UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF PHARMACY SCHOOL OF MEDICINE AND SURGERY

PhD IN PHARMACEUTICAL SCIENCES
XXXIV CYCLE

"Endogenous cross-talk between hydrogen sulfide and nitric oxide: role of L-serine and L-cysteine in inflammatory vascular diseases"

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1. INTRODUCTION

1. 1. Hydrogen sulfide as gasotransmitter

Gasotransmitters are small endogenous gas molecules involved in the regulation of several physiological and pathological processes. This class of gaseous molecules includes nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO). Typically, gasotransmitters are generated endogenously by specific enzymatic systems and they can easily penetrate the cell membrane, to interact with their targets, due to their high solubility in both water and lipids (Yang G et al., 2016). H_2S is a newly discovered gaseous molecule signaling and for years it has been considered a toxic gas because massive exposure to H_2S (> 5000 ppm) can cause death in humans due to the suppressive effect on carbonic anhydrase and cytochrome c oxidase (Cao X at al., 2019). Nowadays, it is recognized that mammalian cell produces H_2S, which are equipped with an enzymatic system to break down this gasotransmitter to avoid an accumulation of high levels of this gas. The ability of H_2S to regulate a lot of physiological or pathological processes is related to its capacity to modify different targets, mainly through the sulfhydration of protein at cysteine residue, or by the interaction with the metal center of proteins. Several studies have demonstrated that anomalous metabolism or altered levels of H_2S may be implicated in different pathologies, including cardiovascular and neurodegenerative diseases or cancer (Cao X et al., 2019). Interestingly, growing evidence has suggested that crosstalk between H_2S and the other gasotransmitters, NO and CO, exists since they share many properties, modes of action, or regulatory targets (Wu D et al., 2018). At present H_2S is considered a key mediator, playing an important role in several physiopathological processes.

1.2. Chemical and biochemical proprieties of H_2S

H_2S is a colorless flammable gas characterized by a strong rotten egg smell under standard conditions of temperature and pressure.

H_2S is easily soluble in water and lipids, due to its low molecular weight, so it can easily come across cellular plasma membrane without the need of a transporter (Wang R., 2012).

H_2S is a weak acid and in the aqueous solution that quickly reaches the equilibrium of H_2S/ hydrosulfide anion (HS^-)/ sulfide anion (S^{2-}) species:

$$H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2\ H^+$$

At physiological pH (7.4) about 20% of H_2S exists as an undissociated form and nearly 80% as HS^- and hydrogen ion (H^+), while S^{2-} is negligible. In mitochondrial matrix where pH is alkaline pH (8), about 92% of H_2S reaches HS^- and the remaining 8% corresponds to H_2S. However, H_2S and HS^- coexist in an aqueous solution it makes difficult to separate their effects and to establish which of them is involved in the signaling process (Bełtowski J et al., 2015). From the chemical point of view, H_2S is the simplest thiol and it can be only oxidized, due to the lowest state of oxidation of sulfur atom (-2). Consequently, H_2S and, all the intermediate derived from it, can exert biological effects by the reaction with the reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Cao X et al., 2019). Others H_2S targets in mammalian cells are the metal center that is included in proteins, as biological irons. In addition, recent studies demonstrated another interaction between H_2S, and a biological target called persulfidation (Cao X et al., 2019). With this interaction H_2S can interact with protein thiol group forming protein persulfide, inducing functional changes of protein targets (Bełtowski J et al., 2015).

1.3 Biosynthesis of H_2S in mammalian cells

In mammalian cells, H_2S biosynthesis occurs via enzymatic and non-enzymatic pathways (Figure 1), however, the non-enzymatic pathway is responsible for a small portion of H_2S production (Rose P et al., 2016). The three enzymes involved in the endogenous generation of H_2S are cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST). In the cytosol, H_2S can be produced mainly from L-cysteine (L-Cys) by CBS and CSE, which are two pyridoxal-5'-phosphate (PLP) dependent enzymes (Rose P et al., 2016; Lechuga TJ et al., 2019). CBS and CSE are also involved in the transsulfuration pathway where homocysteine is converted in L-Cys (Kabil O et al., 2014).

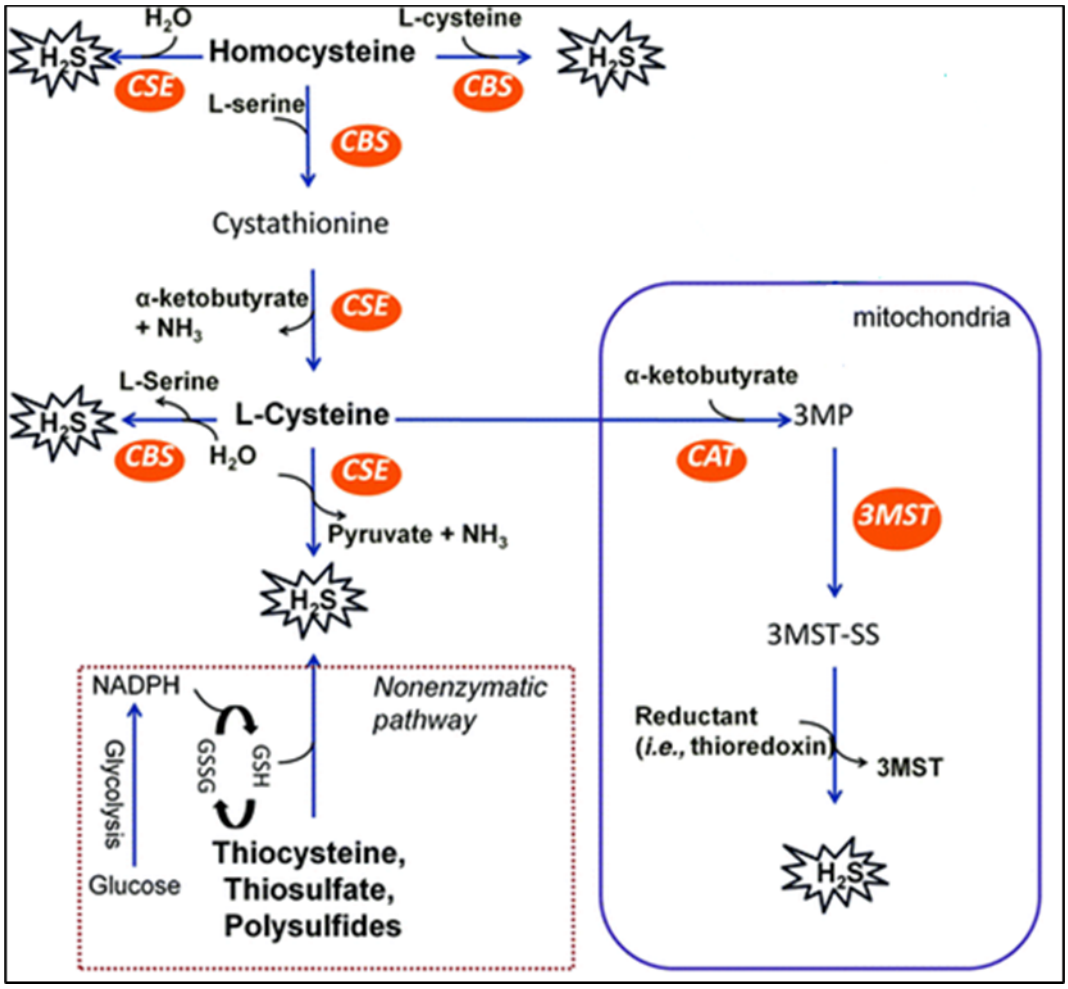

Figure 1. Endogenous H_2S production in mammalian cells. The enzymatic and non-enzymatic pathways involved in H_2S production. H_2S is produced through an enzymatic pathway by CBS, CSE, and 3MST coupled with CAT. The non-enzymatic pathway involves thiosulfate, polysulfide, and thiocysteine, which in presence of reducing equivalent, as NADPH, are reduced into H_2S and other species (Adapted from "A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer?"; Cao X et al., 2020).

3MST is a PLP-independent enzyme mainly located in mitochondria, that cooperates with cysteine aminotransferase (CAT) for H_2S synthesis (Shibuya N et al., 2013). However, in certain conditions such as hypoxia where the activity of 3MST is suppressed, CBS and CSE can translocate in mitochondria to generate H_2S (Teng H et al., 2013). The distribution and the occurrence of CBS, CSE, and 3MST in cells, tissue, and organs are an evolving question since these can vary under different pathophysiologic conditions. In addition, the activity of these enzymes could be tightly regulated and affected by pathological conditions (Cao X et al., 2019). The distribution of H_2S-synthesizing enzymes in organs and tissue has been largely evaluated in different organisms by immunohistochemical analysis, real-time PCR, and by evaluating the H_2S generating activity of all three enzymes. Ultimately, a small amount of endogenous H_2S is derived through non-enzymatic pathways, involving the reduction of sulfur species (Cao X et al., 2019). H_2S is produced as an intermediate or end-product of microbial metabolic pathways, within the gastrointestinal microbiota (Giuffrè A et al., 2018). Indeed, it was reported the intestinal microbiota not only regulates H_2S homeostasis in the gut but also in other many organs and tissue (Giuffrè A et al., 2018; Shen X et al., 2013). Another source of H_2S is represented by persulfides and polysulfides, which are derived from the dietary intake or can be endogenously generated (Giuffrè A et al., 2018).

