beds, most notably the liver. In contrast to fenestrated continuous endothelium, liver sinusoidal ECs possess larger fenestrations (100 to 200 nm in diameter).

1.1 Endothelium function in healthy status: role of endothelial Nitric Oxide Synthase (eNOS)

In physiological conditions, the endothelium plays a leading role in maintaining vascular hemodynamic balance by producing and secreting different vascular mediators, including endothelin-1 (ET1), angiotensin II (ATII), nitric oxide (NO), prostacyclin (PGI2) and hydrogen sulfide (H2S) [14,15,16] which dynamically regulate systemic pressure and blood flow. These chemically different compounds are not deposited in granules, but continuously synthesized on demand and, by acting on specific receptors located on endothelial cells or at the level of genes transcription, they exert their biological effects. The first demonstration that relaxation of muscle cells in response to cholinergic agonist acetylcholine depended on the integrity of the endothelium dates back to 1980 by Furchgott and Zawadzki; 7 years later it has been discovered that the mediator of acetylcholine-induced vasodilation, defined endothelium-derived relaxing factor (EDRF), was nitric oxide (NO), a free radical with a very short half-life [17,18] . NO is produced by nitric oxide synthase (NOS), a homodimer enzyme, which converts the amino acid L-arginine into citrulline and NO. Molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) are necessary as co-substrates, while flavin adenine dinucleotide [19], flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH4) are cofactors [20, 21]. Three isoforms of NOS were cloned, which differ in tissue expression, in the amount of NO produced and in the regulatory mechanism. The endothelial isoform of nitroxide synthase (eNOS) as well as the neuronal one (nNOS), is constitutively expressed and resides in specialized membrane domains, called caveolae, where it is bound to an inhibitory protein, caveolin-1. eNOS is mostly expressed in endothelial cells, however, it has also been detected in cardiac myocytes, platelets, certain neurons of the brain, in syncytio-trophoblasts of the human placenta and in LLC-PK1 kidney tubular epithelial cells[22,23]. In basal conditions eNOS releases NO constitutively [24,25], and following stimuli such as acetylcholine, angiotensin II, bradykinin, thrombin, substance P or histamine, the detachment of caveolin-1 from the enzyme takes place followed by the interaction with Ca^{2+}/calmodulin, which leads to a rapid activation of eNOS [26,27] (**Fig.1**). Mechanistically, the recruitment of calmodulin and heat shock protein 90 (hsp90) to eNOS can displace caveolin-1from eNOS, thereby leading to its

activation[28, 29]. However, eNOS can also be activated by stimuli that do not produce sustained increase of intracellular Ca^{2+}, but still induces long-lasting release of NO. The best established of such stimulus is fluid shear stress. This activation is mediated by phosphorylation of the enzyme [30,31] . Indeed, the eNOS protein can be phosphorylated on several serine (Ser), threonine [32], and tyrosine (Tyr) residues. Phosphorylation at Ser1177 stimulates the flux of electrons within the reductase domain, increases the Ca^{2+}enzyme sensitivity, and represents an additional and independent mechanism of eNOS activation [31,33] (**Fig. 2**). However, it has been demonstrated that under non-stimulated conditions Thr495 residue is phosphorylated interfering with the binding of calmodulin to the eNOS calmodulin-binding domain. In fact, dephosphorylation of Thr495 is associated with stimuli that elevate intracellular Ca^{2+}concentrations and increase eNOS activity. Substantially when Thr495 is dephosphorylated[31] more calmodulin binds to eNOS. However, dephosphorylation of Thr495 has also been shown to favor eNOS uncoupling with consequent production of reactive oxygen species (ROS) [34] .

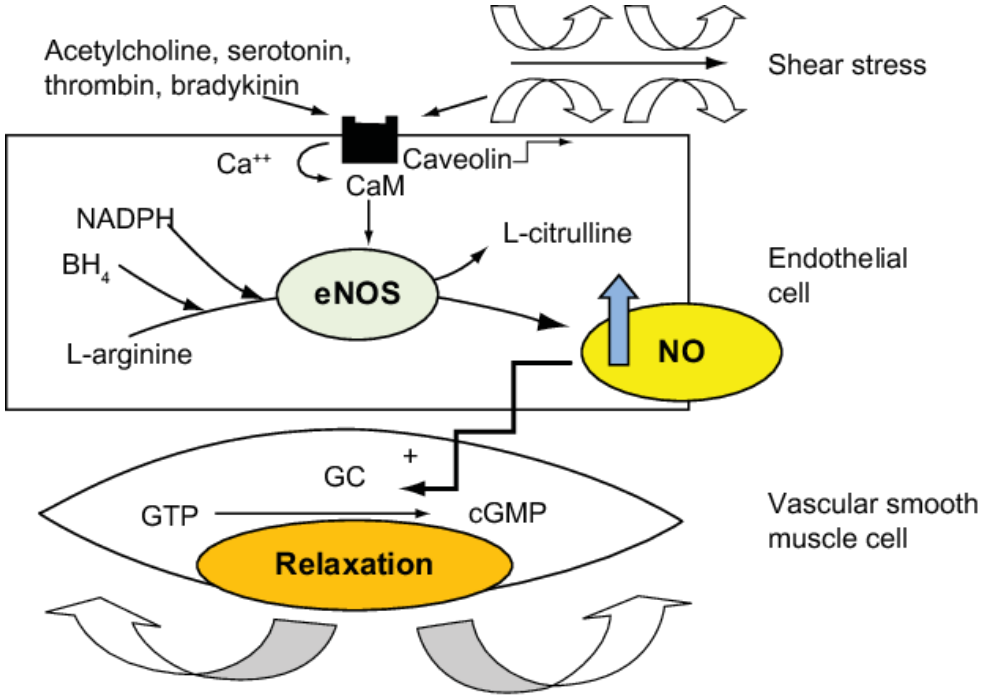


Figure 1. **Synthesis of NO by endothelial cells**. NO is produced by the action of eNOS on L-arginine. Several cofactors, including tetrahydrobiopterin (BH_4) and nicotinamide adenine dinucleotide phosphate, are required to generate this reaction. eNOS is activated in response to vasodilator agonists or shear stress that, by increasing intracellular Ca^{2+}, promote the dislodgement of eNOS from the inhibitor caveolin-1 protein allowing the availability of eNOS for Ca^{2+}/calmodulin (CaM) binding. NO diffuses to vascular smooth muscle and produces relaxation through activation of guanylate cyclase, thus augmenting intracellular cyclic guanosine monophosphate (cGMP) activates downstream signaling inducing vasorelaxation. (*from Giani & Dominici Vascular Health and Risk Management 2012)* ·

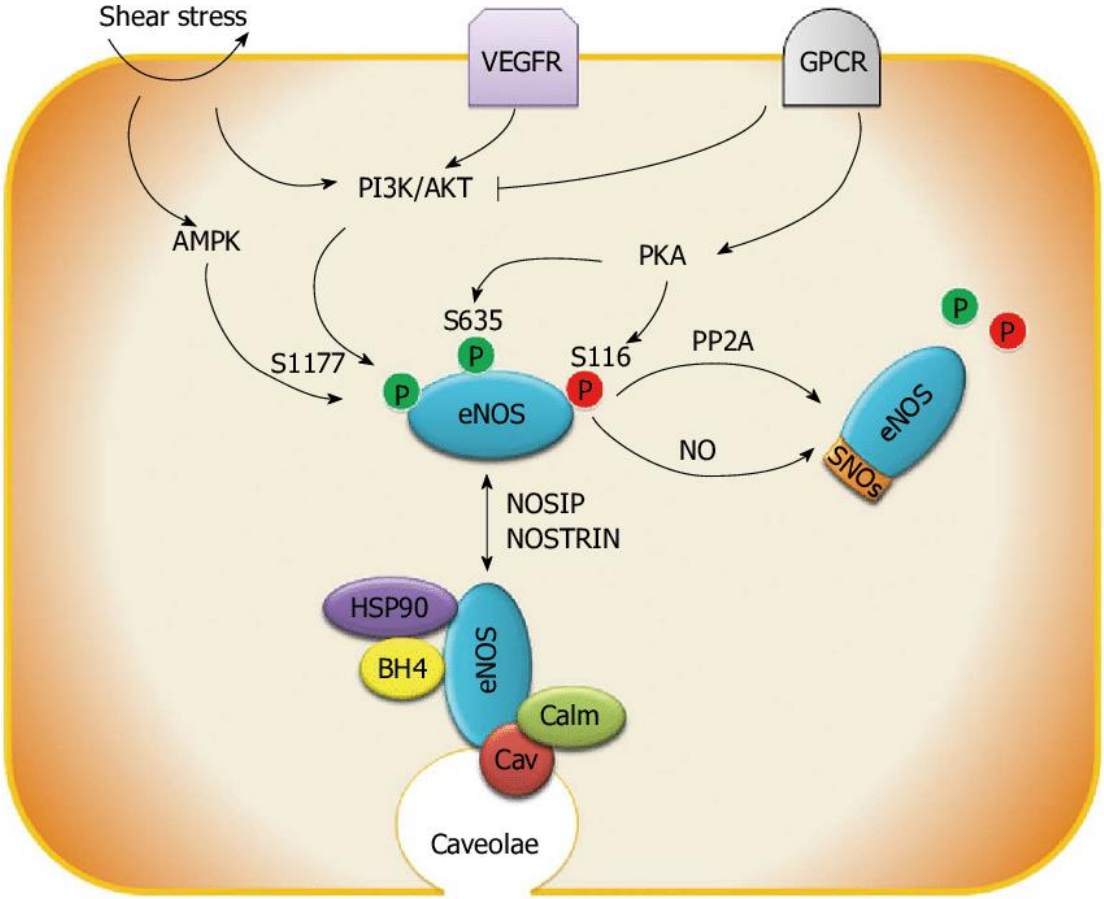


Figure 2. **Post trasductional regulation of eNOS synthase activity**. eNOS phosphorylation can be triggered by shear stress, vascular endothelial growth factor (VEGF), endothelin 1 (ET-1) and other factors though adenosine monophosphate-activated protein kinase (AMPK), protein kinase B (AKT) and protein kinase A [35] pathways, whereas protein phosphatase 2 (PP2A) de-phosphorylates eNOS. (*From Shuo et al. World J of Gastroenterol 2013)*

Endothelial derived-NO exerts important effects on vasculature: it relaxes smooth muscle vessels by stimulating soluble guanyl cyclase and increasing cyclic GMP in smooth muscle cells[36 , 37]; NO is a potent inhibitor of platelets aggregation and adhesion to the vascular wall [38,39, 40, 41].

NO also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and production of matrix molecules [42] .Furthermore, NO inhibits the adhesion of leukocytes to the endothelium by either interfering with the capability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface, or by suppressing CD11/CD18 expression on leucocyte[43,44,45]. Impairment of endothelial barrier can promote pro-inflammatory events. Endothelium-derived NO prevents endothelial cell apoptosis induced by pro-inflammatory cytokines and pro-atherosclerotic factors including reactive oxygen species (ROS) and angiotensin II (AT). The suppression of apoptosis may also contribute to the anti-inflammatory and anti-atherosclerotic effects of endothelium-derived NO [46] . NO is also involved in the regulation of vascular smooth muscle proliferation; in this context it is known that this gas-transmitter inhibits DNA synthesis, mitogenesis and proliferation of vascular smooth muscle cells [47,48, 49, 50] and these antiproliferative effects are notorious to be mediated by cyclic-GMP [51]. Endothelial NOS-derived NO plays a critical role in post-natal angiogenesis, mediating signals downstream of angiogenic factors, which has been found to be critical for collateral formation and angiogenesis post-ischemia[52]. Furthermore, the positive effects of NO on endothelial cell survival are likely to also contribute to the pro-angiogenic effects of NO[46].

The third isoform of NOS is defined inducible iNOS[53]. It is not usually expressed in cells, but its expression can be induced by bacterial lipopolysaccharide, cytokines, and other agents. Although primarily identified in macrophages, iNOS expression can be stimulated in virtually any cell or tissue, provided that the appropriate inducing agents have been identified [23 , 22] . Once expressed, iNOS is constantly active and not regulated by intracellular Ca^{2+} concentrations. When induced in macrophages, iNOS produces large amounts of NO, which represents a major cytotoxic principle of those cells[54]. Due to its affinity to protein-bound iron, NO can inhibit key enzymes that contain iron in

their catalytic centers. These include iron–sulfur cluster-dependent enzymes (complexes I and II) involved in mitochondrial electron transport[54]. In addition, higher concentrations of NO, as produced by induced macrophages, can directly interfere with the DNA of target cells and cause strand breaks and fragmentation[55,56]. A combination of these effects is well-known to form the basis of the cytostatic and cytotoxic effects of NO on parasitic microorganisms and certain tumor cells. Interestingly, non-immune cells can also be induced by cytokines to release a large amounts of NO enough to affect the neighboring cells[22].

1.2 Endothelium and coagulation cascade

The endothelium not only plays a leading role in hemodynamic balance, but also actively participates to coagulation. In fact, in physiological conditions, the endothelium exhibits a luminal surface with an anticoagulant and antithrombotic phenotype, which, following pro-inflammatory stimuli or vascular damage, evolves into the pro-thrombotic and pro-coagulant phenotype [57]. The main anti-platelet factors released by endothelial cells are PGI2 and NO (Cines, 1998 #1495) whose effect is mediated by the increase in cGMP in platelets [58]. Furthermore, ectonucleotidases are exposed on the endothelial surface [59] which hydrolyze ATP and ADP, two powerful platelet aggregating agents. The generation of thrombin, a serine protease that plays an important role in the activation of platelets and coagulation factors, is a crucial factor in the regulation of the antithrombotic-pro-coagulant balance of the endothelium. Endothelial cells express both tissue factor pathway inhibitor (TFPI), which binds Factor Xa, preventing thrombin formation [60,61]; and thrombomodulin, which interacts with thrombin forming a complex that activates the anticoagulant protein C [62]. Furthermore, the endothelial surface is rich in glycosaminoglycans that binds antithrombin, thus constituting the main site of thrombin inactivation [63]. The induction of TF endothelial expression is the crucial event that transforms the endothelial surface from anticoagulant to pro-coagulant. In fact, TF is not present on the endothelium in a physiological state [64] , and it has been shown that agonists, such as thrombin, cytokines, endotoxins, shear stress, hypoxia, oxidized lipoproteins, and many other factors

stimulate the synthesis of TF *in vitro* [65].These events are responsible *in vivo* for the activation of Factor VIIa-dependent coagulation. TF expression is rapidly induced by vascular damage [66]and has been highlighted on the endothelial cells of atherosclerotic plaques [67] demonstrating the involvement of TF in pathological states.

1.3 Molecular basis of endothelial dysfunction of Inflammatory Based Vascular Disease (IBVD): inactivation of bioactive nitric oxide and eNOS uncoupling

Vascular diseases (VD) represent a heterogeneous group that affect heart and vascular tree, in which many different factors contribute to the onset of the development and severity of the diseases. It is a well consolidated concept that the combination of genetic predisposition associated to unhealthy environment and lifestyle can favor appearance of VD. Many are considered the risk factors for VD development included hyperlipidemia, hyperglycemia, cigarette smoking, obesity, hyper-homocysteinemia. All of them could be the cause of VD, alone or in combination, giving a wide range of VD in terms of the symptoms of initiation, the tissues affected (heart, kidney, coronary arteries ect), the severity and the disease progression. All the pathologies included in the VD, i.e. atherosclerosis, heart failure, diabetes, hypertension, are characterized by oxidative stress and inflammatory processes [68] thereby sustaining the concept of inflammatory-based vascular disease (IBVD). Other key players are the reactive oxygen species (ROS). Physiologically, ROS such as peroxinitrite ($ONOO^-$), superoxide (O_2^-), hydroxyl, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), oxygen radicals and non-radical oxidizing agents, respectively, are signal molecules that are all involved in the vascular homeostasis. They regulate oxygen sensing, apoptosis, cell proliferation, defense from microbial injury and inflammatory reactions [69,70]. These molecules are produced within the vessels by endothelium, smooth muscle cells and in adventitia [71] by several enzymatic systems including mitochondrial enzymes, xanthine oxidase, lipoxygenase and NADPH oxidase. Clearly, the vascular tissues have an antioxidant system that continuously counterbalances ROS formation: superoxide dismutase, catalase, thioredoxin peroxidase, glutathione peroxidase, heme

oxygenase all of them are enzymes that exert anti-oxidant activity [69,70]. In any circumstance in which ROS production overwhelms the endogenous antioxidant system, the body is subjected to *oxidative stress*. All the IBVD have in common the oxidative stress and the first target is the endothelium with an impairment of NO biosynthesis by eNOS, this represents the main consequence of oxidative stress to the endothelium. Indeed, it has been shown that in presence of high levels of ROS, the eNOS co-factor BH4 is readily oxidated [72]. This event induces the eNOS uncoupling with consequent generation of O_2^- instead of NO [73, 74] . Due to the enhanced oxidative stress observed in vascular disease, an increased degradation of NO by its reaction with $O^{2-\cdot}$will occur. However, oxidative stress has also been shown to convert eNOS from an NO-producing enzyme to an enzyme that generates $O^{2-\cdot}$. This process has been largely referred to eNOS uncoupling (**Fig. 3**). Mechanisms implicated in eNOS uncoupling include oxidation of the critical NOS cofactor BH4, depletion of L-arginine, and accumulation of endogenous methylarginines. More recently, S-glutathionylation of eNOS has been proposed as an another mechanism leading to eNOS uncoupling[75,76]. These evidences have been confirmed in patients with heart failure with preserved ejection fraction, where the oxidation of BH_4 in BH_2 is associated to eNOS uncoupling [77] (**Fig. 3**). The NO impairment has a wide echo on many aspects of vascular function; in healthy status, over its capacity to dilate vessels, NO reduces oxidation of low-density lipoproteins (LDL), reduces platelet reactivity and leukocyte stickiness providing a vascular protective action. Therefore, the impairment of NO release due to endothelial dysfunction induces vasoconstriction, pro-coagulability, arterial stiffness that sustain the vicious IBVD circle.

Another element that contributes to the impairment of NO biosynthesis associated to IBVD is the protein caveolin-1 (CAV-1). In resting condition eNOS is bound to CAV-1, a resident protein of caveolae that keeps eNOS in a low activated state. When intracellular Ca^{2+} rises, Ca^{2+}-calmodulin complex binds eNOS and, together with hsp90, displace eNOS from the negative control of CAV-1, leading to the enzyme activation[28,29]. Several studies have demonstrated that, in IBVD, the reduced production of NO is due to an increase of CAV-1 expression that retains the eNOS in

a less-active state [78] . Indeed, CAV-1 over-expression has been found in hyperlipidemia [79, 80]diabetes [81,82, 83,84, 85], atherosclerosis [86,87]and pulmonary hypertension [88,89].