1.3.1 Cystathionine β-synthase

CBS was the first H_2S-producing enzyme identified in 1969 (Braunstein AE et al., 1969). In general, CBS has been reported to be the most abundant H_2S-producing enzyme in the nervous system in physiological conditions, although it is expressed in other many organs and systems, including the liver, pancreas, and kidney (Cao X et al., 2019). In addition, recently it has been reported that the expression of CBS in the carotid, uterine, mesenteric, and umbilical arteries (Saha S et al., 2016). CBS condenses homocysteine with L-serine (L-Ser) to produce L-cystathionine and H_2O in the transsulfuration pathway, which represents a critical step to produce L-Cys (Cao X et al., 2019). L-Ser can be replaced in these reactions by L-Cys, resulting in L-cystathionine and H_2S formation (Bełtowski J., 2015). In the presence of L-Cys,

via a β-replacement reaction, where L-Cys is converted to L-Ser and lanthionine, CBS generates H_2S (Singh S et al., 2009). In addition, CBS may also catalyze the alternative reaction between two molecules of L-Cys to form H_2S and lanthionine, or the reaction between two molecules of homocysteine to generate H_2S and homolanthionine (Singh S et al., 2009). The presence of both homocysteine and L-Cys is required for H_2S production by CBS (Singh S et al., 2009). Human CBS (Figure 2) is a homotetramer protein of 552 amino acid-long monomers, each one consisting of three catalytic domains: a C- terminus domain, that includes two motifs known as CBS1 and CBS2, contains an S-adenosyl-L-methionine (SAM) – binding domain, and, a central catalytic PLP binding domain, and finally an N-terminal domain binding a heme group (Giuffrè A et al., 2018). CBS activity can be tightly regulated on the catalytic sites of the protein by several cofactors and activators. The binding of NO or CO to the CBS heme group containing Fe (II), reduces CBS activity and consequently H_2S generation (Taoka S et al., 2001). Both SAM glutathionylation enhanced CBS activity and consequently H_2S production (Ereño-Orbea J et al., 2014).

1.3.2 Cystathionine γ-lyase

CSE is the other PLP-dependent enzyme involved in H_2S production. It is widely expressed in the cardiovascular and respiratory systems, liver, kidney, uterus, and pancreatic islets (Cao X et al., 2109). CSE and CBS shares some of the reaction involved in H_2S production. As CBS, CSE utilizes homocysteine substrate to generate H_2S, α-ketobutyrate, and ammonia. Alternatively, CSE can also produce H_2S, along with pyruvate and ammonia, by using L-Cys as substrate. Since the physiological concentration of L-Cys is higher than homocysteine levels, the main mechanism of H_2S production by CSE is the α – and β- elimination of L-Cys (Chiku T et al., 2009). Conversely, in pathological conditions such as hyperhomocysteinemia, where the levels of homocysteine are increased, about the 90% of H_2S produced by CSE derived from α – and β- elimination of homocysteine (Chiku T et al., 2009). CSE (Figure 3) is a homotetrameric enzyme consisting of 405-amino-acid-long monomers, each one composed of two structural domains: a PLP-binding catalytic domain and a smaller C-terminal domain.

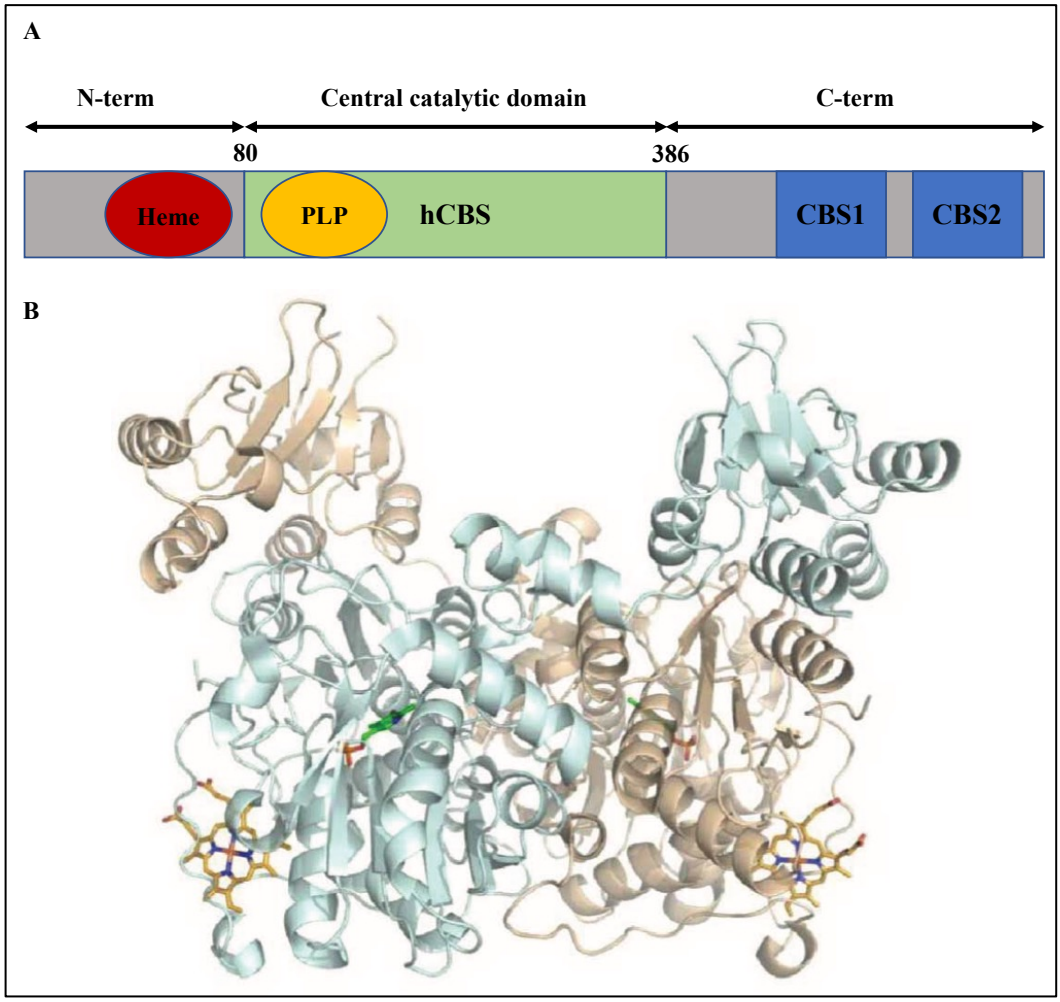

Figure 2. CBS protein modular organization (A). Structure of human CBS homodimer where the sticks in green represent the active site (PLP) where H_2S is produced, while orange sticks represent the heme regulatory site, where NO and CO bind the enzyme to regulate protein inhibition (B). ("Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology"; Giuffrè A et al., 2018).

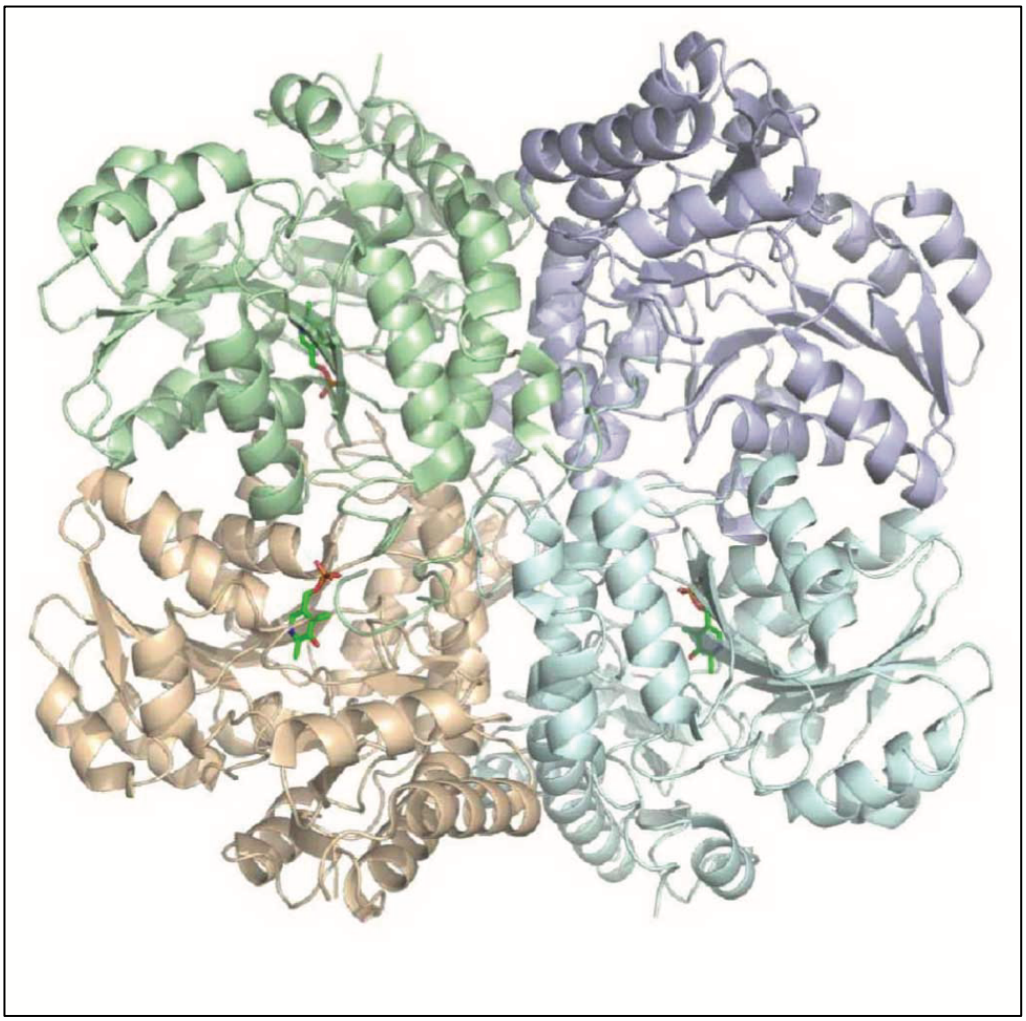

Figure 3. Structure of human CSE homotetrameric enzyme. The green chain represents the site where H_2S is produced ("Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology"; Giuffrè A et al., 2018).