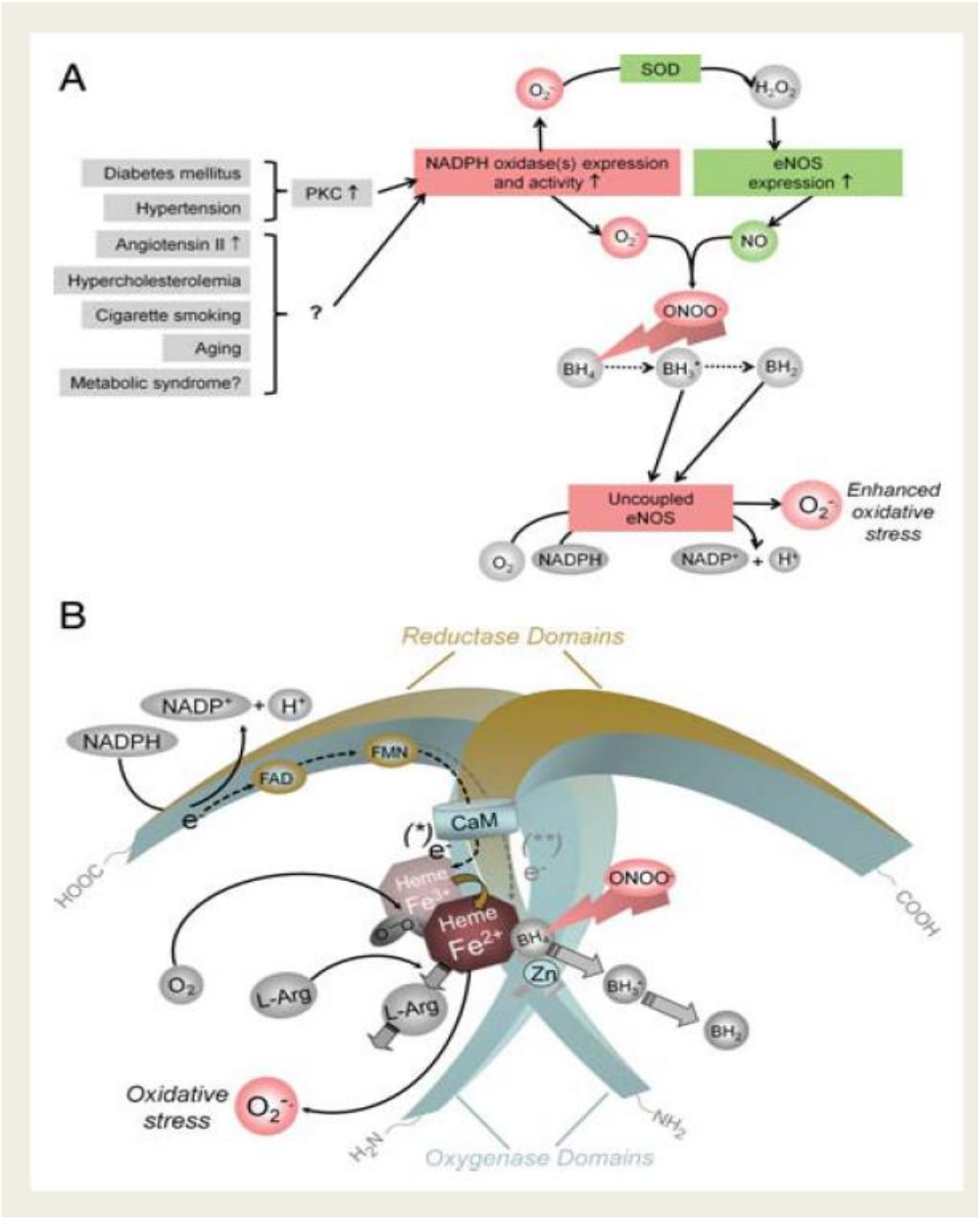


Figure 3. **Potential mechanisms by which cardiovascular risk factors lead to oxidative stress and endothelial NOS uncoupling**. (A) in many types of vascular disease, NADPH oxidases are upregulated generating superoxide ($O_2^{\cdot-}$). H_2O_2, the dismutation product of $O_2^{\cdot-}$can increase eNOS expression via transcriptional and post-transcriptional mechanism (SOD

superoxide dismutase). The product of NADPH oxidases and endothelial NOS, $O_2^{-\cdot}$And NO rapidly recombine to form peroxinitrite (ONOO-). This can oxidize the essential cofactor of eNOS (6R-)5,6,7,8,-tetrahydrobiopterin (BH4) to trihydrobiopterin radical ($BH3^{\cdot}$) and BH2. Consequently, functional eNOS is converted in a dysfunctional$O_2^{-\cdot}$generating enzyme contributing to vascular oxidative stress. (*from Forstermann & Sessa Eur Heart J 2012*)

1.4 Glucose metabolism: insulin and glucagon

Glucose regulation is due to an integrated action of many hormones released from pancreas and gut, that tightly work together to keep plasmatic glucose levels in a physiological range (70-120 mg/dl). Blood glucose concentration can increase after meals arriving to 101-125 mg/dl which represents only an "impaired fast glucose" but also can exceed to values higher than 126 mg/dl meaning that the physiological regulation of blood glucose has been modified inducing a pathological state, called diabetes mellitus. Insulin is a peptide hormone consisting of 51 amino acids arranged in two chains, A and B, held together by two disulfide bridges (**Fig.4**).

Its production is preceded by pro-insulin, molecule consisting of a single polypeptide chain that is broken down, in the Golgi apparatus, into insulin and peptide C. The insulin is then stored in the same cells in the form of ready-made granules to be secreted.

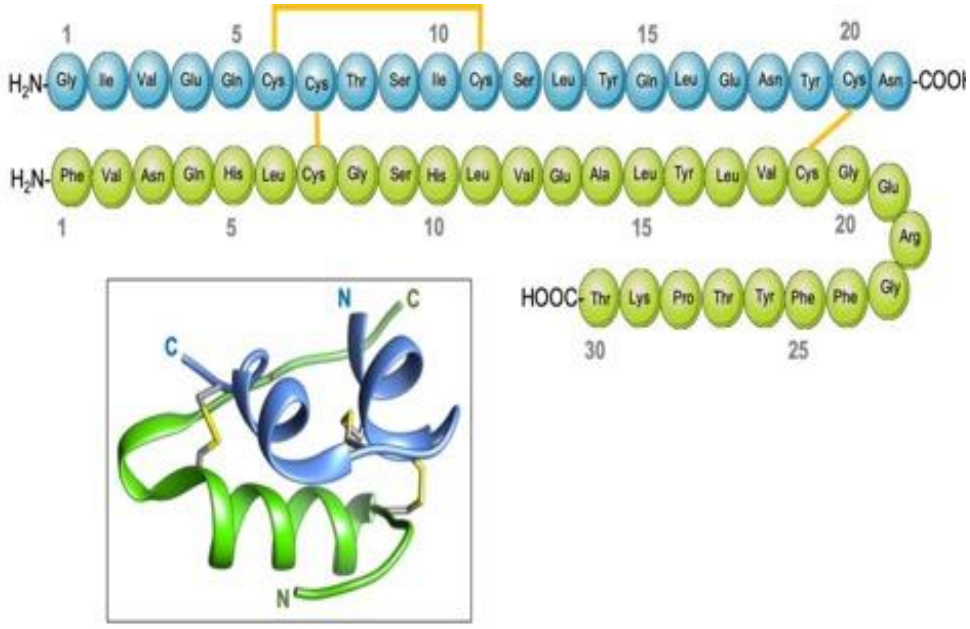


Figure 4. Human insulin structure

Insulin secretion occurs when the plasmatic concentration of glucose or other sugars (mannose, sorbitol), some amino acids (arginine, leucine), hormones such as glucagon-like-peptide 1 (GLP-1) and fatty acids rise up. The increase in blood glucose levels induces the entry of insulin into β-pancreatic cells, through a specific transporter known as GLUT-2 (Glucose transporter). Inside the β cell, glucose is phosphorylated by glucokinase, the enzyme that catalyzes the first step of glycolysis. This glycolytic process determines the final production of ATP which, in the β cell, acts as a second messenger, inhibiting the activity of ATP-dependent potassium channels. These channels are formed by two separate proteins, one of this, SUR1, is a regulatory protein and is the molecular target for a widely used class of oral hypoglycemic agents named sulfonylureas. ATP binding to the SUR1 regulatory subunit inhibits the flow of potassium K + ions towards the outside of the cell. This event causes an increase of potassium [K +] intracellular levels causing the membrane depolarization that in turn opening the voltage-gated calcium channels, resulting in the influx of Ca^{2+} into the cell (when the membrane potential drops below - 40 mV). The rapid increase in intracellular calcium stimulates the exocytosis of the insulin contained in the granules that represent the ready-release pool. Conversely, when blood sugar decreases, the transport of glucose through GLUT2 is reduced and the enzymatic activity of glucokinase is slowed down. This leads to a reduced production of ATP and to a restoration of the membrane potential which returns to values of - 60 mV. The voltage-gated calcium channels close and insulin release is reduced (**Fig. 5**).

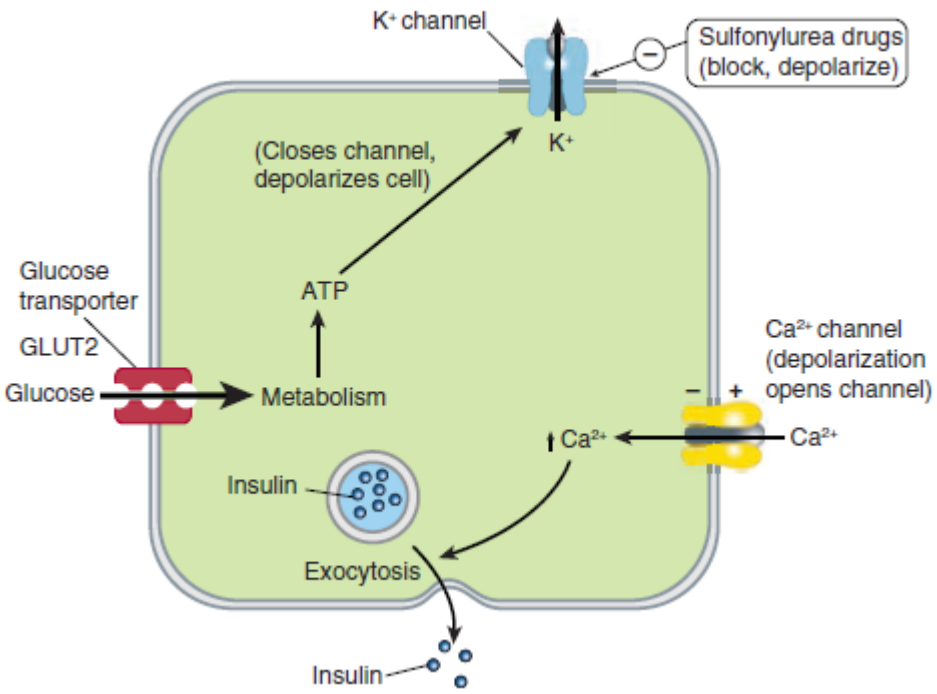


Figure 5: **Molecular mechanism of insulin secretion from beta pancreatic cells**

In the bloodstream, insulin interacts with specific receptors expressed in various tissues. The insulin receptor consists of two dimers, each characterized by an extracellular α subunit and a transmembrane α subunit, with the carboxy-terminal domain extending into the cytosol. The α chains recognize and interact with the insulin circulating in the extracellular environment while the intracellular domains of the β chains contain the active site of the tyrosine kinase. The binding of insulin to the α subunit determines a conformational change responsible for the approach of the two β subunits with subsequent phosphorylation and activation of the tyrosine residues of the receptor. Then, the active site of the kinase phosphorylates tyrosine residues of other proteins that act as signal transducers including the substrate of the insulin receptor (IRS). These molecules, in turn, participate in a cascade of intracellular

phosphorylation thanks to which the effects mediated by the hormone are achieved (**Fig. 6**). In particular, IRS binds to the enzyme phosphatidyl-inositol-3-kinase (PI3K), a dimer consisting of a p85 regulatory subunit and a p110 catalytic subunit, necessary for most of the anabolic and anti-apoptotic actions of insulin. PI3K converts the membrane lipid phosphatidylinositol 4,5 bisphosphate into phosphatidylinositol 3,4,5 triphosphate (PIP3). The latter does not become a substrate of phosphatase but acts as a binding site for Akt / protein kinase B and phosphatidylinositol-3-dependent phosphate kinase-1 (PDK1), which are approached precisely following the interaction with PIP3[90].

One of the main intracellular effects activated by insulin and mediated by Akt / PKB is the translocation of glucose transporters (GLUT) towards the cell membrane. There are several isoforms of GLUTs: GLUT-1 and GLUT-3, present in the brain and fundamental for the uptake of glucose and for its passage through the blood brain barrier; GLUT-2, mainly located in the liver, kidneys and pancreatic β cells; GLUT-4, present in the muscles; and finally GLUT-5, located in the intestine [90]. A further intracellular effect of insulin is represented by the activation of the enzyme glycogen-synthetase that promotes the deposition of glucose in the form of glycogen, contributing to the homeostasis of blood glucose concentrations and activation of fatty acid metabolism, as well as stimulation of cell growth and division. Furthermore, Akt/PKB stimulates the production of NO by activating the enzyme eNOS with consequent vasodilation; blocks BAD, a member of the Bcl2 family involved in the apoptotic process; activates FOXO, a transcription factor that negatively regulates gluconeogenesis, detoxification from reactive oxygen species (ROS), stimulation of the cell cycle and cell survival [91,92] . The hepatic degradation of insulin and the expulsion of this hormone by the kidney reduces its blood concentrations and its hypoglycemic effect. In addition, high amounts of glucocorticoids in the blood circulation reduce the affinity of insulin while the abnormal phosphorylation of serine and threonine residues of the IRS proteins is strongly implicated in insulin resistance phenomenon associated to type 2 diabetes. The action of insulin is contrasted by another hormone, glucagon: a single peptide of 29 amino acids produced and secreted

by pancreatic α cells (**Fig.7**). Its receptors are coupled to a Gs protein and following the binding with the hormone, an increase in the activity of adenylate cyclase is observed with a consequent increase in the production of cAMP which, in the liver, facilitates the catabolism of glycogen and increases ketogenesis. and gluconeogenesis.

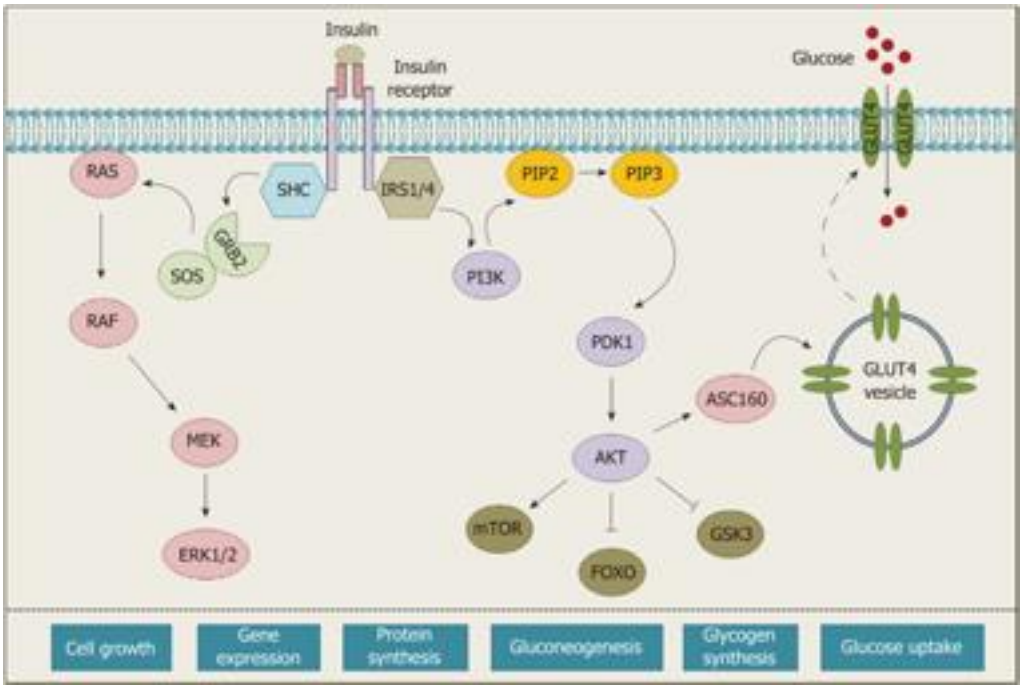


Figure 6. **Insulin receptor and its mechanism of signal transduction** (*from Singh et al., World J Nephrol. 2019)*

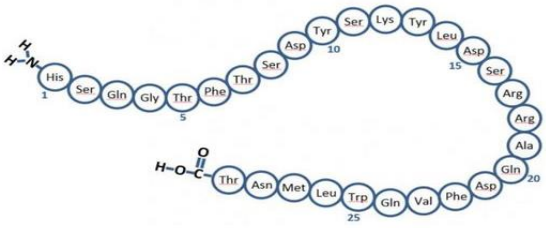


Figure 7. **Human glucagon structure**

The immediate result of the glucagon release is therefore an increase in blood glucose levels at the expense of hepatic glycogen reserves. Glucagon is extensively metabolized in the liver, kidney and in several tissues.

The alteration of the physiological balance insulin-glucagon (**Fig.9**) represents the main event in diabetes appearance; the reduction of insulin production and the consequent increase of glucagon levels, causes an increase in blood glucose concentration values, establishing the hyperglycemic state of diabetes. The World Health Organization, (WHO) defines diabetes as a chronic condition characterized by high concentration of sugar in the blood due to a relative or absolute deficiency in the human insulin secretion or function. The use of adjective mellitus derived from the Latin "sweetened with honey" and refers to the large amount of glucose that is found in the urine of diabetic patients.

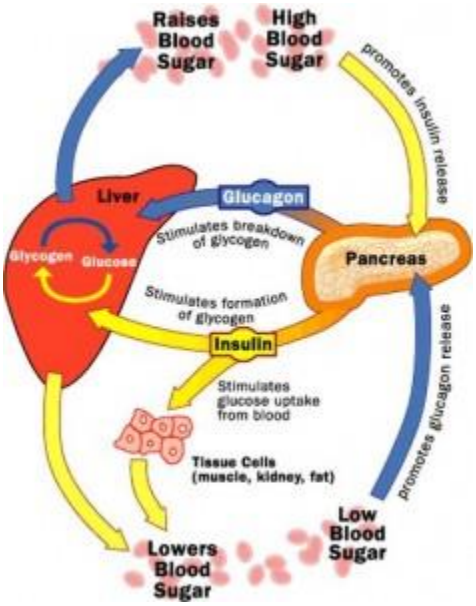


Figure 8. **Insulin and glucagon regulate the glycemia levels**

1.5 Diabetes

Diabetes Mellitus (DM) can be classified in two most important type:

-Type 1 diabetes mellitus (T1DM), also called insulin-dependent diabetes mellitus (IDDM) where hyperglycemia is due to an absolute lack of insulin

-Type 2 diabetes mellitus (T2DM), also called non-insulin-dependent diabetes mellitus (NIDDM) where hyperglycemia is due to a relative insufficiency of insulin production and/or to insulin resistance of peripheral tissues.

Depending on the type of diabetes, drug treatment is different. In type 1 diabetes, administration of insulin as replacement therapy is a mandatory option, while in type 2 diabetes, oral hypoglycemic drugs represent the gold standard therapy.

1.5.1 Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes is usually diagnosed in children and young adults. It accounts for 5-10% of all diabetes cases [93] , and it has been estimated that about 80.000 children all over the world develop the T1DM each year.

T1DM is a particular form of diabetes characterized by the autoimmune destruction of β-pancreatic cells. In absence of β-cells, insulin is neither produced or released, and circulating insulin concentrations are progressively reduced until to an absolute absence. In this condition, tissues fail to take up and store glucose, amino acids and lipids, even when there are high circulating levels of these molecules. Moreover, glycogenolysis and gluconeogenesis proceed unchecked in the liver, promoting the release of glucose to the blood even if the blood sugar levels are already high. Contextually muscle tissue breaks down protein and release amino acids which travel to the liver as fuel for gluconeogenesis. Instead in adipose tissues, triglycerides are broken down and release into the circulation. The derived fatty acids are then break down into the liver for promoting gluconeogenesis and for releasing ketone bodies that, accumulating in the plasma, increasing the blood acidity, which, in turn, leads to nausea, vomiting, confusion and eventually come. All these events lead to a dramatically increase of blood glucose concentration, with a reduction of glucose

reabsorption from kidney and a consequent osmotic diuresis as well as “sweeting” of urine. This phenomenon causes the well-known polyuria and consequent polydipsia.

1.5.2 T1DM: insulin therapy

Various insulin preparations are commercially available: ultra-fast insulin; rapid insulin; insulin with an intermediate duration of action; and finally, long-acting insulin. As for those with ultra-fast action, it is possible to include the insulin Lispro, Aspart and Glulisine, containing small amounts of Zinc to increase their stability. Ultra-fast insulin is administered before meals and has a very fast absorption; in fact, it comes into action 10-15 minutes after injection and reaches the peak of maximum concentration in 30 minutes, showing a post-prandial action similar to the typical one observed with endogenous insulin. Rapid insulin is obtained by recombinant DNA technique, has a short duration of action (5-8 hours) and is identical to human insulin. It is stabilized, thanks to the presence of zinc, in hexamerics, after subcutaneous administration, a little at a time they begin to fragment into dimers and, finally, into active monomers. As a result, this type of insulin should be taken at least 30-45 minutes before meals.