It has been reported that CSE activity is influenced by intracellular Ca^{2+} levels (Mikami Y et al. 2013). More in detail, CSE efficiently produces H_2S in presence of PLP at a physiological concentration of Ca^{2+}, whereas H_2S production is suppressed when intracellular Ca^{2+} levels are increased (Mikami Y et al. 2013). The exact mechanism involved in the regulation of CSE activity by intracellular Ca^{2+} levels needs further investigation.

1.3.3 3-mercaptopyruvate sulfurtransferase

3MST is the most recently discovered enzyme involved in H_2S biosynthesis (Shibuya N et al., 2009). The presence of 3MST has been reported in different organs and systems, including cardiovascular, urogenital systems, kidney, liver, and pancreas (Cao X et al., 2019), and it is mainly localized in mitochondria. 3MST cooperates with CAT for H_2S production where CAT catalyzes the conversion of L-Cys into 3-mercaptopyruvate and L-glutamate. After that, 3MST in presence of dithiol-containing reductants, including thioredoxin or dihydrolipoic acid, converts 3mercaptopiruvate to pyruvate and H_2S (Yadav PK et al., 2013; Mikami Y et al., 2011). Recently, another source of 3 mercaptopyruvate and consequently of H_2S, was found in the brain and kidney. D-cysteine, by the action of D-amino oxidase (DAO), is converted in 3mercaptopiruvate that may be used by 3MST to produce H_2S (Shibuya N et al., 2013). Since DAO is exclusively located in the brain and kidney, this pathway that produces H_2S occurs exclusively in these two organs (Cao X et al., 2019). 3MST activity, distinct from CBS and CSE, seems to be affected by its redox state instead of by the interaction with other factors (Nagahara N., 2013). Human 3MST is a protein composed of 297 amino acid residues assembled as a monomer (Yadav P. K et al., 2013). The 3MST monomer (Figure 4) consists of two structurally related domains, an N-terminal and a C-terminal domain linked by a 26 amino acid linker, that can interact with both domains (Giuffrè A et al., 2018). In the structure of 3MST, three redox-sensitive cysteine (Cys) were reported on its catalytic site, such as Cys 154, Cys 247, and Cys 263, that regulate the enzyme activity (Nagahara N., 2013). It has been reported that shear stress significantly decreases 3MST activity and H_2S production by the oxidation of this thiol (Nagahara N., 2013).

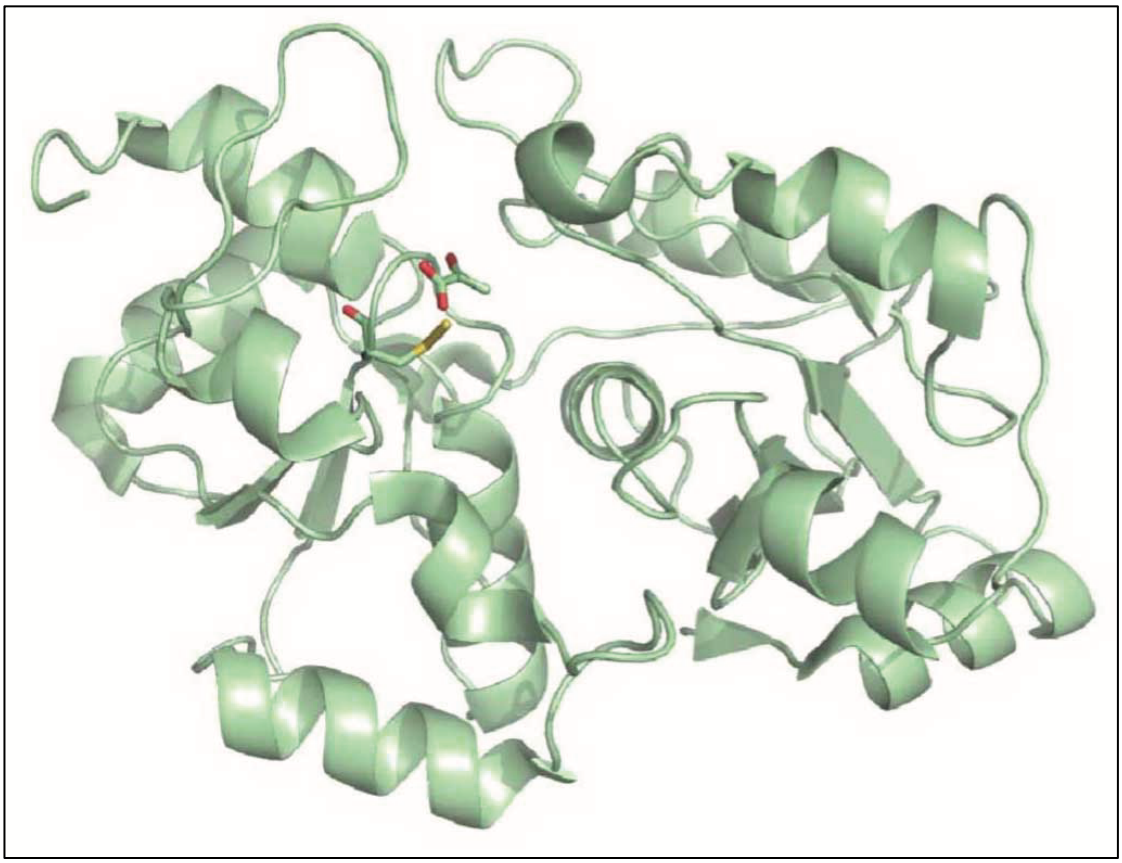

Figure 4. Crystallographic structure of 3MST monomer ("Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology"; Giuffrè A et al., 2018).

1.3.4 H_2S Non - enzymatic production

In mammalian cells, H_2S can be generated via a non- enzymatic pathway (Figure 1) from sulfane sulfur via in presence of reducing equivalents, including nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), which are supplied by oxidation of glucose (Searcy DG., 1998; Cao X et al., 2019).

Reactive sulfur species in persulfide, thiosulfate, or polysulfide can be reduced to H_2S and other metabolites in the presence of NADPH or NADH (Olson KR et al., 2013). This mechanism occurs in hyperglycemia, which enhances H_2S generation via non-enzymatic pathways (Wang Q et al., 2009).

1.4 Metabolism of H_2S

According to the current knowledge, H_2S must be metabolized because H_2S accumulation may result in organ toxicity. The main mechanism of H_2S toxicity is associated with the inhibition of the mitochondrial respiratory chain by the binding to cytochrome c oxidase (Bełtowski J., 2015). Three main mechanisms were indicated for H_2S metabolism in mammalian cells, including oxidation, methylation, and expiration (Figure 5). The most important mechanism involved in H_2S metabolism is oxidation, which occurs in mitochondria. H_2S is quickly oxidized to thiosulfate ($S_2O_3^{2-}$), a reaction associated with mitochondrial electron respiratory chain and catalyzed by superoxide dismutase. $S_2O_3^{2-}$ is further converted to SO_3^{2-} by thiosulfate cyanide sulfurtransferase (TST), which transfers a sulfur atom from $S_2O_3^{2-}$ to cyanide or other sulfur acceptors. Next, SO_3^{2-} is oxide to SO_4^{2-} in a reaction catalyzed by sulfide oxidase (SO). The conversion of $S_2O_3^{2}$ to SO_3^{2-} and SO_4^{2-} occurs in the presence of sulfide- detoxing enzyme. Finally, sulfate can be excreted via urine as the main product of H_2S metabolism (Cao X et al., 2019). Nevertheless, the urinary concentration of sulfate does not serve as a marker of endogenous H_2S because sulfate in urine can derive from the direct oxidation of L-Cys (Cao X et al 2019). Oxidation of H_2S may occur in all types of mammalian cells and mitochondria can oxide H_2S only at low concentration (less than 10 μM), because the higher concentration of H_2S, similarly to the other gasotransmitters,

inhibits cytochrome c oxidase (Bełtowski J., 2015). Different from oxidation, the methylation of H_2S mainly occurs preferably in the cell cytosol. In the methylation pathway, H_2S is converted into methanethiol that in turn can be converted into non-toxic dimethylsulfide by thiol S-methyltransferase (TMST). Only a minimal amount of H_2S may arrange through this pathway in physiological conditions (Bełtowski J., 2015). H_2S can escape from mammalian tissue, in the particular mammalian lung. It seems that the expiration of H_2S may occur when a large amount of this gas is generated in many pathological conditions associated with excessively H_2S production, including septic shock or chronic obstructive pulmonary disease (Cao X et al., 2019).