Among the intermediate-acting insulins, Neutral Protamine Hagedorn (NPH) sees the association of insulin with protamine, which is degraded by proteolytic enzymes after subcutaneous injection allowing the absorption of insulin. It has a duration of action that can be up to 12 hours and is usually mixed with rapid insulin, lispro or aspart.

Long-acting insulins include insulin Detemir and Glargine. Detemir presents myristic acid instead of Threonine, in position 30 of the B chain; this increases the possibility of self-aggregation and binding to plasma albumin this consents to have a duration of action of more than 24 hours. Glargine, on the other hand, has two more arginine molecules in the carboxy-terminal part of the B chain and Glycine in position 21 is replaced with Asparagine. It precipitates after subcutaneous administration and therefore the insulin molecules dissolve slowly from this deposit producing a low but constant release of insulin for up to 24 hours.

1.5.3 T2DM therapy

Therapy of type 2 diabetes includes several classes of drugs with different mechanisms of action.

BIGUANIDES

Even though the exact molecular mechanism is not clearly identified, biguanides, including metformin, phenformin and buformin are widely use in T2DM therapy. They act on the synthesis and release of glucose in the liver by inhibiting gluconeogenesis and glycogenolysis. They favor the entry of glucose into cells, particularly in muscle tissue, and are responsible for a reduction in plasma glucagon levels. Defined as euglycemic, metformin the leading exponent of this class, represents the drug of choice for the treatment of type 2 diabetes and is one of the most effective and sold oral antidiabetic drugs in the world. At the start of therapy, about 10% of patients report diarrhea and gastrointestinal disturbances as side effects. The occurrence of episodes of severe lactic acidosis has been described, contraindicating its use in patients with high risk of acute renal failure or with chronic renal failure.

SULPHANILUREE and MEGLITINIDES

Sulfonylureas are also called insulin secretagogues because they act by increasing the secretion of insulin from the pancreas in a glucose-independent manner. In fact, they interact with a receptor associated with the K + ATP-dependent channels present on the membrane of β cells, called SUR-1 (Sulphonyl-Urea Receptor-1): the binding of sulfonylureas to the receptor determines the closure of the channel, depolarization with consequent entry of calcium ions into the cell and mobilization of vesicles containing preformed insulin. They also reduce the secretion of glucagon, the hyperglycemic hormone.

Sulfonylureas are classified into various generations, in which the difference between the first and the last is an increase of potency and a reduction of side effects, mostly consisting of hypoglycemia, hot flashes and sometimes hematological toxicity.

Tolbutamide, Chlorpropamide and Tolazamide belong to the first generation, while Gluburide, Glibenclamide and Glipizide are part of the second generation.
As for the Meglitinides, Repaglinide and Nateglinide share the same site and mechanism of action of the sulfonylureas. The first is frequently associated with hepatic toxicity while the second with a very low incidence of hypoglycemia.

THIAZOLIDINDIONS

Pioglitazone and Rosiglitazone are part of the thiazolidinedione class; they act by reducing the insulin resistance that characterizes type 2 diabetes by binding to PPAR-γ (peroxisome proliferator-activated receptor-γ) receptors, involved in the modulation of the genes of lipid and glucose metabolism, in the transduction of insulin signal and in the differentiation of adipocytes. Their action is mainly expressed in adipose tissue where they favor the access of glucose and its use for energy purposes. Side effects include fluid retention, edema, heart failure, weight gain and increased risk of fractures.

INHIBITORS OF ALPHA-GLUCOSIDASE

Alpha-glucosidase is an enzyme that breaks down complex carbohydrates and disaccharides into monosaccharides, thus facilitating their absorption. Inhibitors of this enzyme, therefore, delay the absorption of carbohydrates in the gastrointestinal tract and consequently reduce postprandial glycemic excursions.
Acarbose is an effective alternative in the oral therapy of type 2 diabetes; when added to metformin, it causes a reduction in glycated hemoglobin of 0.6-0.7%. Acarbose, like metformin, and unlike other drug therapies, has no negative effects on body weight and does not cause hypoglycemia. It can also be associated with insulin, with favorable effects on glycemic control and body weight.

1.6 Ceramide and Sphingosine-1-phosphate (S1P): bioactive sphingolipids in vascular tone and blood pressure regulation

Sphingolipids (SLs) are important constituents of biological membranes and lipoprotein, representing one of the major classes of eukaryotic lipids [2]. Within this family the most studied and characterized SLs are sphingosine (SP), ceramide (Cer), sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (Cer1P). These molecules act on distinct protein targets including kinases, phosphatases, lipases and membrane receptors, exerting distinct cellular functions, such as cell proliferation, differentiation and cell morphology, apoptosis and endothelial cell permeability[94].The most interesting and recognized SLs biosynthetic pathway is the *"the novo"* synthesis (**Fig. 9**). It starts at the cytosolic leaflet of the ER, where a set of four enzyme groups coordinately generate ceramides of different acyl chain lengths from non-sphingolipid precursors.

Sphingolipid *de novo* biosynthesis is initiated and rate-controlled by serine palmitoyl-transferase (SPT) [95, 96]composed of two major subunits, SPT long chain base subunit 1 (SPTLC1) and SPTLC2. SPT catalyzes the condensation of serine with a fatty acyl-

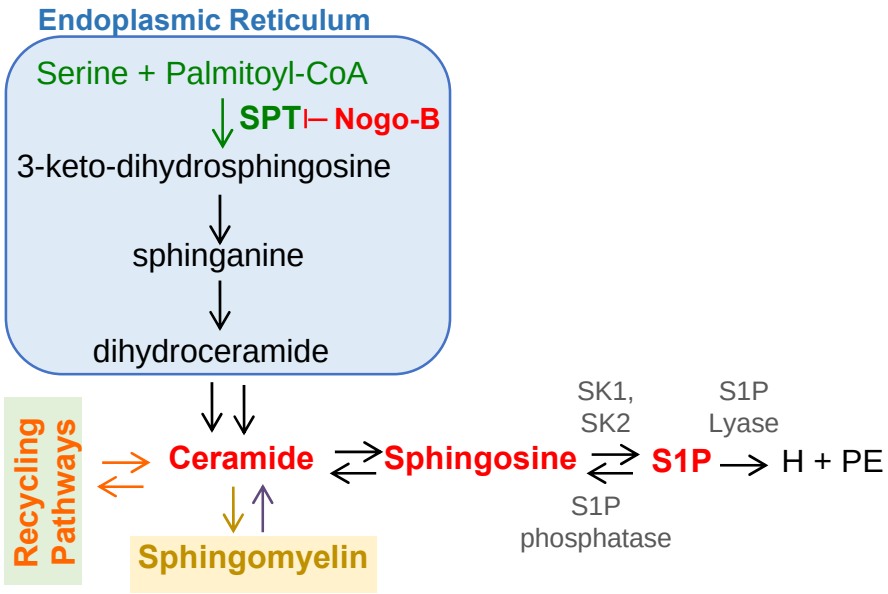


Figure 9. Schematic representation of sphingolipid *de novo* pathway. SPT catalyzes the condensation of serine and palmitoyl-CoA in 3-keto-dihydrosphingosine, first and rate-limiting step of this pathway taking place in the membrane of the ER. Ceramide can be transformed in complex sphingolipids, such as sphingomyelin, or degraded to form sphingosine, which can be phosphorylated by sphingosine kinases (SK) 1 and 2 to form S1P

CoA (**Fig.9**, step in green). Recently, a third subunit was identified in mammals, SPTLC3[97], however the stoichiometry of the complex is still unclear [98, 99,100]. The lethality of the homozygous knockout mice for SPTLC1 or SPTLC2 emphasizes the requirement of this pathway for survival. Because SL are involved in many physiological and pathological processes, the expression and regulation of SPTLC1/2 enzyme has attracted much attention. In 2010 Weissman's group discovered an important regulatory system of SPT, via ormdl proteins, linked to childhood asthma[101-103]. Ceramide, central product of this pathway, is a proapoptotic signaling lipid and a precursor to higher order sphingolipids (i.e. sphingomyelin and glycosphingolipids). Sphingosine, the breakdown product of ceramide, can be phosphorylated by two kinases resulting in the S1P[104, 105], a very potent signaling molecule involved in a variety of processes[106], including lymphocyte trafficking, vascular development and inflammation (**Fig. 10**) (Jung et al., 2012; Yang et al., 2004). Although there has been much attention to the involvement of sphingomyelin to ceramide degradation pathway (**Fig. 10**) in cardiovascular disease, very little attention has been paid to the function of *de novo* pathway (**Fig. 10**) in these pathological conditions.

In this regard, Cantalupo and co-authors discovered a novel mechanism by which SL biosynthesis is regulated[3] . Highly expressed in the vasculature, Nogo-B, a membrane protein of the ER, binds to and inhibits SPT, the first and rate-limiting enzyme of the SL *de novo* biosynthesis (**Fig. 10**).

Mice lacking Nogo-B are protected from inflammation[4, 107], hypertension[3, 108] and heart failure[4], in part via increase of endothelial S1P-S1P1 signaling. From these studies emerged the concept that vascular SL homeostasis needs to be tightly regulated to preserve vascular health, and when this mechanism is deranged contributes to the onset of vascular dysfunctions. The protective role of Nogo-A/Bin Nogo-A/B deficient mice in myocardial permeability, inflammation, and fibrosis has been assessed, showing that Nogo-B binds to and inhibits SPT(Cantalupo, 2015 #1993) , thereby controlling local sphingolipid and S1P production, which preserves blood flow through activation of the $S1P_1/S1P_3$/eNOS [3] pathway. In addition, it has also shown that Nogo-B enhances

endothelial barrier functions through $S1P_1$-Rac activation [109, 110], and exerts anti-inflammatory effects [111, 112,] (Peng, 2004 #2031) .

1.7 The role of S1P and Ceramide in vascular tone regulation

Ceramide and S1P are bioactive and interconvertible lipids, exerting mainly opposite biological functions. S1P signaling contributes to maintain CV homeostasis by preserving the endothelial barrier function [110]and vascular tone. The effects of S1P are mainly mediated by five cell surface G-protein coupled receptors known as S1P1-5[113]. The most abundant in EC, S1P1 is a potent activator of eNOS[108, 114] . S1P2 and S1P3 expressed in vascular SMC mediate vasoconstriction to S1P, although deletion of *S1p2* or *S1p3* gene does not impact BP, likely due to compensatory mechanisms [108] Olivera et al., 2010). S1P3 is also involved in the myogenic response of MA[108]. In healthy vessels, at low concentrations, S1P-induced vasodilation prevails on vasoconstriction[108] . EC are also an important source of plasma S1P[115, 116], which is secreted via a specific transporter, spinster-2 (Spns2)[116] . Once outside of the cells, S1P can signal to its receptors in autocrine/paracrine manner or bind to circulating carriers. Previously, we demonstrated that endothelial-derived S1P is a critical regulator of blood flow and pressure, via S1P1-eNOS signaling[108]. Di Lorenzo's Lab discovered how SL biosynthesis and signaling is regulated, which was a breakthrough. Nogo-B, a membrane protein of the ER highly expressed in blood vessels, functions as "brake" of SPT, limiting the *de novo* biosynthesis of SL[3]. While the sphingomyelins are not much affected by the loss of Nogo-B, endothelial S1P and ceramide, with higher turnover, are elevated as a result of higher SPT activity. Systemic and endothelial loss of Nogo-B enhances endothelial-derived S1P and S1P1-NO signaling axis protecting the mice from hypertension, heart failure and inflammation[3, 4, 107]. These findings revealed a key role for endothelial Nogo-B-S1P signaling in CV homeostasis.

Ceramides can regulate multiple cellular processes including stress responses and apoptosis, via activation of different targets, such as protein phosphatase PP2A and PP1[117, 118]and PKC[119]. Contrary to S1P, the regulation of vascular tone by ceramide signaling is poorly understood and controversial. Two studies point toward a ceramide-dependent vasorelaxation[120] in endothelial denuded aortic ring, via RhoA-mediated inhibition[121]. Other studies claimed ceramide-dependent vasoconstriction of pulmonary arteries in response to hypoxia via PKCζ[122] , and denuded canine cerebral arteries, via PKC as well[123]. However, high concentrations of ceramides are known to be toxic (i.e. 10μM) and eliciting non-specific effects. These controversial findings could be due to different animal models, vascular beds (thoracic aorta, venules, cerebral arteries(Jang, 2005 #2166) [121, 123, 124] ; ceramide species and concentrations; and lastly, because ceramide and S1P are interconvertible, the formation of S1P could account for vasodilating effects of ceramide[125].

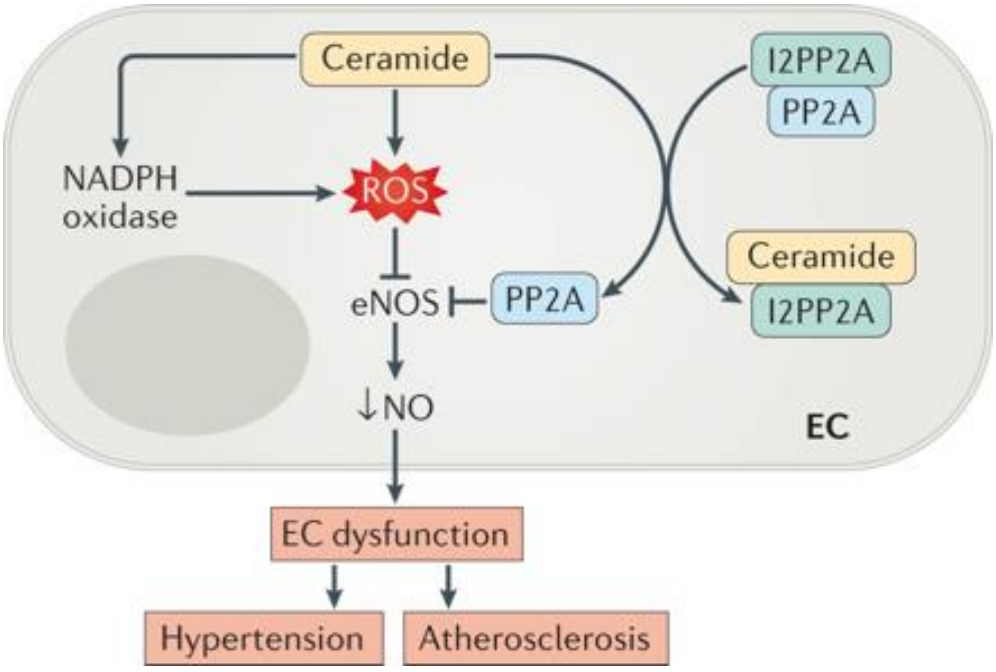


Figure 10. **Ceramide activity on eNOS signaling**. Accrual of ceramide initiates PP2A colocalization with eNOS and increased association between PP2A and eNOS at the plasma membrane promotes dissociation of an Akt-Hsp90-eNOS complex that is required for eNOS phosphorylation and activation. (*Adopted from Ran Hee Choi et al., 2021*).

More clear evidence on the role of ceramide in endothelial dysfunction in type 2 diabetes are provided by two seminal studies from the Symons's group. In the first study, Zhang et al., 2012 demonstrated that accrual of ceramide initiates PP2A colocalization with eNOS and increased association between PP2A and eNOS at the plasma membrane promotes dissociation of an Akt-Hsp90-eNOS complex that is required for eNOS phosphorylation and activation, so PP2A activation precipitates vascular dysfunction in diet-induced obesity. In the following study Leena P. et al., 2015 the same group demonstrated that A novel small-molecule inhibitor of PP2A attenuated PP2A activation and prevented disruption of the Akt-Hsp90-eNOS complex in the vasculature, preserved arterial function, and maintained normal blood pressure in obese mice. From these studies emerged the concept that in obesity ceramide accrual occurs in the vasculature and is causal of dysfunction. However limitation of the aforementioned studies are 1) the accrual of ceramide was reported in the aorta[6] and does not necessarily extend to resistance arteries, accountable for BP regulation; 2) the concept that in obesity ceramide increases in the endothelium of the vasculature and is causal of dysfunction has been extrapolated by *in vitro* experiments (i.e. cultured EC)[6, 7] which do not necessarily recapitulate the *in vivo* conditions. Along the same line, literature investigation on type 1 diabetes and sphingolipids is still poorly understood.

1.8 Aim of the study

The literature examined clearly suggests that SLs, and particularly ceramides, are involved in the development of metabolic co-morbidities of obesity included IBVD, hyperglicemia and dyslipidemia. The oversupply of fat to tissues induces cellular dysregulation with consequent deleterious activity on insulin sensitivity, vascular reactivity, and mitochondrial metabolism [126]. In this context, it has been demonstrated that ceramide diminishes insulin synthesis by decreasing its mRNA levels in pancreatic islets, and that in β cells, saturated FA impair insulin gene expression and increase ceramide levels [127] . Elevated levels of lactosylceramide have been found also in

mitochondria from diabetic heart tissue suggesting that it represents the primary SL responsible of mitochondrial defects in diabetic heart [128] . Starting from these findings, the first part of my PhD has been focused on the possible role of SPT/Nogo-B signaling in vascular homeostasis and its possible involvement, in terms of SLs/ceramides dysregulation, in the IBVD associated to type 1 diabetes.

2 Materials and Methods

2.1.1 Animals

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals and approved by the Committee Centro Servizi Veterinari of the University Federico II and the Weill Cornell Institutional Animal Care and Use Committee; this work was carried out with NOD/Ltj mice purchased from Charles River Laboratories (Milan, Italy) and Jackson Laboratory (Bar Harbor, Maine). All experiments were performed by using 7- to 27-week-old female mice. Animals were bred and housed in our animal facility having free access to water and food.

2.1.2 NOD/ShiLtJ mice

NOD/ShiLtJ mice strain is a polygenic model for autoimmune type 1 diabetes (IDDM) (Makino et al., 1980). Diabetes in NOD mice is characterized by severe hyperglycemia and insulitis, a leukocytic infiltration of the pancreatic islets. Marked decrease in pancreatic insulin content occurs in females around 12 weeks of age and several weeks later in males. Immune phenotypes in the NOD background consists of defects in antigen presentation, T lymphocyte repertoire, NK cell function, macrophage cytokines production, wound healing, and C5 complement. Female NOD mice develop spontaneously and progressively type 1 diabetes disease starting from the 7 to 27 weeks of age. As previously shown, in these a progressive endothelium impairment related to hyperglycemia levels takes place (Bucci, 2004 #1721) .

For our purposes, NOD mice, starting from 6 weeks of age, glycemia values have been monitored weekly to establish the disease progression, classified as follows according to the glycemic levels:

-NODI glycemia< 150 mg/dL = healthy normoglycemic mice;

-NODIII: glycemia> 400 mg/dL = diabetic mice.

The progression of diabetic pathology in these animals is associated with vascular disorders as it happens in humans (Bucci, 2004 #1721) .

2.2 Glycemia measurement

Animals were deprived of food for 2 h from 1 p.m. to 3 p.m. to normalize glucose plasma levels. Then, glycemia value was measured on the blood drop from tail vein following a superficial incision. According to glycemia values the mice were classified into two different groups NODI and NODIII.

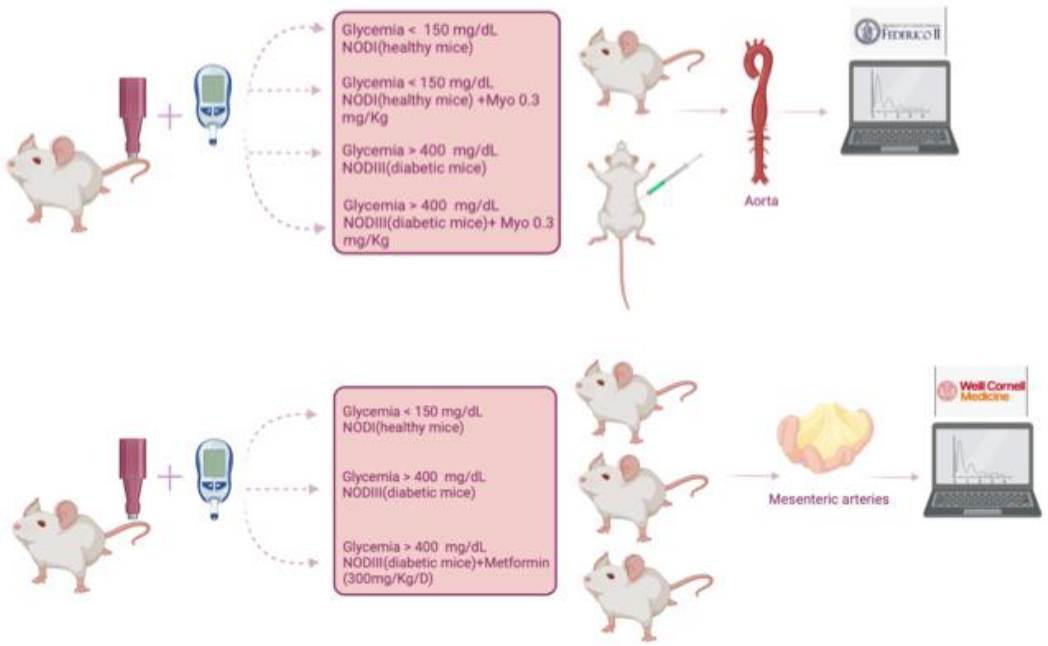


Figure 11. Representation of NOD mice diabetes-development, treatment with myo and metformin and tissues harvested to achieve the two aims of study.

(A) NOD mice according to glycemia levels were divided in two groups, normoglycemic and diabetic, they were subjected to only one dose of Myo, 24h. They were sacrificed and aorta harvested to evaluate vascular reactivity.

(B) NOD mice were divided in three groups, NODI, NODIII and NODIII+metformin, once they have been hyperglycemic for 4 weeks, they were sacrificed and mesenteric arteries harvested to test vascular reactivity.

2.3 Vascular reactivity on aortas rings

NOD mice were sacrificed at different stages of pathology, aorta was rapidly harvested, and adherent connective and fat tissue were removed. Rings of 1–1.5 mm length were cut and placed in organ baths (3.0 mL) filled with oxygenated (95% O2–5% CO2) Krebs' solution. The rings were connected to an isometric transducer (7006, Ugo Basile, Comerio, Italy) and changes in tension were continuously recorded with a computerized system (PowerLAb, ADInstrument). The rings were initially stretched until a resting tension of 1.5 g was reached and then were allowed to equilibrate for at least 45 min; during this period the tension was adjusted, when necessary, to 1.5 g and the bath solution was periodically changed. In each set of experiments, rings were firstly challenged with phenylephrine (PE, 1μM) until the responses were reproducible. To verify the integrity of the endothelium, cumulative concentration-response curves to ACh (10 nM–30 μM) were performed with PE pre-contracted rings. Rings not reaching a relaxation response of at least 75% were discarded.

The composition of Krebs solution was as follows (in mM): NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25 and glucose 11.

2.4 Myriocin treatment

NODI and NODIII mice have been treated with the myriocin, a selective inhibitor of SPT (myriocin, Sigma) at dose of 0.3 mg/kg by intraperitoneal injection or vehicle (0.4% fatty acid–free BSA). Following 24h, mice were sacrificed, and aorta harvested for *ex vivo* pharmacological studies or conscious systemic blood pressure measurement were performed.

2.5 Metformin treatment

In a separate set of experiments, once glycemia reached values over 150 mg/dL, NOD mice were treated with metformin (Cayman chemical company #13118) at dose of 300 mg/Kg/daily in drinking water and replaced every week. To avoid different hyperglycemia conditions among mice, as endpoint for *ex viv*o experiments was established a value of glycemia >400mg/dL for a time window of 4 weeks. Then, mice

were sacrificed, and mesenteric arteries were harvested for *ex vivo* pharmacological studies.

2.6 Experimental protocol for vascular reactivity on aortas rings

In each set of experiments, rings were firstly challenged with PE (1 µM) until the responses were reproducible. To verify the integrity of the endothelium, cumulative concentration-response curves to ACh (10nM30 µM) were performed on PE pre-contracted rings. Then, Isoprenaline (Iso) cumulative concentration-response curve (10nM-30 µM) was performed in both NODI and NODIII aortas. In a separate set of experiments, once rings have been standardized, PE and 5-HT cumulative response curves were performed.

Aortic rings harvested from NODI mice were incubated with Ceramide C-2 10µM or vehicle (dmso) for 15 minutes, then cumulative concentration response curves to vasoconstrictor agents PE and 5-HT have been performed, as well as to Ach and Iso as endothelium-dependent vasorelaxant agents.

2.7 Experimental protocol for vascular reactivity on mesenteric arteries

Second order of mesenteric arteries (MA) harvested from all groups of mice were carefully cleaned by all fat tissue and were further cut into rings (2mm in length). MA were mounted in a small vessel four-chamber wire myograph for measurement of isometric tension (Danish Myo Technology, model 620). After a 15-min equilibration period in Krebs solution as previous described, bubbled with carbogen at 37 °C and pH=7.4, arteries were stretched to their optimal lumen diameter for active tension development that is 0,25 g. After a further 15-min equilibration period, arteries were challenged with PE (1µM) and washed. To verify the integrity of the endothelium, cumulative concentration-response curves to ACh (10nM- 30µM) were performed on PE pre-contracted arteries.

2.8 Experimental protocol and drug treatments on mesenteric arteries

Once verified the endothelial function, PE (1nM- 100 µM) and thromboxane analog cumulative response curves (U46619,1 nM−1 µM) have been performed. To assess vascular reactivity, on U46619 pre-constricted arteries Ach (1nM- 10 µM) and sodium nitroprosside SNP (0.1 nM –30 µM) cumulative concentration-response were performed. In a separate set of experiments, arteries were exposed to eNOS inhibitor L-NAME at concentration of 100 µM for 20', thereafter Ach cumulative concentration-response curve was performed. In a further set of experiments MA from NODI and NODIII treated with metformin were incubated with ceramide (0.3µM) for 15-20 minutes, or with a combination of ceramide C-6 (0.3µM) and Okadaic Acid (90nM) for 15-20 minutes, thereafter Ach cumulative concentration response curve was performed.

2.9 Quantitative real-time PCR

The mRNA levels of SPT and Nogo-B have been evaluated in aorta of both NODI and NODIII mice by quantitative real-time PCR. Total RNA from perfusate and tissue was extracted according to the TRIzol reagent protocol (Thermo Scientific). Reverse transcription was then performed using 100 ng of RNA and Maxima Reverse Transcriptase (200 U/µl) supplied with 5× buffer (250 mM Tris-HCl (pH 8.3 at 25 °C), 375 mM KCl, 15 mM MgCl2, 50 mM DTT; Thermo Scientific, USA). The real-time PCR analysis was done with an iCycler Applied Biosystems 7700, using SYBR green PCR Master Mix (Qiagen, USA).

2.10 Mass spectrometry-based metabolomics, statistics and analysis

NODI and NODIII aorta were homogenized using a potter in 1 ml of pre-chilled methanol/water 1:1 solution, containing 10 nmol of internal standard, and centrifuged at 10,000 g for 10 min at 4°C (Ser et al., 2015). The resulting supernatants were collected and transferred into Eppendorf tubes and stored at -80°C. The pellets were extracted in Dichloromethane and recentrifuged at 10,000 g for 10 min at 4°C. The

resulting supernatants were collected and transferred into Eppendorf tubes and vacuum dried. Analyses were performed according to previous protocol (Sommella et al., 2019). Data were acquired on a SolariX XR 7T (Bruker Daltonics, Bremen, Germany). The instrument was tuned with a standard solution of sodium trifluoracetate. Mass Spectra were recorded in broadband mode in the range 100–1500 m/z, with an ion accumulation of 20 ms, with 32 scans using 2 million data points (2M). Nebulizing (N2) and drying gases (air) were set at 1 and 4 mL/min, respectively, with a drying temperature of 200 °C. Both positive and negative ESI ionization was employed. Five replicates of each injection were carried out. The instrument was controlled by Bruker FTMS Control, MS spectra were elaborated with Compass Data Analysis version 4.2 (Bruker); identification of compounds based on accurate MS measurements was performed by Compound Crawler ver. 3.0 and Metaboscape 3.0 (Bruker). Metabolites signals were normalized using internal standards.

2.11 Microsomal isolation from mouse lung

Microsomal fractions were prepared from lungs of NODI, NODIII, and NODIII mice treated with metformin[129]. Briefly, lungs were homogenized in microsome preparation buffer (50 mM HEPES pH 7.4, 0.25 M sucrose, 5 mM EDTA pH7.4 with NaOH, the homogenates were centrifuged for 15 min at 18,000 × *g* at 4 °C, and the resulting supernatants were ultracentrifuged for 1 h at 100,000 × *g*. The microsomal pellets were then resuspended in SPT reaction buffer (0.1 M HEPES pH 8.3, 5 mM DTT, 2.5 mM EDTA pH 7.4, 50 μM pyridoxal 5′-phosphate

2.12 Serine palmitoyl-transferase (SPT) activity

SPT activity in lung microsomes was measured[129]. Briefly, to 100μL of microsomal suspension were added 0.45 μM [^{3}H]serine and 0.2 mM palmitoyl-CoA. The samples were incubated for 15 min at 37 °C and the reaction was stopped with 50 μl of $NaBH_4$ (5 mg/ml) to convert the reaction product 3-ketosphinganine into Sphinganine. Radiolabeled lipid products were extracted by using a modification of Bligh and Dyer's

method[61]. Briefly, 0.75 ml $CHCl_3$ and methanol (1:2) was added, followed by 0.25 mL of $CHCI_3$ and 0.25mL of NH_4OH (1:1). After centrifugation, the lower layer containing lipids was collected and the organic solvent was removed under a gentle stream of nitrogen gas. The samples were dissolved in $CHCl_3$ and analyzed by thin-layer chromatography.

For the measurement of SPT activity in HUVECs, the cells were grown to confluence in 60mm dishes. 24 hours before collections HUVECs were incubated in endothelial growth basal medium (EBM2) with 10% charcoal-stripped FBS and, where indicated, 25mM D-glucose and 1mM metformin. After washing twice with PBS, the cells were suspended in SPT reaction buffer and sonicated for 15 s. Cell lysates (200 μg) were mixed with 0.45 μM [^{3}H]serine and 0.2 mM palmitoyl-CoA. SPT reaction and analysis were carried on as indicated for microsomes.

2.13 Western blot

Aortas were snap-frozen in liquid nitrogen, pulverized, homogenized in RIPA buffer, and proteins were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Primary antibodies included the following: Nogo-B (#AF-6034; R&D, Minneapolis, MN, dilution 1:1,000); SPTLC1 (#611304; BD, Biosciences, San Jose, CA; dilution 1:1,000); phospho-S239-VAPS and VASP (#3114 and #3132, Cell Signaling Technology, dilution 1:1000); β-actin (#A2228; Sigma Aldrich, St. Louis, MO; dilution 1:2,000).

2.14 Blood pressure measurements in conscious mice

Systolic, diastolic, and mean blood pressure was measured in conscious mice using the pneumatic tail-cuff method (MRBP System, Life Science, Woodland Hills, California). Briefly, animals were placed in a plastic chamber maintained at 34 °C and a cuff with a pneumatic pulse sensor was attached to the tail. After 2 week of training, three consecutive measurements were performed per mouse, and the values were averaged.

In another set of experiments, mice were injected with myriocin (0.3 mg/kg i.p.) or vehicle (0.4% fatty acid free BSA) and blood pressure was measured 1 d later.

2.15 NOx assay

Mouse aortic tissues harvested from NODI, NODIII and NODIII treated with myo, were homogenized in modified RIPA buffer (50-mM Tris–HCl pH 8.0, 150-mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1-mM EDTA, 1% Igepal; Roche Applied Science, Monza, Italy) and protease inhibitor cocktail (Sigma-Aldrich) and then incubated in a microplate with cadmium (Sigma-Aldrich; 50 mg per well) for 1 hr to convert the inorganic anion nitrate (NO_3^-) to nitrite (NO_2^-). After centrifugation at 8000× g, total NOx content was determined using a fluorometric method by Promega Glomax explorer (Madison, WI) and calculated against a standard curve of sodium nitrite ($NaNO_2$, 50–2,000 nM; Sigma-Aldrich). Each independent experiment was performed in duplicate. Data, expressed as mean ± SEM, were reported as nmol/mg of protein.

2.16 H_2O_2 measurement

Aorta from NODI, NODIII and NODIII treated with myo, were homogenized in modified RIPA buffer (50-mM Tris–HCl pH 8.0, 150-mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1-mM EDTA, 1% Igepal; Roche Applied Science, Monza, Italy) and protease inhibitor cocktail (Sigma-Aldrich). Briefly, samples were mixed with a colorless Colorimetric Substrate and the reaction initiated by addition of horseradish peroxidase. The reaction was incubated at room temperature for 15 minutes. The HRP reacts with the substrate in the presence of hydrogen peroxide to convert the colorless substrate into a pink-colored product. The pink product was read at 560 nm by using a spectrophotometer. The levels of H_2O_2 were calculated against a calibration curve of hydrogen peroxide (3.125-100 µM) according to the manufacturer's instruction (arbor assay).

2.17 Statistical analysis

Data are expressed as mean ± s.e.m. One-way or two-way ANOVA with *post hoc* Bonferroni's test and Two-way ANOVA with Sidak post-test were run for all statistical analyses except where a Student's *t*-test analysis was used. Differences were considered statistically significant when $p < 0.05$. All tests were two-sided. GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA) was used for all statistical analysis.

2.18 Drugs

C6 Ceramide (d18:1/6:0) (# 62525) from Cayman chemical

Okadaic Acid | CAS 8111-17-8 | Cayman Chemical

U-46619 | CAS 56985-40-1 | Cayman Chemical

C-2 Ceramide | CAS 3102-57-6 | Cayman Chemical

(R)-(−)-Phenylephrine hydrochloride | CAS 61-76-7 Sigma

Acetylcholine chloride >=99%TLC | 60-31-1 | Sigma

Sodium Nitroprusside, Dihydrate | 13755-38-9 – Sigma

Nω-Nitro-D-arginine methyl ester | 50912-92-0 | Sigma

Sucrose >= 99.5 % | 57-50-1 | Sigma

Sodium nitrite ACS reagent, >= 97.0 % | 7632-00-0 | Sigma

D-(+)-Glucose >=99.5%GC | 50-99-7 | Sigma

Sodium bicarbonate ACS reagent, >= 99.7 % - Sigma

Potassium chloride 99.999 % trace metals | 7447-40-7 | Sigma

Magnesium sulfate BioXtra,>=99.0% | 10034-99-8 | Sigma

Sodium chloride ACS reagent, >= 99.0 % | 7647-14-5 | Sigma

Potassium phosphate monobasic | CAS No.7778-77-0 | Sigma

Calcium chloride anhydrous, beads, | 10043-52-4 | Sigma

Sodium nitrite ACS reagent, >= 97.0 % | 7632-00-0 | Sigma

Bovine Serum Albumin fraction V, ≥96% (GE) | 9048-46-8 | Sigma

Nogo-B (#AF-6034; R&D, Minneapolis, MN, dilution 1:1,000)

SPTLC1 (#611304; BD, Biosciences, San Jose, CA; dilution 1:1,000)

phospho-S239-VAPS and VASP (#3114 and #3132, Cell Signaling Technology, dilution 1:1000)

β-actin (#A2228; Sigma Aldrich, St. Louis, MO; dilution 1:2,000)

Sodium dodecyl sulfate >= 90 % | 151-21-3 | Sigma

Tris hydrochloride | 1185-53-1 | Sigma-Aldrich

DTT 1,4-Dithiothreitol | 3483-12-3 | Sigma-Aldrich

3 Results

3.1. Myriocin significantly reduces endothelium-dependent vasorelaxation in healthy mice (NODI)

In NODI mice treated with the SPT inhibitor myriocin (myo), Acetilcholine- (Ach) (**Fig. 12A**) and Isoprenaline- (Iso) (**Fig. 12B**) induced vasorelaxation were significantly reduced compared to NODI treated with only vehicle (Ach E_{max}=66,9±3.5 vs 50±5.9 % of relaxation vehicle vs myo, respectively; IsoE_{max}=41.0±4.4 % relaxation vs. 56.15±7.8 % relaxation; myo vs vehicle respectively).

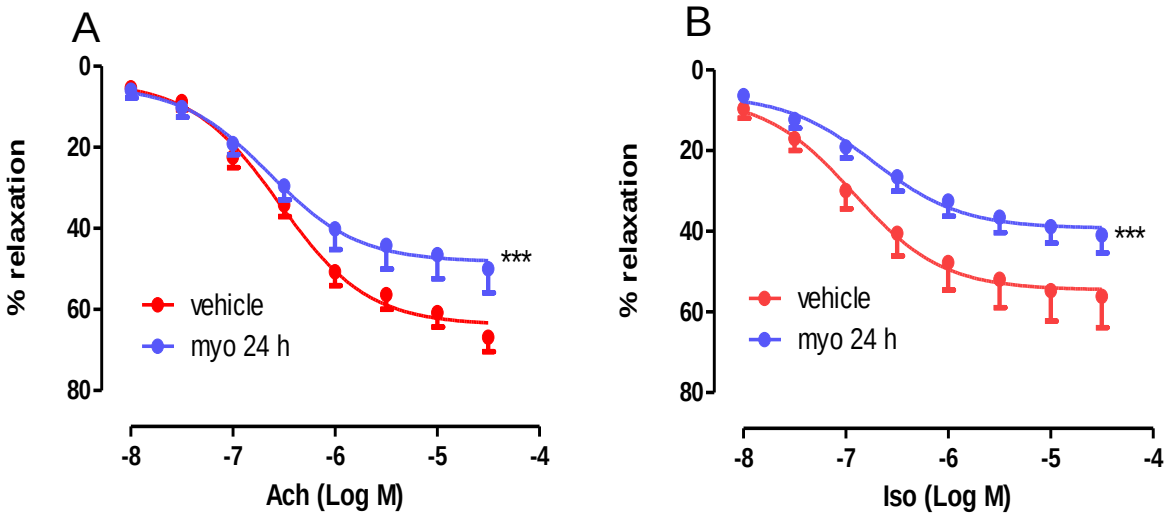


Figure 12. **Myriocin significantly reduces endothelium-dependent vasorelaxation in healthy mice (NODI)**. Myriocin administration have been performed in NODI mice by i.p. injection at dose of 0.3 mg/kg. Following 24h, aorta has been harvested, cleaned and cut in rings and then mounted in isolated organ bath for evaluation of vascular reactivity. Once the rings have been standardized, Ach- and Iso- cumulative concentration response curve have been performed on stable tone of PE (1 µM), (n=5 mice; *** = p<0.001 vs. vehicle)

3.2 Myriocin significantly reduces both PE- and 5-HT-induced vasoconstriction in healthy mice (NODI)

In NODI mice treated with myo, PE-(**Fig. 13A**) and 5HT- (Fig. 13B) induced contraction were significantly reduced compared to NODI mice treated with only vehicle (PE E_{max}= 1507,7±156,8 vs. 908,9±107dine/mg, vehicle vs. myo, respectively; 5HT E_{max}=1283.3±154.49 dine/mg vs. 1700.7±294.13 dine/mg; myo vs. vehicle, respectively).