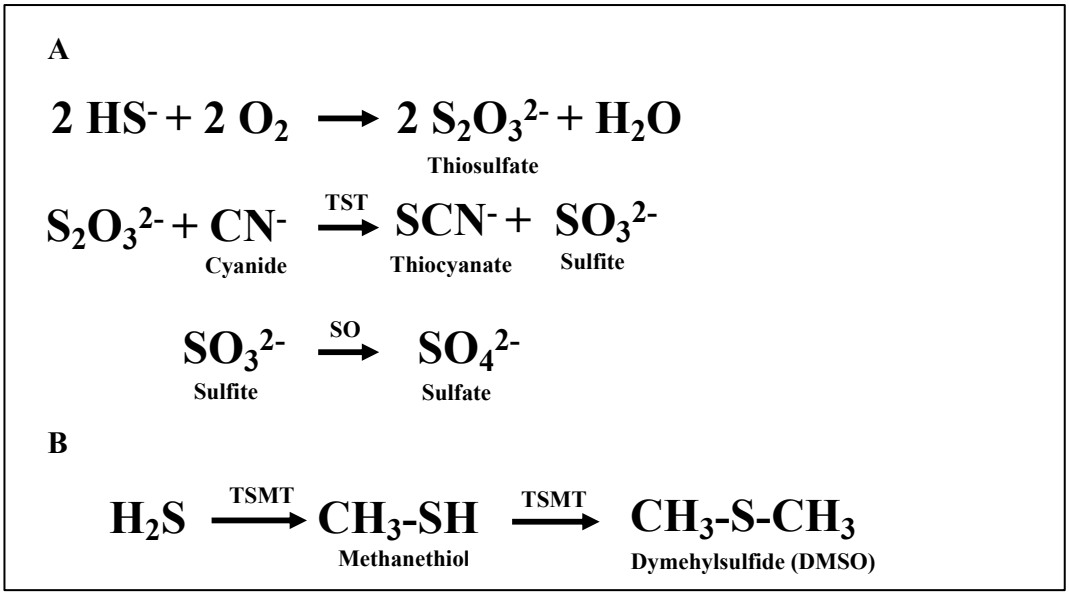

Figure 5. Main mechanisms involved in H_2S metabolism. Mitochondrial oxidation (A); Cytosolic methylation (B).

1.5 H_2S in the cardiovascular system

1.5.1 Role of H_2S in the cardiovascular system

H_2S is involved in the regulation of several pathophysiological processes in the cardiovascular system (Pan LL et al., 2017). A large number of studies investigating the therapeutic value of H_2S revealed that physiological levels of H_2S have a pivotal role in cardiovascular homeostasis, and the use of H_2S donors or H_2S inhibitors could avoid the development of many cardiovascular diseases, including heart failure, atherosclerosis, hypertension, or endothelial dysfunctions (Calvert JW et al., 2010). Moreover, several mechanisms are involved in the H_2S cardiovascular protective effects, which include antioxidant, anti-apoptotic, or anti-inflammation activity and ion channel regulation (Pan LL et al., 2017). In vascular smooth cell muscle (VSMCs), H_2S is involved in the regulation of cell apoptosis, relaxation, contraction, and switch of phenotype (Pan LL et al., 2017). In endothelial cells (EC), H_2S exerts a proliferative and pro-angiogenic action and it can attenuate endothelial inflammation (Pan LL et al., 2017).

1.5.2 Endogenous production of H_2S in the vasculature

Endogenous H_2S is produced through an enzymatic pathway involving CBS, CSE, and 3MST. All these enzymes are expressed in the cardiovascular system (Yang G et al., 2015; Pan LL et al., 2012) either in VSMCs or EC (Zhao W et al., 2001; Ciccone V et al., 2021; Skovgaard N et al., 2011). Nevertheless, CBS expression is more predominant in the brain, nervous system, and liver compared to CSE which is mainly localized in the cardiovascular system (Pan LL et al., 2012).

Changes in expression or activity of H_2S producing enzymes or significant changes in endogenous H_2S levels have been correlated to cardiovascular diseases (Li N et al., 2016).

1.5.3 Physiological regulation of vascular tone by H_2S

One of the earliest demonstrations of the vasodilatory effect of H_2S has been reported by Zhao et al. (Zhao W et al., 2001). This study showed that the vasodilatory effect of H_2S, on phenylephrine (PE) pre-contracted rat aorta ring,

mainly occurred through the opening of adenosine triphosphate-sensitive potassium channels (K^+_{ATP}) identifying it as the first gaseous opener of K^+_{ATP} in VSMCs (Zhao W et al., 2001).

H_2S relaxes blood vessels, such as the aorta, gastric artery, mesenteric artery, and internal mammary artery (Zhao, W et al., 2001; Sun HJ et al., 2020). The mechanism involved in the H_2S relaxing effect of the blood vessel is related to the activation of K^+_{ATP} channels in VSMCs, and this action is endothelium-independent (Tang G et al., 2005). The involvement of K^+_{ATP} channels was confirmed by using glibenclamide, an inhibitor of K^+_{ATP} channels, that blocked partially the H_2S relaxing effect on blood vessels (Webb GD et al., 2008). More in detail, H_2S causes sulfhydration of the Cys 43 of a subunit of K^+_{ATP} channels called Kir6.1. The sulfhydration of Cys43 promotes the capacity of Kir6.1 to bind adenosine triphosphate (ATP), resulting in the opening of K^+_{ATP} channels in VSMCs (Mustafa AK et al., 2011).

However, growing body evidence suggests that several multidimensional mechanisms are involved in the H_2S vasorelaxant effect (Krüger-Genge A et al., 2019). H_2S can activate calcium-activated potassium channels (K_{Ca}^{2+} channels) and large-conductance calcium-activated potassium channels (BK_{Ca}^{2+} channels) resulting in vasodilation (Krüger-Genge A et al., 2019). In addition, the activation of voltage-sensitive potassium channels (K_v channels) and $K_v7.4$ voltage-gated potassium channels expressed in VSMCs seem to be the target of H_2S in the regulation of vascular tone. Transient receptor potential cation channel V4 (TRPV4) is modified by H_2S sulfhydration. Moreover, L-type Ca^{2+} channels, Cl^-/HCO_3^- channels (Krüger-Genge A et al., 2019), and phospholipase A_2 (PLA_2) (d'Emmanuele di Villa Bianca R et al., 2011) are implicated in the H_2S relaxing effect. Activation of endothelial nitric oxide synthase (eNOS) and the inhibition of cyclic guanosine monophosphate (cGMP) degradation are other pathways involved in the regulation of vascular tone by H_2S (Cao X et al., 2018). It has been demonstrated that H_2S acts as a phosphodiesterases (PDEs) inhibitor elevating the levels of cGMP and cyclic adenosine monophosphate (cAMP) (Bucci M et al, 2010). Besides, H_2S increases the level of cGMP, through the inhibition of PDEs and in turn activates the protein kinase G (PKG) that phosphorylates the

vasodilator-stimulated phosphoprotein (VASP) resulting in vasodilation. This vasorelaxant effect is reversed by the inhibition of PKG or eNOS, indicating that H_2S and NO cooperate, with different mechanisms, to maintain elevated levels of cGMP that activate PKG leading to a vasorelaxation (Bucci M et al., 2010; Coletta C et al., 2012). Moreover, H_2S can affect the NO/cGMP pathway by promoting eNOS phosphorylation at Serine 1177 (King AL et al., 2014). In addition, by using a purified recombinant human soluble guanylate cyclase (sGC) it has been shown that H_2S converted ferric to ferrous sGC enhancing NO-donor-stimulated sGC activity and reducing the heme-independent activator BAY 58-266 triggered cGMP formation. This finding suggests an additional mechanism of crosstalk between the NO and H_2S pathways at the level of redox regulation of sGC (Cao X et al., 2018; Lv B et al., 2020). Another mechanism to stimulate eNOS activity by H_2S is represented by the direct sulfhydration. Indeed, H_2S sulfhydrilated Cys 443 of eNOS promoting eNOS dimerization with an increase of NO production (Wang R et al., 2012; Kanagy NL et al., 2017). Thereafter, metabolic/mitochondrial effects could be implicated in H_2S induced vasorelaxant effect. It has been demonstrated that in mesenteric arterioles sodium hydrogen sulfide (NaHS) (10 -100μM) vasorelaxant effect occurs through the inhibition of mitochondrial complexes I and III (Hedegaard ER et al., 2016). Under certain conditions, H_2S can not only relax blood vessels but also contract blood vessels (Caprnda M et al., 2017). H_2S showed a biphasic effect i.e., a contraction at a lower concentration followed by vasodilation at a higher concentration (Caprnda M et al., 2017; Lv B et al., 2020). In rat mesenteric arterial bed has been observed that lower concentration of H_2S (10μM and 100μM) lead to a contractile effect in a concentration-dependent manner, conversely higher H_2S concentration (1mM) caused the relaxation of blood mesenteric arterial bed (Kanagy NL., et al 2017; d'Emmanuele di Villa Bianca R et al., 2011). The biphasic effect of H_2S showed in rat mesenteric bed occurred through the release of arachidonic acid by PLA_2, which led to a vasocontraction followed by vasodilation mediated by the metabolites derived from cytochrome P450 (d'Emmanuele di Villa Bianca R et al., 2011). More recently it has been demonstrated that the H_2S contractile effect in mice aorta requires endothelium/NO signaling and involves e inosine 3',5'-cyclic monophosphate (cIMP) as a second

messenger (Mitidieri E et al., 2021). H_2S at nanomolar concentrations promotes, within vessels, rapid phosphorylation of PDE4A and PDE5 which in turn leads to enhanced degradation of both cAMP and cGMP but not cIMP. Therefore, the reduced availability of both cAMP and cGMP uncovers cIMP signaling which leads to a contractile effect; indeed, the proof of concept was given by the exogenous administration of an analog stable of cIMP that gives a contacting effect with a simile profile of NaHS in pre-contracted aorta rings (Mitidieri E et al., 2021).

1.5.4 Effect of H_2S on VSMCs – Proliferation, apoptosis, and autophagy

Experimental evidence has suggested that VSMCs proliferation is inhibited by H_2S. NaHS, an exogenous source of H_2S, in a dose-dependent manner; indeed, in CSE knockout ($CSE^{-/-}$) mice VSMCs proliferation resulted significantly increased (Du J et al., 2004). The main mechanism involved in the inhibition of cell proliferation by H_2S is the suppression of the activity of mitogen-activated protein kinase (MAPK) (Yang G et al., 2004; Du J et al., 2004). However, several pathways and signals molecule have been identified to contribute to the regulation of cell proliferation by H_2S, including MAPK/TXNIP, and ERK1/2 (Sun HJ et al., 2020). In the vasculature, H_2S can act as an anti-apoptotic or pro-apoptotic mediator. Several studies reported the contribution of H_2S as an enhancer of cell apoptosis (Yang G et al 2004). In human aortic smooth muscle cells, the apoptotic effect induced by H_2S occurred through ERK that in turn activates the caspase-3 (Yang G et al., 2004). In contrast, other studies demonstrated that H_2S inhibited apoptosis (Yang G et al., 2004). H_2S inhibited apoptosis caused by high glucose levels, through the decrease of the ratio of Bcl2-associated x/B 2 and inhibiting caspase 3 activity of primary human umbilical vein endothelium cells (Guan Q et al., 2012; Lv B et al., 2020). Another mechanism involved in the inhibition of apoptosis by H_2S is the suppression of endoplasmatic reticulum stress (ERS) in a rat model of chronic obstructive pulmonary disease (COPD); NaHS treatment caused a reduction of the expression of ERS marker i.e., caspase-12 and glucose-regulated protein 78 (Ding HB et al., 2018). All these data suggest that H_2S may act as a pro-proliferative or anti- proliferative and as a promoter or inhibitor of apoptosis under

different pathological conditions. H_2S is reported to be both a promoter and an inhibitor of autophagy, which represents an essential mechanism for maintaining cellular homeostasis for cells development and differentiation and programmed cell death. H_2S reduces autophagy through the reduction of activation of the AMPK/mammalian target of rapamycin (mTOR) pathway, exerting a protective role in vascular function (Qiu X et al., 2018). In addition, NaHS treatment on VSMCs cultured in high glucose conditions, reversed the upregulation of autophagy and consequently reduced the activation of the AMPK/mTOR pathway, which is associated with autophagy (Qui X et al., 2018).

1.5.5 Effect of H_2S on EC- Proliferation, and angiogenesis

Several studies reported a pro-angiogenic and pro-proliferative role for H_2S in EC. Cytoprotective effects occur at low concentrations of H_2S, conversely at higher concentrations usually occur cytotoxic effects (Szabo C et al., 2011). Indeed, administration of a low dose of NaHS promotes angiogenesis, while a high dose of NaHS exerts an inhibitory effect on angiogenesis. Treatment of RF/6A cells with the H_2S donor, NaHS (10 and 20 μM) promoted cell proliferation, adhesion, and migration. These effects were mediated by AKT phosphorylation as the cell proliferation, adhesion, and migration were reversed by a phosphoinositide 3-kinase (PI3-K) inhibitor LY 294002 or wortmannin. On the other hand, higher concentrations of NaHS showed an opposite effect on RF/6A cells (Cai WJ et al., 2007). PI3K/AKT pathway, MAPK pathway, and K^+_{ATP} channels were involved in the regulation of the pro-angiogenic effect of H_2S, too (Szabo C et al., 2011). H_2S also exerts a proliferative effect on EC mainly through the activation of the mammalian target of rapamycin and the vascular endothelial growth factor 2 pathway (Yang G et al., 2004).

1.5.6 Crosstalk between H_2S and NO in the cardiovascular system

As gasotransmitters, H_2S and NO not only share activity but also many biological effects. Growing experimental evidence showed a crosstalk between H_2S and NO since their physiologic and pathologic function and signaling can be overlapped. Thereafter, they can influence each other's production or physiological response

and they also produce a novel species through biochemical interaction (Lo Faro ML et al., 2014; Wu D et al, 2018).

The binding of NO to CBS reduced CBS activity and H_2S generation (Taoka S et al., 2001), while recombinant eNOS activity was inhibited by H_2S (Kubo S et al., 2007). However, NO and H_2S inhibit each other's production and several studies reported that they can promote their respective production. In EC H_2S increases NO production through calcium release and activation of eNOS/AKT pathway, and calcium chelator inhibited H_2S induced NO production (Kida M et al., 2013). In addition, in $CSE^{-/-}$ mice, H_2S reduction led to a decrease of NO levels, eNOS dysfunction, and oxidative stress (Wu D et al., 2018). Thus, eNOS activation and NO generation are associated with H_2S cytoprotective function (King AL et al., 2014; Wu D et al., 2018).

One of the mechanisms through which H_2S can enhance eNOS activity is S-sulfhydration. H_2S can promote eNOS dimer formation to enhance NO production (Wu D et al., 2018). Several studies have suggested crosstalk between NO and H_2S by using an exogenous source of both gasotransmitters. Recently endogenous crosstalk between NO and H_2S has been shown within the transsulfuration pathway. This study demonstrated that the vasorelaxant effect of L-Cys involves not only H_2S but also L-Ser, which triggers the sphingosine -1 phosphate (S1P) biosynthesis by the interaction with its receptors target mediates NO production. Thus, the relaxing effect of L-Cys is mediated by the contemporary action of NO and H_2S, defining new endogenous crosstalk between the two gasotransmitters within the endothelium (Mitidieri E et al., 2019).

1.6. Role of H_2S in diabetes mellitus

1.6.1 Diabetes mellitus and H_2S - an overview

Nowadays, diabetes mellitus (DM) is a common problem in our society that affects about 10% of the adult population in the world. DM is a chronic metabolic disease characterized by elevated levels of blood glucose or hyperglycemia, resulting from anomalies in insulin secretion or insulin action or both (American Diabetes Association., 2014; Banday MZ et al., 2020). Long-term hyperglycemia can lead to

many diseases, that are associated with DM, including microvascular and macrovascular disorders, diabetic retinopathy, and nephropathy (American Diabetes Association., 2018).

There are two main types of diabetes, classified by ethology and pathology:

- type 1 of DM (T1DM) is an autoimmune disease that occurs when T-cell mediated the destruction of pancreatic β-cell, resulting in a complete lack of insulin and hyperglycemia (Banday MZ et al., 2020; Kahaly GJ et al., 2016). Both genetic predisposition and some environmental factors, including obesity and insulin resistance, are involved in the development of T1DM (Tremblay J et al., 2019).
- type 2 of DM (T2DM), a non-insulin-dependent type of DM, is characterized by insulin resistance and pancreatic β-cell dysfunction and it constitutes about 90% of all the cases of diabetes (Banday MZ et al., 2020; DeFronzo RA., 2004). T2DM usually appears at the age of 30 years and is often associated with obesity. The impaired effect of insulin on glucose metabolism, known as insulin resistance, is usually associated with obesity and could represent the main mechanism involved in the pathogenesis of T2DM.

Insulin secretion and sensitivity play a pivotal role in glucose homeostasis, in particular glucose levels, and their abnormalities result in DM. Current evidence relates DM and its chronic complication, which are also associated with changes of gasotransmitters signals (Qian LL et al., 2018; van den Born JC et al., 2016).

1.6.2 Endogenous production of H_2S in the pancreas

Altered expression of enzymes deputed to H_2S synthetases and H_2S levels have been observed in diabetic animals and humans (Gheibi S et al., 2020). Several studies showed that H_2S is produced in both pancreatic β-cell and in the main target of insulin, such as liver, adipose tissue, and skeletal muscle where it can regulate insulin secretion and insulin sensitivity/resistance (Gheibi S et al., 2020). H_2S producing enzyme, CBS and CSE are expressed in pancreatic β-cell. In mouse β-cell line (MIN6) and fresh mouse β-cell isolated from islets, CBS and CSE mRNA were detected but only CBS has been found at the protein level (Bełtowski J et al.,

2018; Kaneko Y et al., 2016). Many in vivo studies reported that the expression of both CBS and CSE, as well as H_2S biosynthesis was higher in pancreas and liver in streptozocin (STZ) induced diabetic rats (Yusuf M et al., 2005; Zhu L et al., 2020). Similarly, hepatic CBS and CSE activity increased in STZ induced diabetic rats, and this effect was reversed by the treatment with insulin (Bełtowski J et al., 2018; Jacobs RL et al., 1998). Other studies reported that CSE expression is higher than CBS in rat islets at mRNA levels (Wu L et al., 2009). Indeed, following glucose stimulation only CSE expression was significantly increased, suggesting a pivotal role for CSE as an inducible H_2S generating enzyme (Gheibi S et al., 2020; Kaneko Y et al., 2009). More in detail, in cultured insulinoma INS-1 E cell most of the H_2S production was abolished after treatment with propargylglycine (PAG), CSE inhibitor (Yang W et al., 2005). All these dates suggest that differences may exist in the expression and activities of CBS and CSE between the various species (Bełtowski J et al., 2018). In addition, 3MST expression in pancreatic β-cell has been documented, but until now its role has not been deeper examined (Tomita M et al., 2016).