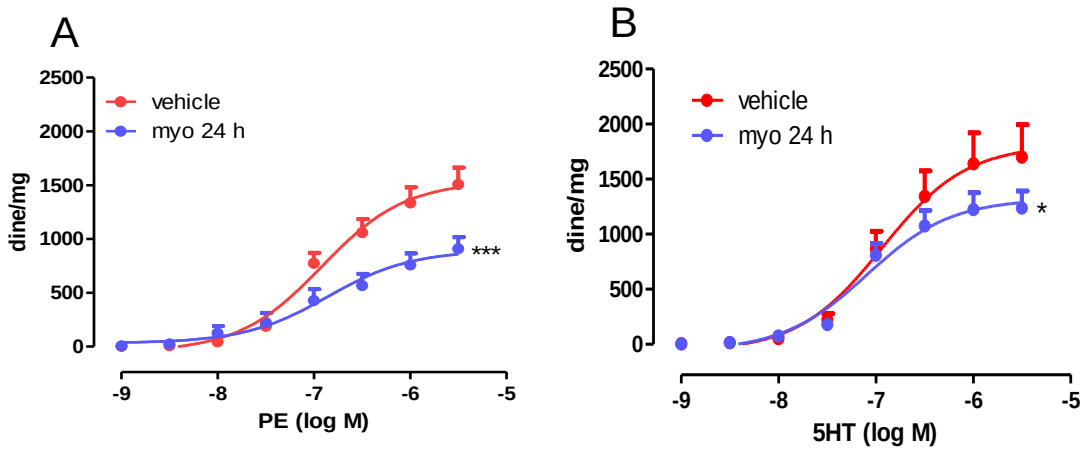


Figure 13. Myriocin significantly reduces both PE- and 5-HT-induced vasoconstriction in healthy mice (NODI). Myriocin administration have been performed in NODI mice by i.p. injection at dose of 0.3 mg/kg. Following 24h, aorta has been harvested, cleaned, and cut in rings and then mounted in isolated organ bath for evaluation of vascular reactivity. Once the rings have been standardized, PE- and 5HT- cumulative concentration response curve have been performed. (n=5 mice; * = p<0,05; ***=p<0.001 vs. vehicle).

3.3 Myriocin significantly ameliorates the impaired PE-and 5-HT-induced vasoconstriction observed in diabetic mice (NODIII)

Aortas of diabetic mice (NODIII) displayed a significant impaired contraction when challenged with both PE (**Fig. 14A**; E_{max}=972.35±189 dine/mg vs1507,7±186.85 dine/mg, NODIII vs. NODI respectively) and 5HT (**Fig. 14B**; $E_{max=}$ 646±100.7 dine/mg vs. 2345.7±324.7 dine/mg; NODIII vs. NODI respectively), confirming the vascular dysfunction associated to hyperglycemia characteristic of this disease (Bucci et al., 2004).Treatment of diabetic mice (NODIII) with the SPT inhibitor myriocin, recovered the PE-induced contraction to the same extent observed in normoglycemic mice (NODI) (**Fig. 14A**; E_{max} = 1808,4±254 dine/mg vs. 1507.704±156.85 dine/mg; myo vs. vehicle, respectively) and significantly improved 5-HT-induced vasoconstriction (**Fig. 14B**; E_{max}=1560.9±223.15 dine/mg vs. 1700.73±294.13 dine/mg; myo vs. vehicle respectively).

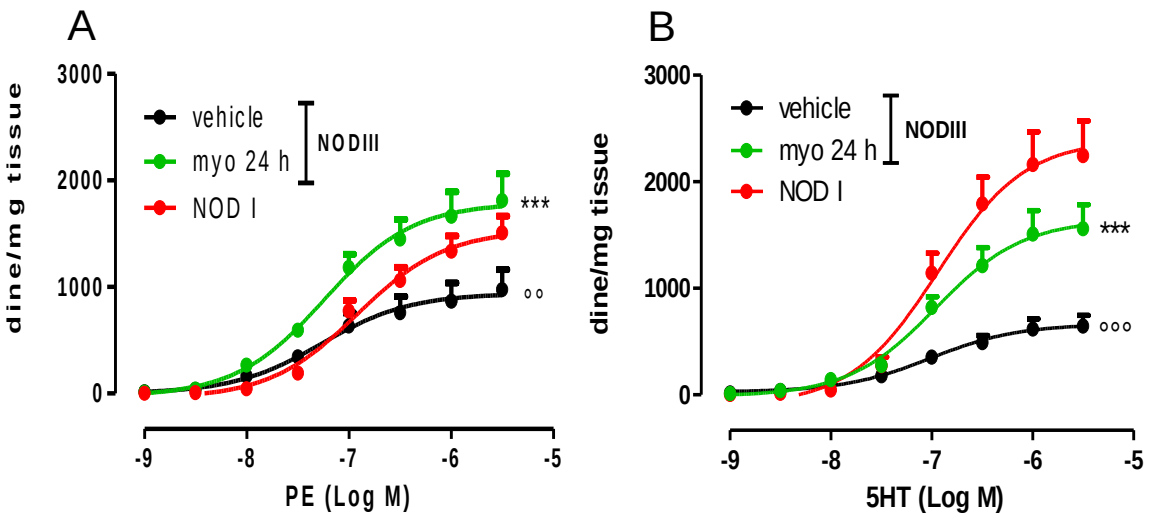


Figure 14. Myriocin significantly ameliorates the impaired PE-and 5-HT-induced vasoconstriction observed in diabetic mice (NODIII)

Myriocin administration have been performed in NODIII mice by i.p. injection at dose of 0.3 mg/kg. Following 24h, aorta has been harvested, cleaned and cut in rings and then mounted in isolated organ bath for evaluation of vascular reactivity. Once the rings have been standardized, PE- and 5HT- cumulative concentration response curve have been performed.(n=5 mice; *** =p<0.001 myo 24h vs. vehicle; °° = p<0.01; °°°= p<0.001 NODI vs. NODIII).

3.4 Myriocin does not affect the impairment of both Ach- and Iso-induced vasodilation in diabetic mice (NODIII)

As expected, aortas of diabetic mice displayed a significant impairment of vasorelaxation following both Ach (**Fig 15A**; E_{max}= 34.8±3.9 % relaxation vs. 66.6±4.01 % relaxation; NODIII vs. NODI respectively) and Iso (**Fig 15B**; E_{max}=32.7±7.49 % relaxation vs. 56.15±7.8 % relaxation NODIII vs. NODI respectively) addition, due to endothelium dysfunction [81] . Conversely to what observed for vasoconstriction, the treatment of NODIII mice with myriocin, did not affect neither Ach-nor Iso-induced relaxation (Ach E_{max}=34.5±4.1 % relaxation vs. 34.8±3.9 % relaxation; myo vs. vehicle respectively; Iso E_{max} = 36.6±6.42 % relaxation vs. 32.7±7.49 % relaxation; myo vs. vehicle respectively).

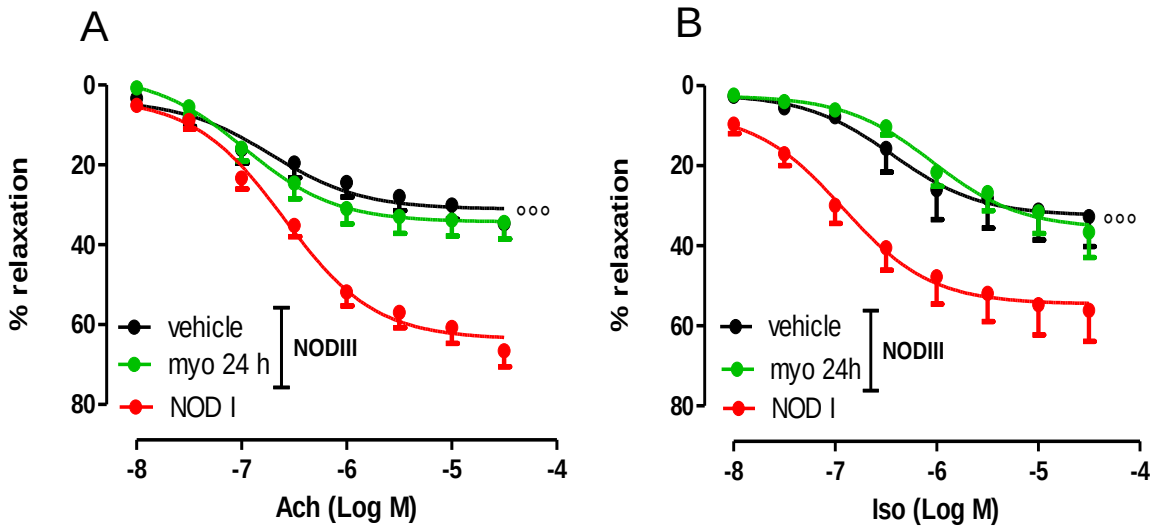


Figure 15. **Myriocin does not affect the impairment of both Ach- and Iso-induced vasodilation in diabetic mice (NODIII)**

Myriocin administration have been performed in NODIII mice by i.p. injection at dose of 0.3 mg/kg. Following 24h, aorta has been harvested, cleaned and cut in rings and then mounted in isolated organ bath for evaluation of vascular reactivity. Once the rings have been standardized, Ach- (A) and Iso- cumulative concentration response curve (B) have been performed on stable tone of PE (1 μM). (n=5 mice; °°° = $p<0.001$ NODIII vs NODI).

3.5 SPT expression is upregulated in aortas of diabetic mice (NODIII)

SPT is the first rate-limiting enzyme of *de novo* biosynthesis of SLs and, in healthy vessels, it bounds to Nogo-B that negatively regulates its activity [3] . To define the possible role of SLs in hyperglycemia-related vascular dysfunction SPT and Nogo-B expression have been evaluated in aortas of both NODI and NODIII mice. As shown in (**Fig. 16A)**, SPT mRNA expression was significantly enhanced in NODIII compared to NODI mice aortas. Conversely, Nogo-B was not significantly different between NODI and NODIII aortas (**Fig. 16B**). As shown in figure 16 D-E, western blot analysis performed on the same tissues confirmed the selective increased expression of SPT in NODIII mice. When the data obtained have been evaluated as SPT/Nogo-B ratio, it appears clear that the unbalance between the two proteins takes place in aortas of diabetic mice toward SPT (**Fig 16C-F**). This finding strongly suggests that modifications of SPT/Nogo-B ratio leads to increased SLs biosynthesis contributing to the endothelial dysfunction related to hyperglycemia.

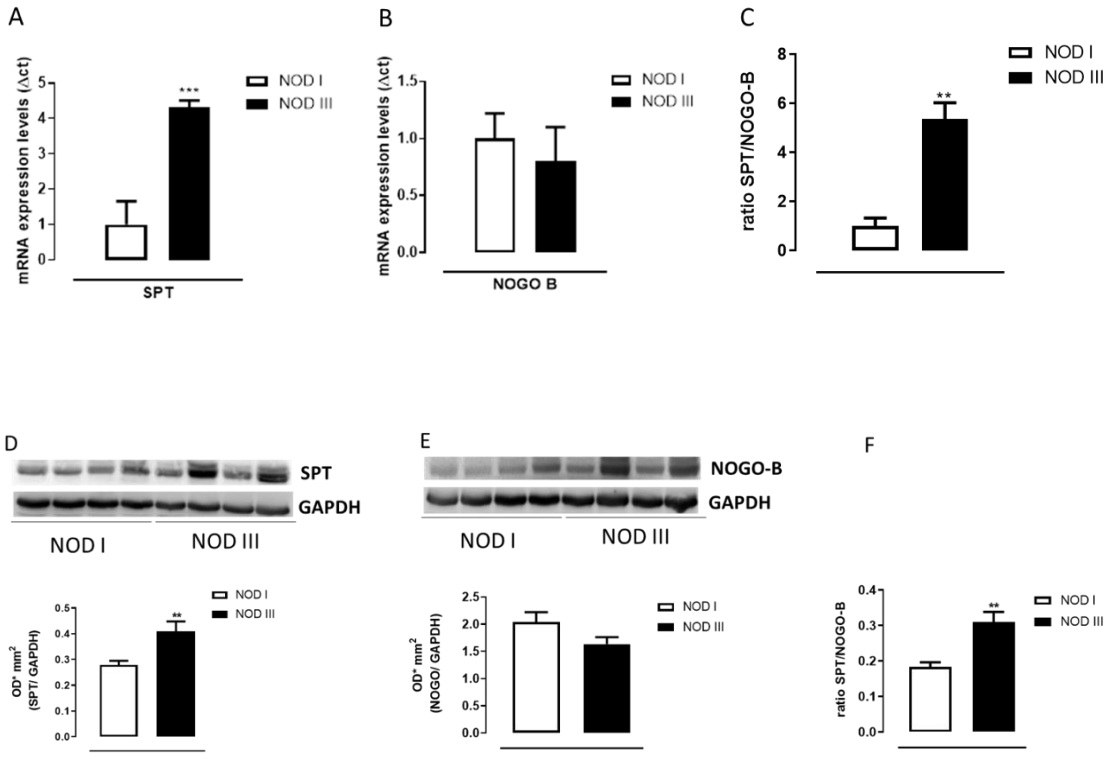


Figure 16. SPT, expression is upregulated in aortas of diabetic mice (NODIII).

PCR and Western Blot analysis have been performed on aorta homogenates harvested from healthy mice (NODI) and diabetic mice (NODIII). (A) SPT mRNA expression, (B) Nogo-B mRNA expression, (C) mRNA SPT/Nogo-B ratio, (D) SPT protein expression, (E) Nogo-B protein expression, (F) protein SPT/Nogo-B ratio (n= 4 mice for PCR analysis, n=7 mice for western blot analysis. ****=** $p<0.01$ NODIII vs NODI; ***=$p<0.001$ NODIII vs NODI).

3.6 Diabetic mice (NODIII) display a mild hypertension that is further increased by myriocin treatment

In diabetic mice (NODIII) the systemic blood pressure (SBP) was significantly increased compare to healthy mice (NODI), as shown in (**Fig. 17A**). Treatment with myriocin (0.3 mg/kg, 24h) further increased SBP, confirming the data obtained in *ex vivo* performed on aorta harvested form NODIII mice treated with myo.

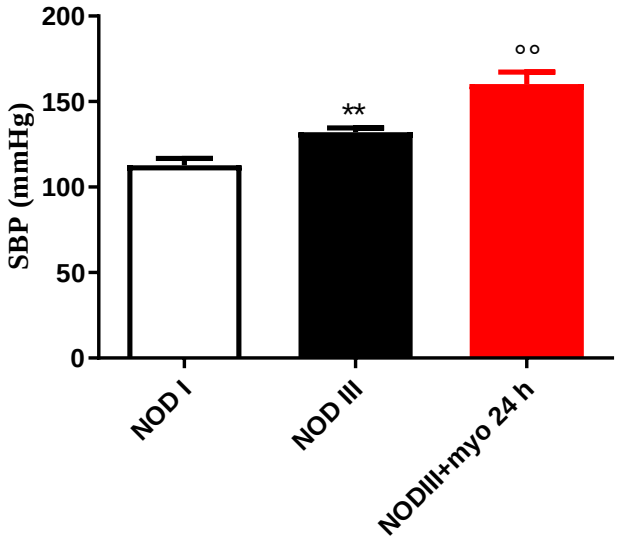
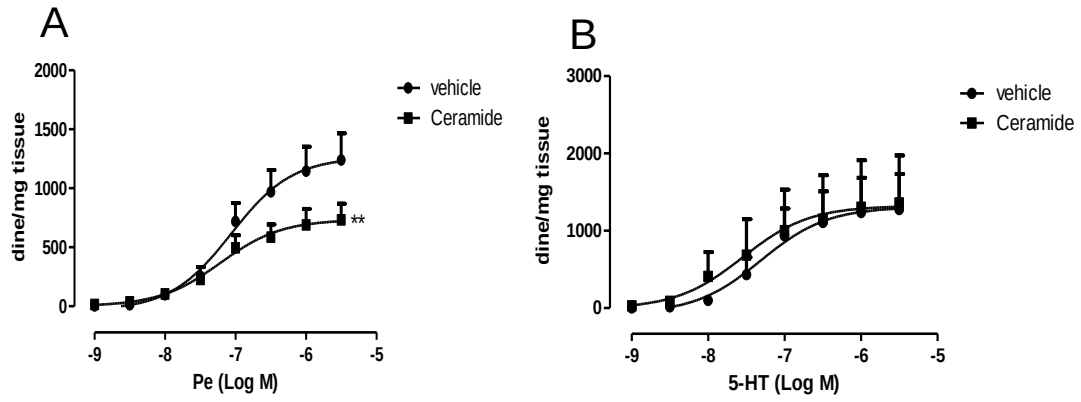


Figure 17. **Diabetic mice (NODIII) display a mild hypertension that is further increased by myriocin treatment.** Systolic blood pressure has been measured by using tail cuff method. After 2 weeks of training to use mice to manipulation, myriocin administration have been performed in NODIII mice by i.p. injection at dose of 0.3 mg/kg. Following 24h, three consecutive measurements of systolic pressure for each mouse have been recorded, and the values averaged. (n= 12 mice; ** = $p<0.01$ NODI vs. NODIII; °° = $p<0.01$ NODIII + myo 24h vs. NODIII)

3.7 Ceramide directly affects vascular reactivity contributing to IBVD appearance

In order to verify if higher levels of ceramide within the vessels could affect vascular reactivity contributing to IBVD appearance, aorta harvested from healthy mice has been exposed to ceramide (10 μM or vehicle, DMSO, for 15 minutes), then vascular reactivity has been tested by using isolated organ bath technique. As shown in **Fig. 18**, incubation of the aortic rings with ceramide leads to significant impairment of vascular reactivity. In particular, PE (**Fig. 18A**), but not 5-HT-induced vasoconstriction (**Fig. 18B**), was significantly reduced, as well as Ach- and isoprenaline-induced vasorelaxation (**Fig.18C-D**). Such pattern of vascular reactivity is almost superimposable to one observed in NODIII mice, in which diabetic condition are severe. This finding strongly suggests a role of SPT-derived ceramide in IBVD caused by hyperglycemia.



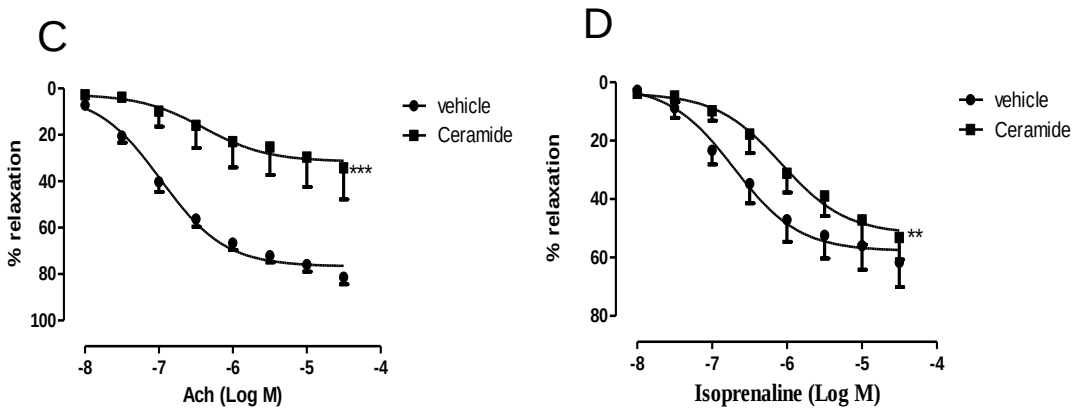


Figure 18. Ceramide directly affects vascular reactivity contributing to IBVD appearance. Aortas harvested from NODI mice have been incubated with ceramide (10 µM) or vehicle (DMSO) for 15 minutes, then vascular reactivity has been evaluated. Once the rings have been standardized, PE (A) and 5-HT (B) cumulative response curves have been performed. In another set of experiments, once the rings have been standardized, Ach (C) and Iso (D) cumulative concentration response curves have been performed on stable tone of PE (1 µM). (n=5 mice; **= p< 0.01, ***= p< 0.001 ceramide vs. vehicle).

To confirm the causative role of ceramide accumulation in diabetic-related vascular dysfunction, aorta and plasma harvested from NODI and NODIII mice have been processed and used for ceramide, glucosylceramide and sphingomyelin (as representative SLs) determination by performing a metabolomic approach (**Fig. 19**). Preliminary data show that ceramide and glucosylceramide levels are significantly higher in NODIII aorta compared to NODI. As opposite, plasma levels of both SL are reduced in NODIII compared to NODI. These results, even though does not reach statistical significance, reinforce the hypothesis that diabetic condition favors ceramide accumulation in vascular wall altering the vascular function. However, further determinations, performed on a wide number of samples, are required to confirm this hypothesis.