1.6.3 Involvement of H_2S on insulin secretion and pancreatic β-cell apoptosis

Once released in β-cell H_2S can influence insulin secretion, modulating circulating glucose levels. In pancreatic cell line MIN6 and isolated pancreatic mice islets cultured in high glucose conditions (10mM), both L-Cys (0.1-10mM) and NaHS (10μM-1mM) caused a reduction of insulin secretion (Kaneko Y et al., 2006; Gheibi S et al., 2020). Conversely, in low glucose conditions, insulin secretion has not been influenced by the presence of L-Cys or NaHS (Kaneko Y et al., 2006; Gheibi S et al., 2020). Furthermore, insulin secretion in HIT-T15 cells was inhibited by about 70% by NaHS (100μM) treatment (Gheibi S et al., 2020). It has been demonstrated that H_2S acts as an endogenous activator of K^+_{ATP} channels also in pancreatic β-cell (Ali MY et al., 2007). The activation of K^+_{ATP} by high pancreatic endogenous H_2S levels inhibits glucose-stimulated insulin secretion (Bełtowski J et al., 2018; Zhu L et al., 2020). The involvement of K^+_{ATP} could be explained as the inhibitory effect of L-Cys or NaHS, on insulin secretion, which is reproduced by

using tolbutamide, a K^+_{ATP} blocker (Kaneko Y et al., 2016). S-sulfhydration of K^+_{ATP} channels has been proposed as an underlying mechanism by which H_2S influences insulin secretion (Carter RN et al., 2006; Gheibi S et al., 2020).

The data about the effect of H_2S on pancreatic β-cell are controversial. It has been found that CSE/H_2S pathway is involved in the regulating of cell functions by stimulating β cell apoptosis and inducing K^+_{ATP} channel activity (Yang G et al., 2007) whereas β-cell apoptosis represents the hallmark of both T1DM and T2DM (Marrif HI., 2016). It has also been demonstrated that exogenous H_2S or CSE overexpression can induce β-cell apoptosis through the stimulation of p38MAPK and ERS, and these effects were abolished by the p38MAPK inhibitor (Yang G et al., 2007; Bełtowski J et al., 2018). All data suggest that H_2S can contribute to the development of deleterious effects of DM, including β-cell loss and insulin deficiency, by the inhibitory effect on insulin secretion or the stimulation of β-cell apoptosis.

In contrast, other studies have demonstrated a protective role of H_2S against β-cell apoptosis induced by oxidative stress or glucotoxicity (Kaneko Y et al., 2009; Taniguchi S et al., 2011). NaHS (100μM) or L-Cys (3mM) attenuated apoptosis in β cells stimulated with glucose (20mM) (Kaneko Y et al., 2009; Gheibi S et al., 2020). As well as NaHS can increase AKT phosphorylation, promoting cell proliferation and survival (Gheibi S et al., 2020). In addition, apoptosis in mice isolated pancreatic β cells induced by sodium palmitate (0.5mM), cytokine or hydrogen peroxide, was inhibited by exogenous H_2S (100μM) (Taniguchi S et al., 2016). This protective role may result from the inhibition of oxidative stress. In contrast, NaHS fails in inhibiting β cell apoptosis induced by ERS inducer, such as tunicamycin (Taniguchi S et al., 2016).

1.6.4 Effect of DM on H_2S circulating levels

The enhanced production of H_2S in the pancreas and liver does not reflect an increase in the plasma concentration of these gasotransmitters, which was similar between diabetic and non-diabetic mice (Zhu L et al., 2020). Maybe the increased H_2S production in the liver and pancreas could represent a local tissue response to DM. Interestingly, NOD (non-obese diabetic) mice, a model of T1DM, showed a

decrease of H_2S plasma levels correlated to the severity of DM, and in NOD III mice the decrease was about 50%, suggesting that the endogenous H_2S production is impaired under hyperglycemia conditions (Brancaleone V et al., 2009). Furthermore, in line with the animal studies, plasma levels of H_2S in diabetic patients were significantly lower when compared with normal subjects (Jain SK et al., 2010). In addition, it has been observed that the decrease of H_2S plasma levels was associated with a history of cardiovascular disease, primarily in patients with T2DM (Jain SK et al., 2010).

1.6.5 Interaction of H_2S with others gasotransmitters in pancreatic β-cell

The regulation of insulin secretion by the interaction between H_2S and other gasotransmitters has been investigated in rat pancreatic β-cell. NO producing enzyme eNOS and neuronal nitric oxide synthase (nNOS), heme oxygenase 1 and 2, (HO-1 and 2), were found together with CSE and CBS (Beltwski J et al., 2018). It has been reported that in cultured pancreatic β-cell the H_2S donor, NaHS promoted NO and CO production, while sodium nitroprussiate, a NO donor, stimulated H_2S and CO synthesis, and finally a donor of CO, CORM-2, stimulated NO and H_2S production. Calmodulin antagonist attenuated these effects indicating the involvement of the calcium-calmodulin pathway (Moustafa A et al., 2014).

1.7. Role of H_2S in inflammation

1.7.1 Inflammation – an overview

Inflammation is a complex physiological adaptive response of vascularized tissue, caused by several noxious stimuli and conditions, including pathogens, external mechanical stimuli, irritant agents, or tissue injury. In clinical practice, the five "cardinal" signs of inflammation are pain, heat, redness, swelling, and in the same case loss of function (Fujiwara N et al., 2005; Whiteman M et al., 2011).

In the wide sense, the inflammatory response leads to removing the pro-inflammatory stimuli promoting the process of healing and finally preserving the whole-body functions (Whiteman M et al., 2011). At the molecular level, several

mediators orchestrate and coordinate the inflammatory response. Indeed, a multitude of pro-inflammatory agents, by activating a precise sensor, initiates the inflammatory response that in turn gives rise to the production of specific mediators, which represent the effectors of inflammation response (Medzhitov R., 2014). Leukotrienes, histamine, bradykinin, interleukins, platelet-activating factor, and ROS can destroy the cause of inflammation, protect the injured tissue, and finally, avoid the spreading of the inflammatory process (Serhan CN et al., 2007).
Generally, the inflammatory response is classified as acute or chronic, based on the duration and kinetics of the reaction (Chen L et al., 2017).

Acute inflammation is represented by the immediate response of the host to the inflammatory stimulus. The first actor triggering the inflammatory process is the tissue-resident macrophages and mast cells, which lead to the production of several inflammatory mediators, including chemokines, cytokines, vasoactive amine (as histamine and serotonin), plasma protease, and eicosanoid (arachidonic acid metabolites). The effects of these mediators lead to the migration of blood components, including plasma and leucocyte, through the postcapillary venules, to the site of infection or injured tissue. Blood vessels, through the vascular endothelium, allow the extravasation of leucocytes to the site of injury. This process occurs through the selective ligation of selectins on endothelial cells with integrins and chemokine receptors present on leucocytes. Once in injured tissue, leucocytes are activated by direct contact with the pathogen agent or through the action of cytokines, produced by tissue-resident cells, and can remove the stimulus or repair the damaged tissue (Whiteman N et al., 2014; Medzhitov R., 2014; Chen L et al., 2017). Leucocytes, in particular neutrophiles, by releasing toxic substances from their granules, for example, ROS or reactive nitrogen species, try to kill the external agent (Serhan CN et al., 2005).

Thus, the resolution of the acute inflammatory response involves the elimination of the inflammatory agent followed by a repair phase, that is principally mediated by both tissue-resident and recruited macrophages. In addition, the change in lipid mediators from arachidonic acid metabolites to lipoxins (anti-inflammatory mediator), and the production of resolvins and protectins, is crucial for the resolution of inflammation (Serhan CN et al., 2007). Therefore, acute inflammation

is characterized by vascular changes, edema, and leucocyte, mainly neutrophiles, infiltration (Whiteman N et al., 2014).

If the acute inflammatory response fails and the inflammatory process persists and became chronic, T cells are also involved. In general, chronic inflammation leads to progressive tissue destruction mediated by inflammatory cells, vascular proliferation, and fibrosis (Pahwa R et al 2021).

Several studies have proposed that gasotransmitters including NO, CO, and more recently H_2S, are involved in the inflammatory response, although the role of H_2S is still controversial.

1.7.2 H_2S and inflammation

In the last decade, many studies indicated a role of H_2S in the inflammatory process, in part via the modulation of K_{ATP} channels, and the pathway CSE/H_2S seems to be mainly involved in the regulation of the inflammatory process (Li L et al., 2009). Alterations of H_2S levels are involved in many diseases, including hypertension, diabetes, and chronic inflammation or models of acute inflammation, as reported in several studies (Whiteman M et al., 2011). These studies reported both pro- and anti-inflammatory roles for H_2S, although its exact role is unclear. For example, H_2S has been reported to be a pro- inflammatory mediator in sepsis; (Whiteman M et al., 2011) that leads to an increase in the production of inflammatory markers, including cytokines, NO, and prostanoids. This results in tissue hypoperfusion and hypotension, which in turn damages the vasculature, in particular the vascular endothelium, impairing tissue respiration and leading to death in several patients (Rangel-Frausto MS et al., 1999). The activation of K^+_{ATP} channels seems to modulate the hypotensive effects, thus H_2S is proposed as a key mediator of this process, due to its interaction with these channels (Zhao W et al., 2001).

In early studies, celiac ligation and puncture (CLP) or lipopolysaccharide (LPS) injection, usually used as a model of endotoxic shock, increased significantly CBS and CSE and H_2S levels in plasma, lung, kidney, pancreas, and liver. Moreover, in patients with septic shock the plasmatic levels of H_2S are increased compared with normal subjects (Li L et al., 2004; Huy Y et al., 2003; Zhang H et al., 2006); in contrast, the administration of DL-Propargyglycine (PAG), a CSE inhibitor, in CLP

or LPS animal model, significantly reduced H_2S levels in plasma and promoted survival (Zhang H et al., 2006). These findings may suggest that H_2S production can be induced by inflammation, as the same happened for NO produced by inducible NO synthase (iNOS) (Huy Y et al., 2003). Furthermore, administration of NaHS, an exogenous source of H_2S, in combination with LPS, caused hypotension, increased liver and kidney inflammation, and aggravate tissue injury (Li L et al., 2005; Bathia M et al., 2012). The same effects were observed in hemorrhagic shock, where H_2S or NaHS treatment, lead to an increase in H_2S levels in plasma and liver, and PAG pre-treatment reduced H_2S production, suggesting a pro-inflammatory role of H_2S. In addition, in CLP or LPS treated animals, PAG administration decreased plasma levels of tumor necrosis factor- α (TNF- α), nitrite and nitrate, and the expression of liver and lung myeloperoxidase (MPO) activity, which are usually used as an index of inflammation (Li L et al., 2005).

Inflammatory swelling, known as edema, represents another model of acute and non-immune inflammation. Edema is the excessive accumulation of serous fluids in the intercellular space of tissue and occurs in the early phase of acute inflammation response, due to the increase of blood vessels permeability (Whiteman L et al., 2014). Induction of edema by carrageenan intra-plantar injection, caused a significant increase of H_2S production and neutrophiles infiltration, and this increase was reduced by PAG treatment (Bathia M et al., 2005), underling a pro-inflammatory role of H_2S. The intracellular signaling involved in the pro-inflammatory action of H_2S was reported in the study which investigated the effect of a sub-plantar injection of NaHS, as an exogenous source of H_2S, or the substate L-Cys in mice. Intraplantar administration of both, NaHS and L-Cys caused paw edema in mice where the pro-inflammatory effect of H_2S involves the PLA_2 (d'Emmanuele di Villa Bianca R et al., 2010).

On the other hand, in the model of gastrointestinal inflammation H_2S seems to exert ani-inflammatory proprieties. It is well known that non-steroidal anti-inflammatory drugs, usually used for the treatment of a wide range of inflammatory disorders, caused ulcer formation and gastric injuries by inhibiting the activity of both cyclooxygenases 1 and 2 (COX-1 and COX-2), which are involved in the prostaglandin's productions. Prostaglandins produced by COX-1 protect gastric

mucosal tissue, regulate the secretion of bicarbonate and gastric bow flood (Whiteman L et al., 2014). Thus, the inhibition of COX activity is essential for the reduction of inflammation, edema, and pain, but the inhibition of COX-1 produced prostaglandins results in an acute inflammatory response that triggers gastric ulceration (Bathia M et al., 2012).

The prolonged use of non-steroidal anti-inflammatory drugs is usually associated with a gastric tissue lesion. In particular, it has been demonstrated that non-steroidal anti-inflammatory drugs decrease CSE expression and activity, and in turn H_2S production. Thus, the inhibition of H_2S production could contribute to the gastric damage associated with the use of non-steroidal anti-inflammatory drugs (Fiorucci S et al., 2005). In contrast, experimental evidence showed that NaHS by reducing leukocyte adhesion to the endothelium and neutrophil infiltration to the mucosa exerted protective effects in the gastrointestinal tissue. Moreover, the expression of intracellular adhesion molecule 1 (ICAM-1) and TNF- α, two inflammatory markers, was reduced following the administration of NaHS, but the synthesis of prostaglandin E2 was not modified (Bathia M et al., 2012).

Similar H_2S protective effects were observed in models of colitis and also in this case the impaired H_2S synthesis may play a key role in the development of this inflammatory disorder. All this experimental evidence, such as the reduction of leukocyte adhesion, the induction of apoptosis in neutrophiles, and chemotaxis, suggested a key role for H_2S in the resolution of gastrointestinal inflammation (Li L et al., 2007).

An involvement of H_2S was also suggested in chronic inflammation disorder, such as rheumatoid arthritis (RA), as RA patients showed elevated H_2S levels in the synovial fluid sample when compared with healthy subjects (Muniraj N et al., 2017). It is not clear if the increased H_2S levels in the synovial fluid sample are an endogenous mechanism that promotes the inflammation process or exerted an anti-inflammatory effect. In a study on synoviocytes isolated from RA patients, NaHS showed a biphasic effect (Kloesch B et al, 2012). Indeed, the treatment of synoviocytes with low NaHS concentration led to the inhibition of pro-inflammatory mediators production caused by LPS, such as interleukin 6 and TNF-α. This effect was also observed in macrophages treated with LPS. In contrast, the

treatment of synoviocytes with a higher concentration of NaHS promoted the LPS-induced production of pro-inflammatory mediators, exerting pro-inflammatory properties (Kloesch B et al, 2012). Another important component of the inflammatory process is represented by the oxidative stress that through the production of ROS at the site of inflammation can promote the inflammatory state (Whiteman M et al., 2011). Particularly, peroxynitrite ($ONOO^-$), hydrogen peroxide, hypochlorous acid (derived from neutrophils), and the up-regulation of NO production by iNOS act as pro-inflammatory molecules. The over-production of this species, during the inflammation state, could remove the external insult but at the same time, it can actively participate in the inflammatory state by damaging the surrounding tissue (Whiteman M et al., 2004; Whiteman M et al., 2005). It has been suggested that H_2S can exert anti-inflammatory proprieties through antioxidant activity (Schreir SM et al., 2010). An exogenous source of H_2S inhibited tissue damage or cellular protein oxidation, caused by hypochlorous acid or $ONOO^-$ in *in vitro* experiments (Whiteman M et al., 2006). In another study, NaHS showed anti-inflammatory proprieties through the scavenge of lipid peroxide and the inhibition of both activity and expression of NAD(P)H oxidase (Muzaffar S et al., 2008). In addition, in neuronal cells, NaHS through its antioxidant effects and the up-regulation of glutathione synthesis inhibited cell death induced by beta-amyloid (Bathia M et al., 2012). In summary, the role of H_2S in inflammation seems to be complex, as experimental evidence suggested this gasotransmitter as an anti- or pro-inflammatory mediator. Endogenous H_2S showed to contribute to the inflammatory state through chemokines, cytokines, adhesion molecules, or leucocyte recruitment. In contrast, treatment with an exogenous source of H_2S as NaHS, suggested an anti-inflammatory role for H_2S, for example through its antioxidant effects. Thus, the experimental results may be different due to the experimental approach, the cell target, and how the cell or the tissue is exposed to H_2S.

1.7.3 Experimental evidence of H_2S and NO cross-talk in inflammation

In literature the investigation of the crosstalk between H_2S and NO in inflammation was less extensive; A study that investigated the crosstalk between these

gasotransmitters showed that in rats treated with LPS, NO reduced the formation of H_2S. In this study, Anuar et al. demonstrated that NO reduced plasma levels of TNF- α, IL-1 β, H_2S synthesis, and CSE mRNA activity and MPO activity (Anuar F et al., 2006; Lo Faro ML et al., 2014). Besides, it has been reported that in RAW264.7 macrophages treated with LPS, NaHS, or H_2S pure as gas, inhibited NO production and iNOS expression through blocking of HO-1. The induction of HO-1 by H_2S occurred via ERK, which in turn led to a reduction of iNOS expression. In addition, this inhibition was augmented by the treatment with L-Cys which is a substrate of CBS for H_2S production (Lo Faro ML et al., 2014).

Similar results were observed by Whitenman et al. in RAW264.7 macrophages treated with LPS by using GYY4137 as an H_2S donor. The H_2S donor inhibited pro-inflammatory mediators, including IL-1beta, TNF- α, and in addition the production of NO, through NF-kB inactivation (Whiteman et al., 2011). In a mice model of inflammatory lung disease, where CSE expression and H_2S levels decreased, the administration of NaHS, an exogenous source of H_2S, inactivated iNOS and reduced neutrophiles infiltration, reducing inflammation (Khattak et al., 2021).

In a mouse model of inflammation of viral myocarditis, Hua et al. showed that NaHS treatment reduced inflammation avoiding cell infiltration in the injured tissue that in turn led to a reduction of cardiac edema. The levels of iNOS mRNA increased in a mouse model of viral myocarditis, and NaHS reduced the overexpression of iNOS mRNA and increase HO-1 (Hua W et al., 2013).

Opposite effects of H_2S were observed after the treatment of mice with PAG as reported by Hua et al. The protective effects of NaHS occur via the reduction of overproduction of NO by iNOS in inflamed tissue and the increase of expression of HO-1. Despite the reduction of iNOS overexpression causing a reduction of inflammation, the administration of H_2S did not improve animal survival (Hua W et al., 2013).

Once again, all this experimental evidence showed how the mutual levels of H_2S and NO play a pivotal role in both physiological and pathophysiological conditions.

1.8 Role of H_2S in metabolic disorder

1.8.1 The metabolic syndrome – an overview

The metabolic syndrome (MetS), also known as syndrome X, was first described in 1988 by Reaven GM, and it is a common metabolic disorder that results from clustering of clinical evidence that may occur altogether, including abdominal obesity, high glucose, insulin resistance, dyslipidemia, and hypertension (McCracken E et al., 2018). MetS, associated with an increase of atherosclerosis as a consequence of chronic inflammation and vascular endothelial dysfunctions (ED) lead to cardiovascular disease (Eckel RH et al., 2005).