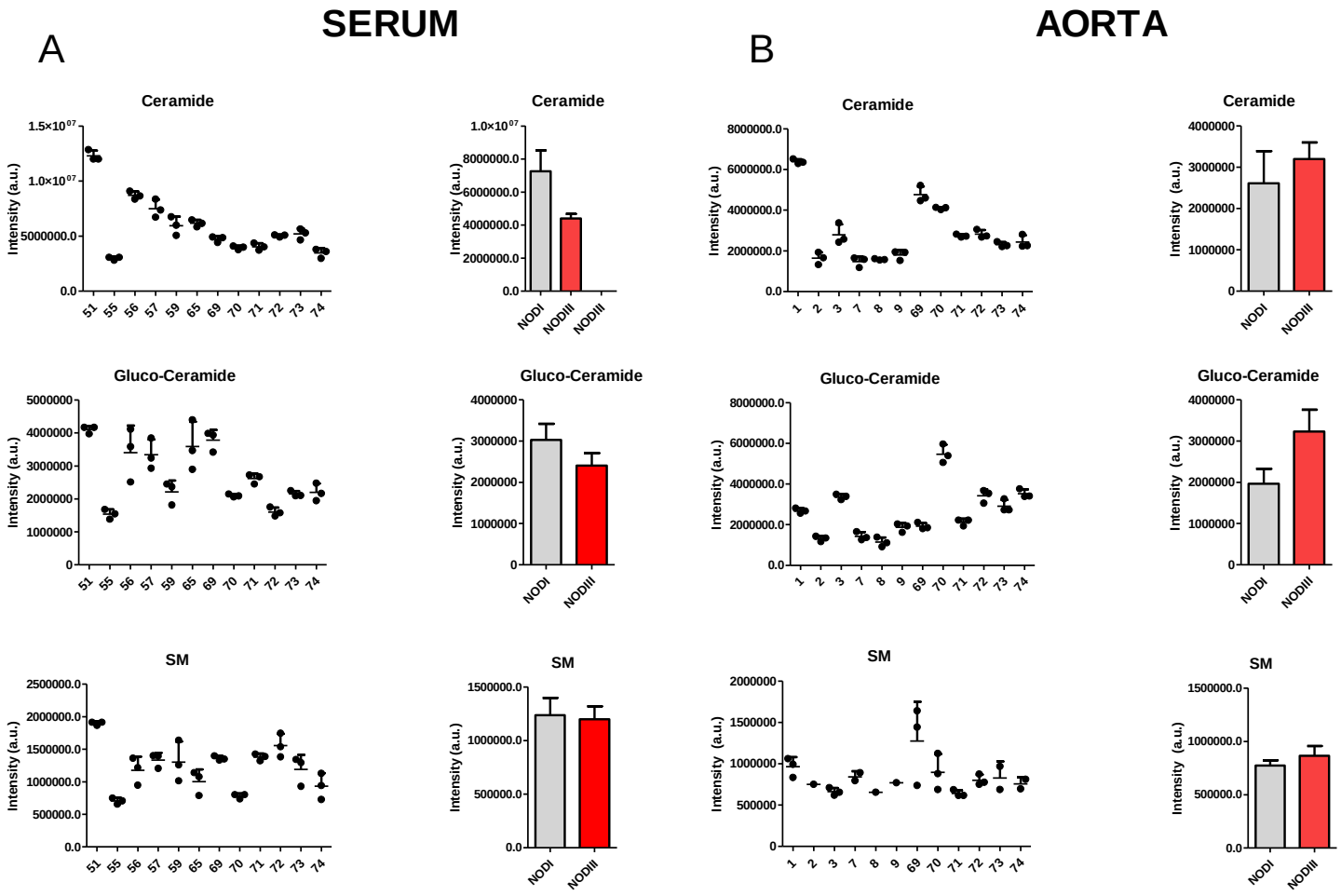
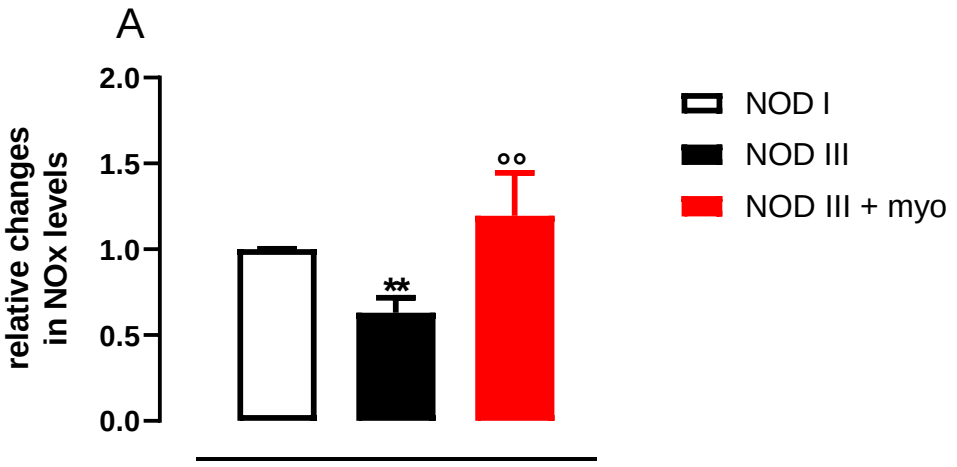


Figure 19. In aortas wall of NODIII mice occurred an increase of ceramide and glucosylceramide *contents.* Evaluation of ceramide, glucosylceramide and sphingomyelin (SM) levels in aorta and plasma harvested from NODI and NODIII. (n=6 mice)

3.8 Ceramide mediates the transition from NO to hydrogen peroxide (H_2O_2)

It has been recently shown that chronic exposure to ceramide switched the vasoactive mediator of flow-induced dilation from NO to deleterious reactive oxygen species such as H_2O_2, resembling what occurs in arterioles from coronary artery disease (CAD) patients [130] . Starting from this evidence, we have evaluated if this noxious mechanism, due to ceramide accumulation, could be confirmed also in our model of type 1 diabetes derived IBVD. Total nitrites (NOx) as an indirect index of NO production, and H_2O_2 levels have been measured in aorta collected from NODI, NODIII, and NODIII mice treated with myriocin (0.3 mg/Kg i.p. for 24 h). The results show a significant reduction (almost 50%) of NOx content in NODIII mice compared to NODI that is recovered in NODIII mice treated with myriocin (**Fig. 20A**). Conversely, H_2O_2 levels in diabetic mice (NODIII) are significantly increased (also in this case almost 50%) compared to normoglycemic mice (NODI) and treatment with myriocin restores H_2O_2 values to one observed in NODI (**Fig. 20B**). These data strongly suggest detrimental role of ceramide in vascular disease associated to hyperglycemia and its contribution in alteration of redox homeostasis between NO and H_2O_2.



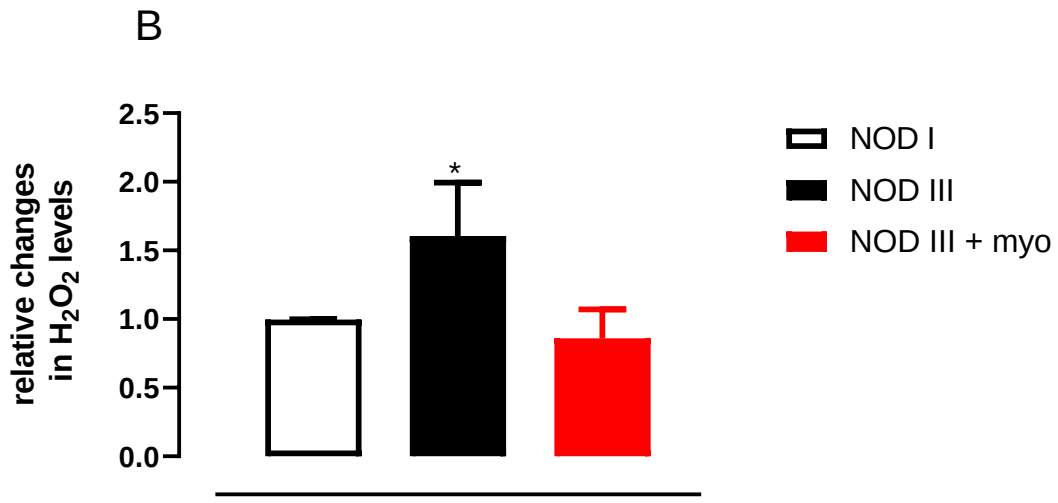


Figure 20: **Ceramide mediates the transition from NO to hydrogen peroxide (H_2O_2)**

NOx (A) levels measured in aorta harvested from NODI, NODIII and NODIII mice treated with myriocin (0.3 mg/kg i.p. 24h before). (A) The NOx levels in NODI are: 1776 ± 240 µM/mg protein. The NOx levels in NODIII mice are: 945 ± 172.1µM/mg protein. The NOx levels in NODIII + myo mice are: 2125 ± 445 µM/mg protein. The data have been expressed as a relative change compared to the levels of NOx detected in NODI aorta, taken as 1. (n=13 mice for NODI; n=10 for NODIII and n=4 for NODIII + myo; ** = $p<0.01$ vs. NODI and °°= $p<0.01$ vs. NODIII. H_2O_2 (B) content in aorta harvested from NODI, NODIII and NODIII mice treated with myriocin. (0.3 mg/kg i.p. 24h before) The H_2O_2 levels in NODI are: 6.28 ± 1.38 µM/mg protein. The H_2O_2 levels in NODIII mice are: 14 ± 3.3 µM/mg protein. The H_2O_2 levels in NODIII + myo mice are: 7.5 ± 1.8 µM/mg protein. The data are expressed as a relative change compared to the levels of H_2O_2 detected in NODI aorta, taken as 1. n=10 mice for NODI; n=4 for NODIII and n=4 for NODIII + myo; * = $p<0.05$ vs. NODI.

3.9 Discussion

The goal of the first part of this project was to investigate the possible role of *de novo* biosynthesis of SLs as novel, non-canonical players, in vascular reactivity, as well as in vascular dysfunction associates to hyperglycemia.

The concept that endothelium holds an essential role in vascular homeostasis is well consolidated; as demonstrated in the last decades, endothelium-derived mediators are released continuously providing the "right tone" of the vascular network, accordingly to blood demand of tissues and organs. Any circumstance in which imbalance between vasodilators and vasoconstrictors agents occurs, as well as chronic disturbances in mechanical shear stress, promotes endothelial dysfunction. The literature of the field has clearly identified in the "Endothelial dysfunction or alteration" as endothelium modifications caused by pro-inflammatory stimuli. This condition is common to various diseases i.e. atherosclerosis, heart failure, diabetes, and hypertension. These diseases can originate from different organism districts, but all lead to inflammatory vascular disorders. The early event involved in endothelial damage, and consequently for unbalance of vascular homeostasis, is the oxidative stress [68] . The reduced biosynthesis of NO, by eNOS, is one of the main consequences of the oxidative stress that in turn promotes vasoconstriction, pro-coagulability and arterial stiffness, all features of inflammatory based vascular disease (IBVD) [131,132, 133]. Several studies have linked endothelial dysfunction and resultant atherosclerosis to insulin resistant states, such as obesity and hyperglycemia induced-diabetes [134,135,136]. Hyperglycemia results from the inability of hyperinsulinemia to compensate for insulin resistance, as commonly seen in the setting of T2D. It can also result from insufficient release of insulin from pancreatic beta-cells (due to their autoimmune destruction), as seen in individuals with T1D, here reduced glucose uptake is observed, and this results in decreased glucose oxidation. As a consequence, tissues become heavily reliant on FAs and lipids as a source of energy[137]. Diabetes-induced endothelial dysfunction[5] coupled to alterations of lipids contents have been implicated in endothelial dysfunction, vascular permeability and cardiovascular disease [138,139,140]. Nevertheless, evaluation of SLs homeostasis within vasculature in the onset and/or development of

T1DM has not been fully elucidated and is still debatable. In this context Holm Laurits J and colleagues[141] have shown a reduction of SLs metabolism in patients affected by T1D; conversely the research group of Kester [142] have shown that in murine model of streptozocin-induced T1DM, the pattern of SLs levels in retina were significantly modified with low levels of ceramide coupled to higher levels of glucosylceramide, suggesting an enhanced conversion of ceramide in glucosylceramide. A study by Hammer and coworkers assessed that during 24 h of partial insulin deprivation in individuals with T1D plasma lipids increased by 50%, but there are not data that deeply elucidate which sub-class of SLs has been involved. Study on streptozocin-induced T1D showed a significant elevations in TAG, ceramide and palmitoyl CoA accumulation[143] , despite no changes in plasma levels. As well as in Akita Ins2+/- mouse model of T1D Basu and coworkers reported elevated levels of palmitoyl CoA, Ceramides, DAG and TAG in hearts compared to WT[144]. Starting from these evidence, we focused our studies on the possible role of SLs in T1DM-related vascular disease. For this purpose we used a mouse model of T1D called NOD mice, a strain that spontaneously develops autoimmune type 1 diabetes in which the progression of diabetic pathology is associated with vascular disorders as it happens in humans [81] . According to glycemia levels, NOD mice were divided in two groups: normoglycemic or healthy mice (NODI) and hyperglycemic or diabetic mice (NODIII) [81] .

The contribution of *de novo* biosynthesis-derived SLs in vascular homeostasis has been confirmed in healthy mice (NODI) through the inhibition of SPT, the first and rate limiting enzyme of the pathway. Indeed, aortic rings incubated with myriocin, a selective SPT inhibitor, displayed both an impaired endothelium-dependent vasorelaxation and agonist-induced vasoconstriction. This evidence is in line with the current literature that reveals a role of SLs in controlling the vascular tone in healthy status. The previous known occurrence of diabetes-induced endothelial dysfunction displayed by diabetic mice (NODIII) was evidenced by an altered thoracic aorta relaxation-response to Ach and Iso, indicating a perturbation in eNOS/NO signaling in hyperglycemic condition, coupled with a significant impairment of agonists-induced contraction. Incubation of aortic rings of NODIII mice with myriocin was able to

restored PE-induced contraction to the same extent observed in NODI but did not affect endothelium-dependent vasorelaxation. Western blot and PCR analysis performed on aorta homogenates revealed a significant increase of SPT expression in NODIII compared to NODI, whilst Nogo-B expression was unaltered. This finding leads to a modification of SPT/Nogo-B ratio in NODIII mice in which the over-expressed portion of SPT is no longer affected by negative control of Nogo-B. This latter evidence could be explained as a compensative, but not resolutive attempt, of the vessels to recover the impaired vasodilatation due to eNOS/NO disruption. Indeed, the lack of myriocin in recovering endothelium-dependent vasorelaxation in diabetic mice could be imputable to the reduced NO biosynthesis, that is the final mediator involved in sphingosine-1-phosphate one of the main SLs-induced vasodilatation [145] .In line with this hypothesis, different studies carried out in Di Lorenzo's Lab revealed a protective function of *de novo* pathway within the vasculature in the pathogenesis of hypertension as well as vascular permeability [3] . Indeed, it has been shown that in the resistance arteries from systemic and EC Nogo-A/B-deficient mice occurs a endurance to development of hypertension and vascular dysfunction revealing a protective function of the *de novo* pathway due to the production of endothelial S1P, its importance the activation of local S1P-$S1P_1$-eNOS signaling.

These results are confirmed *in vivo* by evaluating systemic blood pressure. Indeed, NODIII mice displayed a mild hypertensive phenotype, most likely derived from the reduced NO bioavailability due to endothelial dysfunction, and selective inhibition of SPT by myriocin further increased the systemic blood pressure.

Several studies suggested that elevations in circulating lipid levels lead to the build-up of fatty deposits within the blood vessel lumen and the ectopic formation of ceramides within ECs. EC-derived ceramides are potent regulators of vascular tone. In small coronary arteries from mice in vitro or in human cultured ECs, administration of ceramide analogues impaired EC-dependent vasorelaxation[146], exacerbates vasoconstriction [123] and decreases NO production [147] . Prominent, short chain ceramide analogues are not natural ceramides, but are rapidly deacylated and recycled into long-chain ceramides [148]. Alternative experimental interventions that also increase

endogenous ceramide production in cells or tissues (such as by incubating ECs or isolated vessels with palmitate) recapitulate the effects of ceramide administration by decreasing eNOS phosphorylation, eNOS activity and NO production[6,7,8]. Interestingly, inhibition of ceramide biosynthesis with the use of pharmacological agents (such as myriocin) or genetic modification (such as *Des1* heterozygous knockout) restores eNOS activity, NO production and EC-dependent vasodilatation [6, 7]. Starting from these evidences, we aim to assess if exposure aortic ring of healthy mice (NODI) with exogenous ceramide could affect vascular reactivity recapitulating the vascular dysfunction observed in NODIII mice. For this purpose aorta harvested from healthy mice has been exposed to ceramide (10 μM or vehicle, DMSO, for 15 minutes), then vascular reactivity has been tested by using isolated organ bath technique. The results clearly showed a significant reduction in endothelial-dependent vasorelaxation and a partial reduction in agonist-induced vasoconstriction. Interestingly, the Ach- and Iso- concentration-response curves obtained in aortic rings of NODI mice incubated with ceramide mimicked the ones obtained in NODIII mice strongly suggesting a detrimental role of ceramide accumulation in endothelial function. To further endorse the causative role of ceramide accumulation in vascular dysfunction, aorta and plasma harvested from NODI and NODIII mice have been processed and used for ceramide, glucosylceramide and sphingomyelin (as representative SLs) determination by performing a metabolomic approach by mass spectrometry. Measurement of SLs in aortas of NODIII mice showed an accrual of both ceramide and glucosylceramide compared to NODI, while as opposite, plasma levels of both SLs were reduced in NODIII compared to NODI. These results clearly confirm that in diabetic condition the over-expression of SPT leads to ceramide accumulation within the vasculature altering its function and reactivity.

4 Beneficial effect of metformin on endothelial function

Metformin anti-hyperglycemic effects are mainly due to the inhibition of hepatic glucose output and the improvement of insulin sensitivity[149]. The mechanisms underlying these benefits are complex and not fully understood and to some extend controversial as different doses and duration of metformin treatment can results in different molecular mechanisms.

Some of the proposed mechanisms of action for metformin include[150]the inhibition of complex I in the mitochondrial electron transport chain, thus reducing ATP production[151], and the activation of the AMPK (5'-AMP-activated protein kinase)[152], which is particularly relevant for the endothelial function as AMPK can activate eNOS.

Increasing clinical data support the beneficial role of the hypoglycemic drug metformin on the vasculature disease related to diabetic conditions. Metformin can improve the endothelial function, assessed by vasodilation of brachial artery during reactive hyperemia, in patients with metabolic syndrome[153]. Since 2010 large clinical trial have been conducted to evaluate metformin as adjunct therapy in T1D[154]. The REMOVAL clinical study funded by the Juvenile Diabetes Research Foundation was conducted to address potential cardiovascular effects of metformin in T1D patients. Metformin treatment (1000 mg twice daily, for 3 years), added to insulin therapy, reduced the progression of atherosclerosis in adults of 40 years of age and older with T1D and three or more cardiovascular risk factors [155, 156]. In a follow up study, carotid intima-media thickness, a surrogate of atherosclerosis, was also found reduced after 6 years of metformin treatment of T1D patients [157]. Before the REMOVAL trial, small scale studies reported beneficial effects of metformin on carotid intima-media thickness in patients with metabolic syndrome and T2D[157, 158].

These pre-clinical and clinical data support a beneficial role of metformin on the vasculature, specifically in preserving the endothelial function, in part by AMPK-mediated eNOS activation.

Starting from these evidences, aim of the second part of the project is to investigate the possible protective action of metformin on hyperglycemia-induced endothelium dysfunction, in part, by restoring physiological SL levels.

5 Results

5.1 Metformin administration transiently improves hyperglycemia in T1D

At 10 weeks of age NOD were monitored for the levels of glycemia once per week. After 2h of starvation, glucose was measured on the blood from tail vein following a superficial incision. When glycemic levels were <150mg/dL NOD mice were considered not diabetic (NODI). When glycemia increased to levels of 150-200 mg/dL, the mice were subjected to metformin (300mg/Kg daily) or vehicle treatment. Metformin transiently alleviates hyperglycemia in NOD mice compared to vehicle (**Fig. 21**). This is not surprising since clinical studies also have reported a not sustained lowering effect of metformin on HbA1c in T1D patients[154] .

To avoid the different hyperglycemic levels could account for divergent vascular

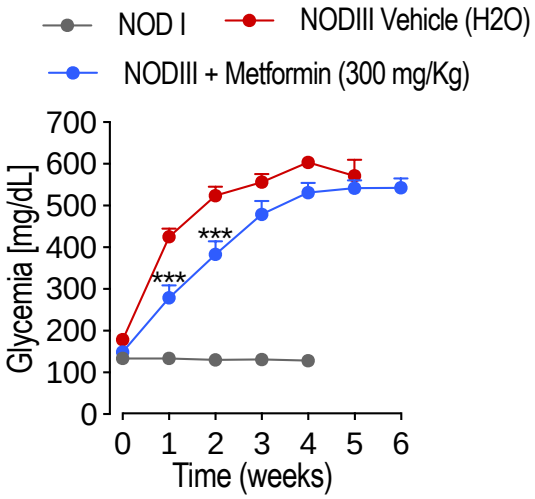


Figure 21. Metformin induces a transient reduction in glycemic levels in NOD mice. Mice presenting glycemic levels <150 mg/dL are considered non-diabetic (NODI). When glycemic levels reached values between 150-200mg/dL, NOD mice were treated with metformin (300mg/Kg/ daily in drinking water) or vehicle [1]. Time 0 corresponds to the beginning of the pharmacological treatment. (n=8 mice; **p<0.01; p<0.001 metformin vs. vehicle.

functions between metformin and vehicle treated groups, we established as end point not the time of treatment but the time that the mice have been with glycemic levels

>400mg/dL, which was set to 4 weeks. As shown in (**Fig.21)**, both vehicle and metformin treated mice have been with glycemia ≥400mg/dL for 4 weeks before vascular studies on MA.

5.2 High glucose upregulate SL de novo biosynthesis

It has been reported that the treatment of cultured cells with high glucose increases the activity of SPT [159 , 160], leading to the accumulation of SL. Therefore, we have exposed human umbilical vein endothelial cells (HUVEC) to high and low glucose, 5 and 25 mM, respectively, and evaluated by Western Blot analysis the expression of the components of serine palmitoyltransferase (SPT) complex, which regulates the first and rate limiting step of the SL de novo biosynthesis. SPT long chain subunit 1 (SPTLC1) was markedly upregulated over time following incubation with high glucose (**Fig. 22**). Interestingly, Nogo-B, an inhibitor of SPT activity was significantly downregulated in the same conditions, suggesting that SL *de novo* biosynthesis was upregulated by elevated glucose.

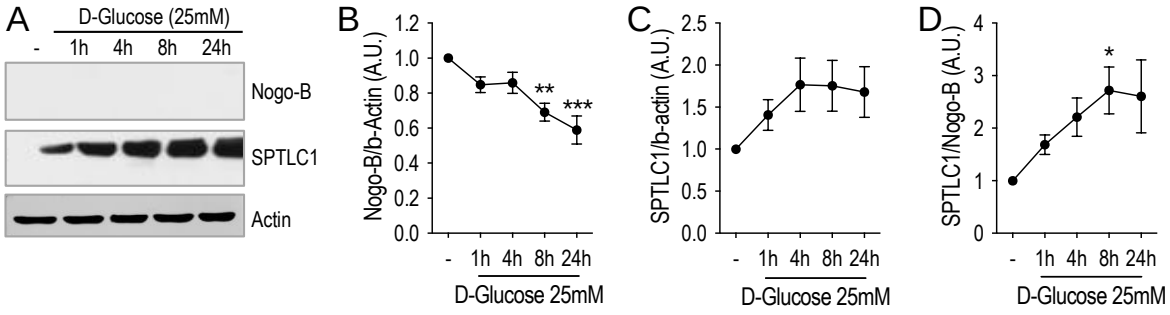


Figure 22: High glucose upregulate SL de novo biosynthesis. (**A**) Western blot and densitometric analysis of (B) Nogo-B, (C) SPTLC1, and (D) the ratio between SPTLC1 and Nogo-B in HUVEC treated with D-Glucose 25mM for the indicated time point (n=6; *$p \leq 0.05$, *p$P \leq 0.01$, ***$p \leq 0.001$).

5.3 Metformin down-regulates the activity of SPT in HUVEC exposed to high glucose conditions as well as in NODIII mice

To assess whether metformin could downregulate SPT activity in high glucose conditions, HUVECs have been exposed to high and low glucose (5 and 25 mM,

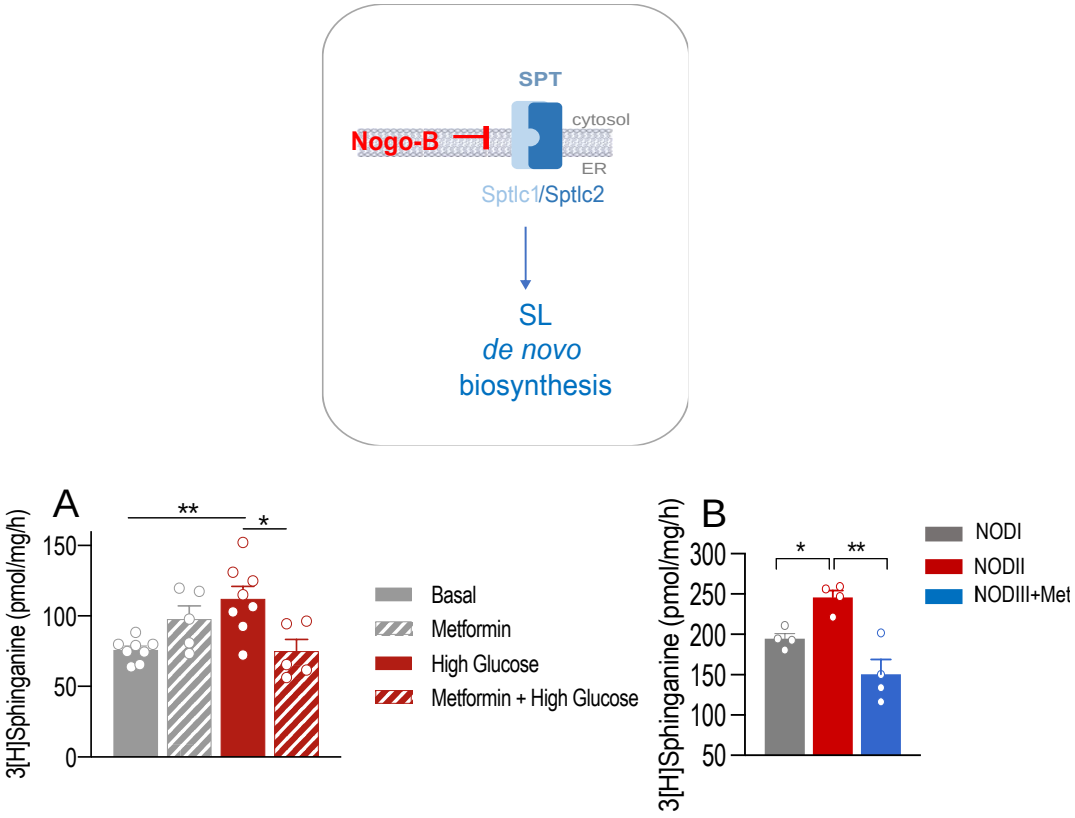


Figure 23. **Metformin downregulates the activity of SPT in HUVEC exposed to high glucose conditions as well as in NODIII mice**. SPT activity measured using [^{3}H]-serine and palmitoyl-CoA as substrates for SPT. Sphinganine — the reaction product — was separated in TLC (thin-layer cromatography) and quantified. (**A**) SPT activity of HUVEC cultured for 24h in normoglycemic (5mM D-glucose) and hyperglycemic (25mM) conditions, with or without Metformin 1mM (n≥5/group). (**B**) SPT activity in lung microsomes from NODI, NODIII and NODIII+Metformin mice (n=4 mice *p≤0.05, **≤0.01).

respectively) in presence and absence of metformin (0.3 mM, 24h). HUVECs showed a significant increase of SPT activity under high glucose concentrations compared to low glucose, suggesting that *de novo* synthesis is upregulated in hyperglycemia

conditions. Interestingly, metformin treatment was able to restore SPT activity to normoglycemic [112] levels **(Fig. 23A)**. To corroborate this finding *in vivo*, we have assessed SPT activity in microsomes from lungs of NODI, NODIII + vehicle, NODIII+ metformin (**Fig. 23B**). Endothelial cells contribute to 20% of lung's weight, therefore the lungs have been used as a source of endothelial cell isolation or as organ of choice, with the heart, to assess SPT activity in mice.

Metformin treatment significantly reduced SPT activity in mouse lung microsomes from NODIII+metformin mice compared with NODIII mice treated with vehicle. Altogether, these *in vitro* and *in viv*o findings put forward a potential new mechanism of action of metformin, which is to downregulate the *de novo* biosynthesis of SL and counterbalance the upregulated of SPT activity caused by high glucose levels.

5.4 Metformin improves endothelial function via eNOS-NO signaling pathway

Next, we assessed whether metformin treatment could improve vascular reactivity of resistance arteries. To this end, mesenteric arteries from the three groups of NOD mice were isolated, cleaned from fat and connective tissue and mounted in the wire myograph system (DMT A620). Severe diabetes (>400 mg/dL) impaired Ach-mediated vasorelaxation of MA from NODIII mice vs. NODI (**Fig. 24B**). Metformin treatment significantly improved Ach-mediated vasorelaxation suggesting vasculo-protective functions of metformin in T1D.

To investigate the role of eNOS-derived NO, in Ach-induced vasorelaxation, MA were incubated with L-NG-nitroarginine methyl ester (L-NAME), a pharmacological inhibitor of NOS. First, in NODIII mice Ach-mediated relaxation was significantly reduced compared to NODI MA indicating an impairment of endothelial functions. Interestingly, metformin treatment did not change Ach-mediated vasorelaxation in presence of L-NAME, suggesting that metformin improves endothelial functions via

eNOS-NO pathway rather than prostacyclin and EDHF, which remained reduced compared to NODI MA (**Fig. 24C**).

Finally, to confirm the endothelium as main target of the beneficial effects of

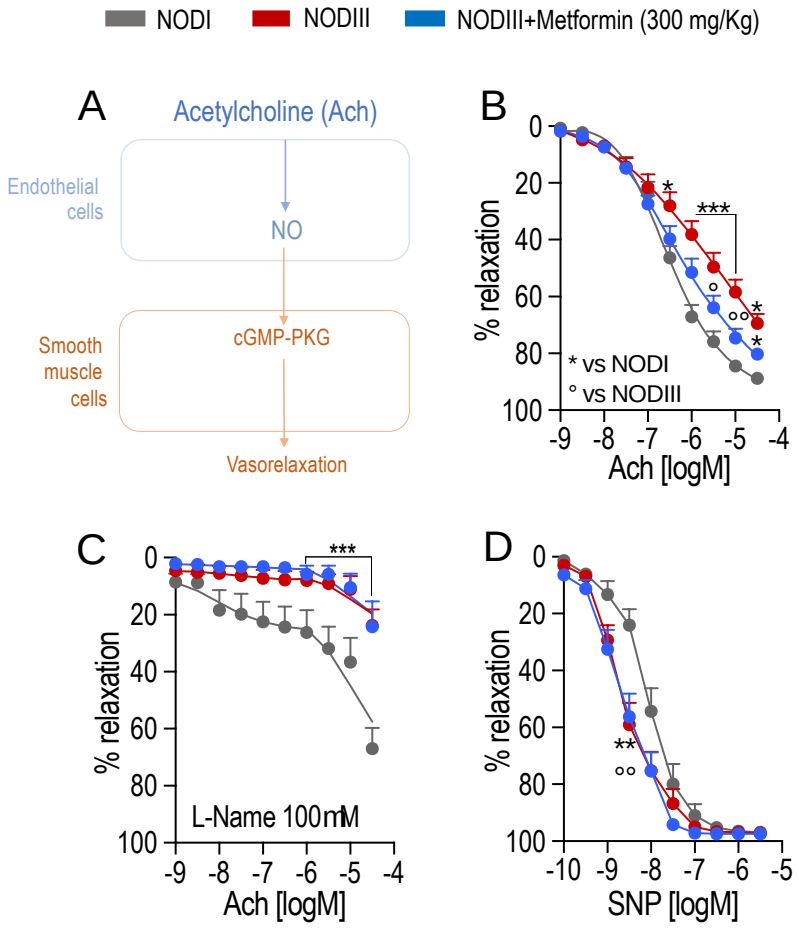


Figure 24. **Metformin improves endothelial function via eNOS-NO signaling pathway.** (A) Schematic representation of Ach-induced NO formation and downstream cGMP signaling. MA from NODI, NODIII + vehicle and NODIII+metformin have been assessed for vascular reactivity in the wire myograph system (DMT A620). Acetylcholine (Ach) concentration-response curves in (B) absence and (C) presence of L-NAME (100 microM). (D) SNP concentration-response curves (n =6 mice; *p< 0.05; **p< 0.01; ***p< 0.001 NODIII vs NODI; °p< 0.05; °°p< 0.01 NODIII+metformin vs NODIII).

metformin rather than smooth muscle cells, we assessed the vasorelaxation induced by SNP, an NO donor, thus bypassing eNOS-NO production step. Interestingly, there were no differences between NODIII MA from mice treated with metformin and vehicle

(**Fig. 24D**), suggesting that metformin did not impact cGMP signaling pathway, but improved the upstream production of eNOS-derived NO in T1D.

5.5 Metformin treatment does not reduce the hyper-contractility of diabetic MA

Next, we have evaluated the vasoconstriction of MA to PE, α_1 adrenergic receptor agonist, and U46619, an analog of thromboxane A2 activating thromboxane A_2 receptor. MA from NODIII mice showed an exaggerated vasoconstriction to both PE and U46619 (**Fig. 25 A-B**).

These findings corroborate published studies showing an increased vascular tone in diabetes [161]. Interestingly, metformin treatment did not ameliorate the hyperresponsive of diabetic MA to PE and U46619 (**Fig 25 A and B**), suggesting that the endothelium is the main target of the beneficial effects of metformin on the vasculature in T1D conditions.

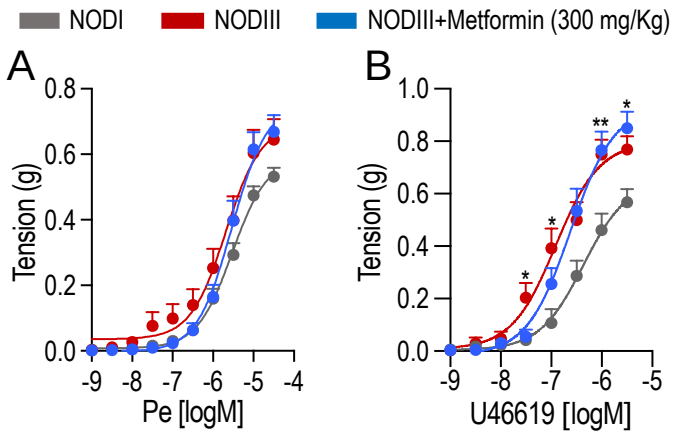


Figure 25. ***Metformin treatment does not reduce the hypercontractility of diabetic MA***
Concentration-response curve to(A) PE and (B) U46619. (n= 6 mice;*p< 0.05; **p< 0.01 NODIII + vehicle vs NODI).

5.6 Metformin treatment increases P-VASP, downstream target of NO signaling, in thoracic aorta

To corroborate the increased endothelial NO production following metformin treatment, thoracic aortas from NODI, NODIII+vehicle and NODIII+metformin have been lysed and assessed for phosphorylation of VASP (Vasodilator-stimulated phosphoprotein) by Western Blot analysis. P-VASP was significantly reduced in diabetic mice compared to NODI (**Fig. 26A**).

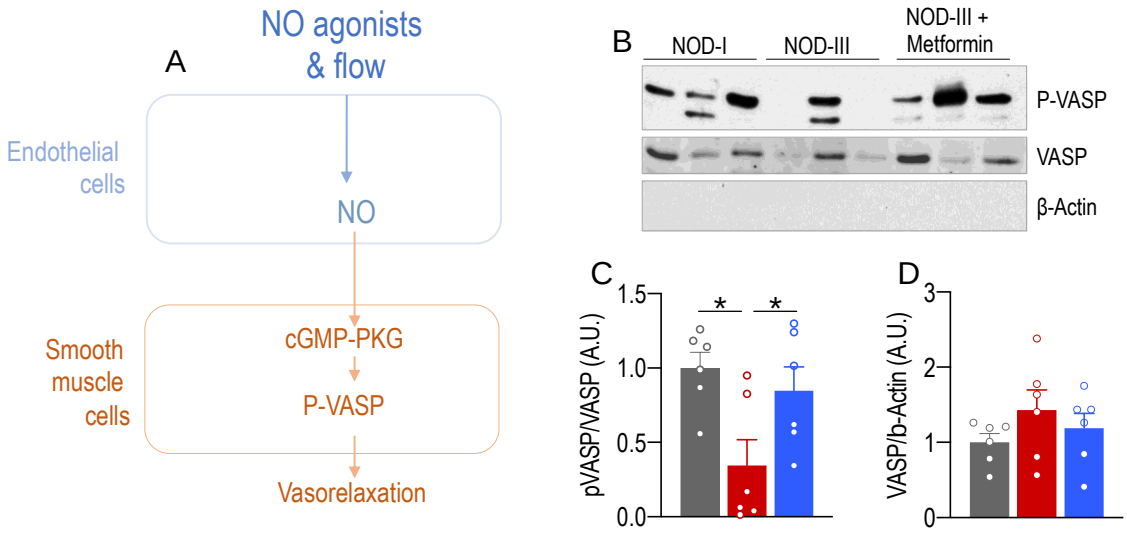


Figure 26. Metformin treatment increases P-VASP, downstream target of NO signaling, in thoracic aorta. (A) NO agonists as well as flow stimulate eNOS-derived NO formation, which rapidly diffuse to the smooth muscle layer underneath to activate cGMP-PKG signaling pathway. Downstream P-VASP is one of the mediators of NO-induced vasorelaxation. (B) Western Blot analysis for PVASP and VASP expression in thoracic aorta lysates from NODI, NODIII+vehicle and NODIII+metformin. Quantification of (B) P-VASP/VASP ratios and (C) VASP/ beta-actin. (n=6 mice; *p<0.05 vs NODI and NODIII+Metformin).

In agreement with vascular reactivity data, metformin restored P-VASP in NODIII mice to NODI levels (**Fig 26A**), whereas the levels of total VASP were unchanged (**Fig. 26B**). These data strongly support that metformin can protect the endothelium from dysfunction by improving eNOS-derived NO signaling.

5.7 Exogenous ceramide recapitulates endothelial dysfunction in healthy vessels through PP2A-mediated dephosphorylation

Based on the *in vitro* and *in vivo* SPT activity, and taking in account the recent relevant literature, we hypothesized that ceramide accrual might contribute to the endothelial dysfunction in T1D by reducing eNOS activation via PP2A-mediated dephosphorylation. To this end, MA from normoglycemic mice have been incubated with ceramide (300nM, 30 min) followed by Ach-mediated vasorelaxation. Interestingly, ceramide treatment was able to impair Ach-mediated vasorelaxation of MA from NODI to the levels of NODIII MA (**Fig. 27**).

To confirm the role of protein phosphatase 2A (PP2A) as downstream mediator of ceramide actions on eNOS, MA have been incubated with okaidic acid (OKA),

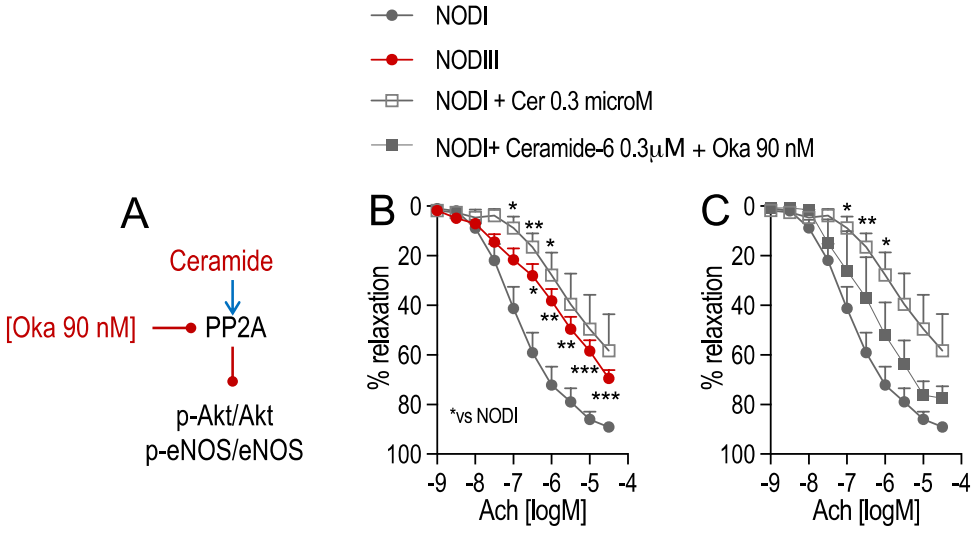


Figure 27. Exogenous ceramide recapitulates endothelial dysfunction in healthy vessels. (A) Ceramide activates protein phosphatase 2A (PP2A), which in turn dephosphorylates eNOS contributing to endothelial dysfunction. Okadaic Acid (OKA) is an inhibitor of PP2A. (B) Ceramide treatment of MA reduces Ach-mediated vasodilation of NODI MA to a similar extend of NODIII MA. (C) Incubation of MA with OKA (90nM) can reinstate Ach-mediated vasodilation following ceramide treatment. (n=3 mice; *p< 0.05; **p< 0.01; ***p< 0.001 NODI).

pharmacological inhibitor of PP2A and ceramide. As shown in (**Fig. 27C**), OKA was able to reinstate Ach-mediated vasodilation, although did not reach the significance because of the low number of mice. These data support the idea that ceramide accrual contributes to endothelial dysfunction via PP2A-mediated eNOS dephosphorylation.

5.8 Exogenous ceramide abolishes the protective effects of metformin on the endothelium of NODIII mice

Metformin treatment restored endothelial-mediated vasodilation in response to Ach in MA (Fig. 24). Because metformin seems to counterbalance the upregulation of SL production in high glucose conditions *in vitro* and *in vivo* (**Fig. 28**), it is possible that metformin restores endothelial functions by reducing vascular ceramide accrual. Thus, we assessed whether exogenous ceramide (300 nM, 20 min) could re-instate endothelial dysfunction in MA of NODIII mice treated with metformin. As shown in **(Fig. 28B)**, in MA harvested from NODIII treated with metformin, a partial recover of Ach-induced vasorelaxation occurred. When MA of these mice were exposed to ceramide the beneficial effect of metformin treatment was lost (**Fig. 28B**) with a further reduction of endothelium-dependent vasorelaxation. Incubationof NODIII+metformin mice with ceramide and OKA restored the endothelium-dependent vasorelaxation to the same extent observed in NODIII treated with metformin.

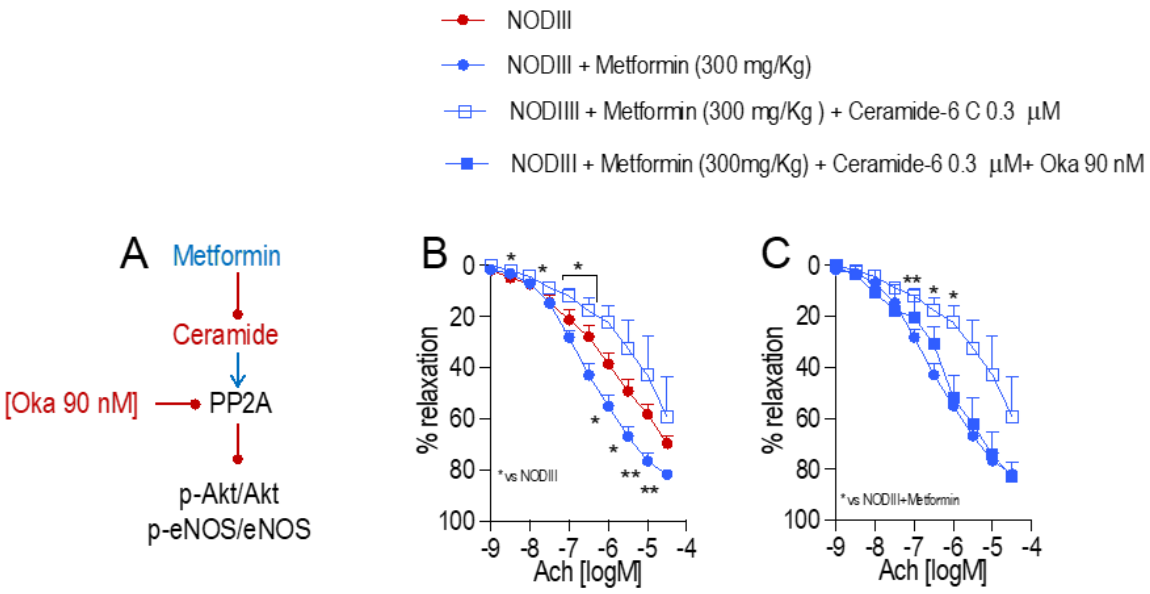


Figure 28. Exogenous ceramide abolishes the protective effects of metformin on the endothelium of NODIII mice. (A) Schematic representation of metformin inhibition of ceramide production and its downstream signaling. (B) Concentration-response curves of Ach were performed on MA from NODIII+metformin, incubated with C6:0-ceramide (0.3 µM) or vehicle, and NODIII MA. (C) Concentration-response curves of Ach were performed on MA from NODIII+metformin, incubated with C6:0-ceramide (0.3 µM), or C6:0-ceramide (0.3 µM) and OKA (90nM), or vehicle. (n =3 mice; *p< 0.05; **p< 0.01; ***p< 0.001 vs NODIII, vs NODIII+metformin).

6 Discussion

Metformin (dimethylbiguanide) has become the preferred first-line oral blood glucose-lowering agent to manage type 2 diabetes (T2D). Its history is linked to Galega officinalis (also known as goat's rue), a traditional herbal medicine in Europe, found to be rich in guanidine, which, in 1918, was shown to lower blood glucose. Guanidine derivatives, including metformin, were synthesized and some (not metformin) were used to treat diabetes in the 1920s and 1930s but were discontinued due to toxicity and the increased availability of insulin. Metformin was rediscovered in the search for antimalarial agents in the 1940s and, during clinical tests, proved useful to treat influenza when it sometimes lowered blood glucose. This property was pursued by the French physician Jean Sterne, who first reported the use of metformin to treat diabetes in 1957. After intensive scrutiny metformin was introduced into the USA in 1995. In 1998 the UK Prospective Diabetes Study (UKPDS) provided a new rationale to adopt metformin as initial therapy to manage hyperglycemia in T2D. This epic study, which redefined the therapeutic strategy for the management of type 2 diabetes, noted that in addition to glucose-lowering effects, weight neutrality and low hypoglycemia risk, long-term metformin therapy might reduce cardiovascular events and improve survival[162]. Following clinical studies confirmed the beneficial cardiovascular effects of metformin treatment[163, 164]. Today, metformin is the most prescribed glucose-lowering drug worldwide with the potentials for other therapeutic applications.

As cited above, one of the main recognized mechanisms of action for metformin include[150] the inhibition of complex I in the mitochondrial electron transport chain, thus reducing ATP production[151], and the activation of the AMPK (5'-AMP-activated protein kinase)[152], which is particularly relevant for the endothelial function as AMPK can activate eNOS. AMPK is a heterotrimeric enzyme (α_1, α_2, β_1, β_2, γ_1, γ_2, γ_3), that is activated both by direct AMP allosteric regulation and by phosphorylation of its α catalytic subunit [165, 166]. AMPK is a major regulator of cellular energy homeostasis coordinating the enzymes involved in carbohydrate and fat metabolism to enable ATP conservation and synthesis. In disease states characterized by an unbalanced metabolic

state, such as T2D, AMPK is down-regulated[167, 168]. Increasing evidence suggests that the role of AMPK goes beyond energy metabolism control, as the enzyme may regulate a wide range of cell functions accounting for a variety of metformin pleiotropic actions [165,169]. For example, AMPK stimulates eNOS activation and NO production[152] , thus supporting a protective role of this kinase on the endothelium. An elegant molecular study *in vitro* by using bovine aortic EC (BAEC) demonstrated that clinically relevant concentrations of metformin (50-500 micromol/l) dose-dependently increased the phosphorylation of eNOS at Ser1179 (equal to human Ser1177) via activation of AMPK[170]. These protective actions of AMPK on the endothelium have been demonstrated in different pre-clinical paradigms[171,152, 172, 173]. Exercise has been demonstrated to activate AMPK-eNOS pathway in the mouse aorta[174].Vasculo-protective effects of different drugs used in metabolic and cardiovascular diseases have been shown to involve AMPK activation in endothelial cells. For instance, in isolated rat mesenteric arteries *in vitro*, the incubation of simvastatin, a cholesterol lowering drugs, increases the activation of eNOS via AMPK[175]. Similarly, it has been shown that rosiglitazone, an anti-diabetic drug targeting peroxisome proliferator-activated receptor gamma, stimulated AMPK-mediated phosphorylation of eNOS at Ser1177[176]. Interestingly, in a mouse model of STZ-induced T1D, Zou and colleagues demonstrated that metformin treatment was able to accelerate wound closure by inducing endothelial cell progenitor's mobilization and AMPK-dependent eNOS activation[177]. Another study demonstrated that metformin was able to restore endothelial functions and improve NO bioavailability in Goto-Kakizaki [9] rats, an animal model of nonobese type 2 diabetes, fed with high fat diet[178]. Therefore, the second part of this thesis has been focused on the possible activity of metformin on SLs biosynthesis, as novel molecular mechanism involved in its beneficial action on vascular dysfunction related to diabetes.

The selection of a T1DM animal model (NOD mice) to investigate the vascular effect of metformin was based on the finding that NOD mice: (1) spontaneously develop an autoimmune diabetes with remarkable analogy to human T1DM; (2) develop a progressive impaired vascular reactivity, which we have previously

characterized. Taking advantage of the fact that metformin does not control glycemia in T1DM, this strain represents an appropriate tool to investigate the effect of metformin on vascular complications, minimizing the confounding variable attributable to the beneficial effect that a reduction of glycemia would have by itself on vessels. Indeed, treatment of NOD mice with metformin transiently alleviated hyperglycemia in NODIII mice compared to vehicle, most likely because, in the onset of autoimmune beta cell pancreatic destruction (the first two weeks of treatment), a residual content of insulin is still available. To evaluate the possible protective action of metformin on vascular reactivity of diabetic mice, mesenteric arteries (MA) harvested from NOD mice have been used. NODIII mice displayed a significant impaired Ach-mediated vasorelaxation of MA from NODIII mice vs. NODI. Metformin treatment significantly improved Ach-mediated vasorelaxation suggesting vasculo-protective functions of metformin in T1D. The further demonstration of the involvement of eNOS/NO signaling in the protective action of metformin is furnished by the experiments performed in presence of the pharmacological eNOS inhibitor L-NAME. Indeed, metformin failed to recover Ach-mediated vasorelaxation in presence of L-NAME, strongly suggesting that metformin beneficial vascular activity is mediates by eNOS/NO pathway. To confirm that metformin action is specifically on endothelial cells, rather than smooth muscle component, cumulative response-curve with an exogenous source of NO (SNP) has been performed on MA. The results clearly show no difference between NODIII MA from mice treated with metformin and vehicle, suggesting that increase of eNOS-derived NO is the mediator of metformin vascular effect. Similarly, metformin did not impact on smooth muscle contractility, as demonstrated by stimulating MA with adrenergic α1 agonist PE or with analog of thromboxane A2. To elucidate the downstream signalling pathways activated in response to an increased NO availability by metformin, evaluation of pVASP/VASP ratio have been performed. Phosphorylation of VASP in vascular extracts is widely used as an index of NO/cGMP pathway activity [179] . In our setting, p-VASP/VASP ratio was significantly reduced in NODIII mice compared to NODI and, in agreement with vascular reactivity data, metformin restored P-VASP/VASP ratio in NODIII mice

to the same extent observed in NODI, further supporting the hypothesis of a beneficial vascular effect of metformin mediated by eNOS-derived NO signaling activation. The finding that aortas harvested from normoglycemic mice exposed to an exogenous source of ceramide displayed an endothelium dysfunction like what observed in NODIII mice, has been also confirmed in MA. Some previous studies demonstrated that ceramide activates protein phosphatase 2A (PP2A),which, by diminishing the phosphorylation of eNOS at s Ser1177, reduces eNOS activity [6 7 180] . In vascular ECs, ceramide regulates PP2A, by disrupting its interaction with inhibitor 2 of PP2A (I2PP2A), releases PP2A from this repressive factor and increases its access to various substrates [181 182] . This sequence of events leads to an increased association between PP2A and eNOS and a concomitant dissociation of eNOS from the activating kinase complex AKT/HSP90 [6 7]. Blocking PP2A activity ameliorates high-fat-induced or ceramide-induced endothelial dysfunction and hypertension in animal models [6,7] .

Here we showed that incubation of MA from healthy mouse with ceramide recapitulated the impairment vasorelaxation observed in aorta of NODIII mice . Moreover, incubation of MA with OKA, a selective PP2A inhibitor, significantly restored Ach-induced vasorelaxation implying that the pathway by which Ceramide impacts negatively on endothelium is PP2A/eNOS.

To further dissect the molecular mechanism of metformin action on endothelial function, a separate set of experiments *in vitro* has been devoted to assess the role of SLs in endothelial cells exposed to high glucose, and the possible beneficial effect of metformin. The data obtained demonstrate that HUVECs exposed to hyperglycemic environment displayed a significant increase of SPT expression, particularly SPT long chain subunit 1 (SPTLC1). Conversely, Nogo-B resulted significantly down-regulated. So, the data *in vitro* on endothelial cells confirm what observed in *ex vivo*: an unbalance SPT/Nogo-B ratio takes place in diabetic conditions, driving enhanced SLs biosynthesis. This hypothesis is confirmed by performing SPT activity assay on HUVEC exposed to high glucose. Indeed, in this condition SPT activity was significantly augmented and the treatment with metformin recovered the SPT activity at the physiological rate. Similarly, in *ex vivo* experiments, performed on microsomes

from lungs of mice, confirmed not only the increased SPT activity in NODIII mice compared to NODI, but also the ability of metformin to completely reverted SPT over-activity to physiological levels.

In conclusion, here we have demonstrated that *de novo* biosynthesis-derived SLs is an emerging class of non-canonical mediators that, within the vessels, actively participate to vascular homeostasis. In endothelium, the rate limiting enzyme of *de novo* biosynthesis SPT is affected by high glucose environment increasing both its expression and activity. This event leads to a lipid mediator accrual, i.e. ceramides, within endothelium causing endothelium dysfunction. In diabetic condition the eNOS/NO/cGMP signaling impairment represents the main cause of reduced vasodilatation and, in our hypothesis, the ceramide accumulation leads to PP2A activation, a phosphatase that acts on eNOS removing a phosphate group, thereby inactivating the enzyme. In this context, the antidiabetic drug metformin, beyond its hypoglycemic activity, exerts a beneficial effect on vasculature ameliorating the endothelium performance. Here we have demonstrated that SLs are candidates as target of metformin action by reducing ceramides detrimental effect. However, further investigation are required to further clarify the molecular mechanism of metformin on endothelium, indeed, the efficacy of metformin in the normalization of aortic and mesenteric artery endothelial dysfunction is presumably not only an expression of one mechanism alone but of diverse and manifold properties of this antidiabetic compound. This finding could not only improve scientific knowledge in the physiological interplay between type 1 diabetes and SLs, but it could also represent a possible pharmacological target in diabetic treatment with Metformin in the future.

7 Abbreviations

- **AGEs**
- advanced glycation end products
- **CCL2 (MCP-1)**
- monocyte chemoattractant protein-1
- **NO**
- nitric oxide
- **NOS**
- nitric oxide synthetase
- **eNOS**
- endothelial nitric oxide synthase
- **SLs**
- Sphingolipids
- **SPT**
- serine-palmitoyl transferase
- **PE**
- Phenylephrine
- **5HT**
- Serotonin
- **Cer**
- Ceramide
- **Iso**
- Isoprenaline
- **Ach**
- Acetylcholine
- **PP2A**
- Protein-phosphatase 2A
- **GlucCer**

- Glucosylceramide
- **PGI2**
- Prostacyclin
- **NADPH**
- Reduced nicotinamide adenine dinucleotide phosphate
- **CaM**
- Calmodulin
- **GTP**
- Guanosine 5’-triphosphate
- **GC**
- Guanylate cyclase
- **cGMP**
- Cyclic guanosine monophosphate
- **VD**
- Vascular disease
- **ROS**
- Reactive oxygen species
- **IBVD**
- Inflammatory-based vascular disease
- **GLUT**
- Glucose transporter
- **SPTLC1**
- Long chain base subunit 1
- **SPTLC2**
    - Long chain base subunit 2
- **S1P**
- sphingosine-1-phosphate

- **FFA**

- free fatty acids
- **NOD mice**
- Non-obese diabetic mice
- **L-NAME**
- N-nitro-L-arginine- methyl ester
- **pVASP**
- phosphorylated vasodilator-stimulated phosphoprotein
- **SNP**
- sodium nitroprusside
- **VASP**
- vasodilator-stimulated p
- **p-VASP**
- vasodilator-stimulated p phosporilation

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List of Thesis Publications

Involvement of 3',5'cyclic inosine monophosphate in cystathionine γ-lyase -dependent regulation of the vascular tone. Br J Pharmacol. 2021 Apr 30. doi: 10.1111/bph.15516
Mitidieri E, Vellecco V, Brancaleone V, Vanacore D, Manzo OL, Martin E, Sharina I, Krutsenko Y, Monti MC, Morretta E, Papapetropoulos A, Caliendo G, Frecentese F, Cirino G, Sorrentino R, d'Emmanuele di Villa Bianca R, Bucci M.

Erucin exhibits vasorelaxing effects and antihypertensive activity by H_2 S-releasing properties Br J Pharmacol. 2019 Mar 2. doi: 10.1111/bph.14645.
Martelli A, Piragine E, Citi V, Testai L, Pagnotta E, Ugolini L, Lazzeri L, Di Cesare Mannelli L, Manzo OL, Bucci M, Ghelardini C, Breschi MC, Calderone V.

Anomalous $K_v 7$ channel activity in human malignant hyperthermia syndrome unmasks a key role for $H_2 S$ and persulfidation in skeletal muscle.
doi: 10.1111/bph.14700
Valentina Vellecco, Alma Martelli, Iris Sofia Bibli , Marianna Vallifuoco, Onorina L Manzo, Elisabetta Panza, Valentina Citi, Vincenzo Calderone, Gianfranco de Dominicis, Caterina Cozzolino, Elisabetta M Basso, Martina Mariniello, Ingrid Fleming, Antonio Mancini, Mariarosaria Bucci, Giuseppe Cirino

Nory inserisci anche il lavoro degli mdx e scrivi under submission

Posters and oral communications

19 – 22 SET 2018

XXI SIF SEMINAR - PhD Students, Fellows, Post Doc and Specialist Trainees

Poster : Anomalous $K_v 7$ channel activity in human malignant hyperthermia syndrome unmasks a key role for $H_2 S$ and persulfidation in skeletal muscle

26 – 27 SET 2018

Convegno Monotematico SIF - Cardiovascular diseases: from population to basic science searching for new therapeutic targets

Poster: Possible role of SPT/Nogo-B signaling in diabetes-related endothelial dysfunction

8 MAR 2021

XXIII SIF Seminar on Pharmacology for PhD Students, Fellows, Post Doc and Specialist Trainees - DIGITAL EDITION

Oral communication: Metformin improves endothelial-dependent vascular function independently from hyperglycemia in a mouse model of type 1 diabetes"

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