A definition for MetS was formulated variously by the World Health Organization (WHO), the National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III), the European Group for the Study of Insulin Resistance, and the Diabetes Federation (IDF). These definitions may differ in the criteria included and the details, but all of them are recognized as essential component obesity (an indicator of insulin resistance or abdominal adiposity), impaired metabolism of glucose, hypertension, and atherogenic dyslipidemia (Samson SL et al., 2014). The global prevalence of MetS can be related to geographic and sociodemographic factors, but also the criteria used for MetS diagnosis. For example, it is estimated, by the International Diabetes Federation, that in Europe 41% of men and 38% of women are affected by MetS (McCracken E et al., 2018). According to the various definition formulated for MetS, many factors are included i.e. family history, increasing age, obesity, high triglycerides, low HDL, high levels of blood pressure, and high fasting glucose level (McCracken E et al., 2018). Among the several mechanisms proposed for the pathophysiology of MetS, the most well-accepted of these is the insulin resistance (as a consequence of obesity) and an increase of fatty acid flux, that is matched with a low grade of inflammation and oxidative stress (Eckel RH et al., 2005; Roberts CK at al., 2013; McCracken E et al., 2018).

1.8.2 H_2S and insulin resistance

In physiological conditions insulin regulates glucose uptake by the liver, skeletal muscle, and adipose tissue, inhibiting hepatic gluconeogenesis and lipogenesis. H_2S

plays a key role in the regulation of insulin sensitivity in several organs and tissue including the liver, skeletal muscle, and adipose tissue. In many pathological conditions, such as MetS, the reduction of responsiveness to insulin by its target tissue causes a reduction of glucose uptake by adipose tissue and skeletal muscle and elevated hepatic glucose production, leading to insulin resistance (Zhang H et al., 2020). Insulin resistance albeit represented by clinical evidence related to MetS plays s a pivotal role in the pathogenesis of MetS.

In the liver, insulin regulates glycogenolysis and gluconeogenesis to maintain normal plasma glucose levels (Czech MP et al., 2017). When insulin resistance occurs, insulin fails to inhibit glycogenolysis and gluconeogenesis in the liver, leading to an alteration of circulating glucose levels in the plasma (Hatting M et al., 2018). In the liver, all three enzymes involved in H_2S production are expressed, and they cooperate to maintain normal H_2S levels in the liver for the regulation of glucose metabolism.

The effect of H_2S on glucose metabolism is controversial. In HepG2 cells, NaHS inhibited glucose uptake and impaired glucose storage (Zhang H et al., 2020). In addition, the overexpression of CSE in HepG2 cells led to a decrease in liver glycogen levels, conversely, CSE silencing increased the level of glycogen in the liver (Zhang H et al., 2020). Furthermore, in $CSE^{-/-}$ hepatocytes were observed a reduction of gluconeogenesis and glucose release whereas in HepG2 cells H_2S promoted gluconeogenesis and glycogenolysis (Zhang H et al., 2020). The inhibition of glucose uptake in HepG2 cells is related to the activation of AMP-activated protein kinase (AMPK) by H_2S. Instead, the interaction of H_2S with another protein target, including the upregulation of phosphoenolpyruvate carboxykinase and the downregulation of glucokinase activity, was involved in the increased glucose production (Zhang L et al., 2013). Thus, H_2S can suppress glucose uptake and glycogen storage, but it can promote gluconeogenesis (Untereiner AA et al., 2016). The reduction of glucose uptake in the liver and the promotion of glucose production can lead in turn to hyperglycemia and the consequent insulin resistance. On the other hand, in literature, it has been reported that H_2S can inhibit gluconeogenesis. The silencing of CSE in HepG2 cells after insulin secretion caused an increase in glucose production, whereas treatment with

NaHS attenuated the glucose production (Zhang L et al., 2013). In addition, CSE knockout mice treated with a high-fat diet showed an exacerbation of insulin resistance and obesity, conversely, a relief of a high-fat diet metabolic disorder was observed in wild-type mice after the treatment with NaHS (Zhang L et al., 2013).
As described above insulin can regulate adipose tissue glucose uptake, and several studies demonstrated a role for H_2S in the regulation of insulin sensitivity in adipocytes. A role for CSE as the major source of H_2S in adipose tissue was assessed by Feng et al, which demonstrated that H_2S or L-Cys caused a basal or insulin-stimulation reduction of glucose uptake in rat adipocytes (Feng X et al., 2009). Conversely, the inhibition of CSE by PAG had opposite action, indicating that H_2S produced in baseline conditions can inhibit glucose uptake (Feng X et al., 2009). In addition, rats fed with a high fructose diet, used as an animal model of insulin resistance and hyperlipidemia, showed an increase in both CSE expression and H_2S production in adipose tissue, that correlated with impairment of glucose uptake induced by insulin (Beltowsky J et al., 2018). TNF-α has detrimental effects on insulin sensitivity and these effects may be mediated by H_2S. In adipose tissue of obese humans or animals, TNF-α is overproduced and it can interact with many components of the insulin pathway. In 3T3-L1 cell TNF –α inhibits the glucose uptake mediated by insulin and increases CSE expression and activity and consequently H_2S synthesis. The inhibition of CSE but not CBS attenuated the detrimental effect on glucose uptake induced by TNF-α (Huang CY et al., 2013). The effects on insulin sensitivity in 3TL-L1 adipocytes treated with a high glucose concentration of glucose were also examined by Manna et al. In 3T3-L1 cells H_2S or L-Cys abolished the detrimental effect of hyperglycemia, including the reduction of glucose uptake mediated by insulin, the reduction of phosphatidylinositol 3,4,5-thriphosphate (PIP3), and the downstream phosphorylated AKT levels and the expression of insulin-sensitive glucose transporter GLUT4 (Manna P et al., 2011; Zhang H et al., 2020). In addition, CSE inhibitor PAG blocked the protective effect of L-Cys and in normoglycemic conditions, both L-Cys and H_2S had no effects on glucose uptake (Manna P et al., 2011). Thereafter, the same group demonstrated that hyperglycemia-induced insulin resistance in the 3T3-L1 cell line may be mediated by the downregulation of the CSE-H_2S pathway because hyperglycemia

decreased CSE expression and consequently H_2S production even in 3T3-L1 cells (Manna P et al., 2011). All these findings suggested that in addition to glucose uptake and insulin signaling H_2S may be involved in many indirect effects on systemic and local insulin sensitivity due to its action in the adipocytes. In adipose tissue, insulin is also involved in the regulation of lipolysis that causes the hydrolysis of triglyceride, stored in the adipocyte lipid droplets, to release glycerol and free fatty acid (FFA). The excessive release of FFA into the bloodstream, due to the enhanced baseline lipolysis rate, contributes to insulin resistance in both liver and skeletal muscle, local oxidative stress, and inflammation (Beltowski J et al., 2018). In addition, insulin lipolysis is also regulated by other several endogenous factors, including hormone-sensitive lipase, which is activated by a cAMP-activated protein kinase, circulating catecholamines that increase cAMP levels, others G-protein coupled receptors, and cytokine. Another mechanism by which insulin can inhibit lipolysis in adipose tissue is through the activation of phosphodiesterase with a reduction in intracellular cAMP levels (Nielsen TS et al., 2014). Insulin deficiency or the reduction of responsiveness to insulin of adipocytes can result in more than lipolysis. So, the enhanced lipolysis can be the cause but also a consequence of insulin resistance. Several studies investigated the role of H_2S in the regulation of lipolysis. It has been reported by Geng et al., that L-Cys or the H_2S donor GYY4137 inhibited lipolysis in adipocytes, whereas the inhibition of CSE by PAG caused an increase of lipolysis (Geng B et al., 2013). However, PAG increased lipolysis and GYY4137 suppressed lipolysis in a vivo study, both PAG and GYY4137 improved insulin sensitivity in mice fed with high-fat diet (HFD) mice (Geng B et al., 2013). The reduction of lipolysis by H_2S was also reported in 3T3-L1 cells, where NaHS or GYY4137 inhibited lipolysis induced by the βadrenoceptor agonist CL-316, conversely, the inhibition of CBS by AOAA or CSE by PAG enhanced lipolysis (Tsay CY et al., 2015).

Skeletal muscles act as the major insulin-responsive organs, regulating systemic glucose metabolism. Xue et al demonstrated the involvement of H_2S in glucose uptake in skeletal muscle. L-6 myotube treated with NaHS showed an increase of glucose uptake, an upregulation of phosphorylated insulin receptor, PI3K, and AKT levels. The knockdown of insulin receptors or the inhibition of PI3K abolished the

upregulation of glucose uptake induced by NaHS (Xue R et al., 2013). In addition, in diabetic rats, chronic treatment with NaHS can improve insulin resistance (Xue R et al., 2013). These findings suggest that H_2S in skeletal muscle promotes glucose uptake by targeting insulin receptor and PI3K pathway and in such a manner ameliorate insulin resistance (Beltwosky J et al., 2018; Zhang H et al., 2021).

Another aspect that should be considered is the mitochondrial dysregulation that plays an important role in the pathogenesis of insulin resistance. H_2S regulates hepatic mitochondrial biogenesis and bioenergetics. The mitochondria are involved in the regulation of energy homeostasis, oxidation of fatty acids, and ROS production. The reduction of mitochondria density and the impaired oxidative phosphorylation, resulting in mitochondria dysregulation, can contribute to a dysregulation of glucose metabolism in the liver, skeletal muscle, and adipose tissue (Beltowsky J et al., 2018; Gonzalez-Franquesa A et al., 2017). Data in the literature suggested that endogenous H_2S can stimulate mitochondrial biogenesis by the activation of peroxisome proliferator-activated receptor-gamma coactivator-relate protein and peroxisome proliferator-activated receptor - α (Untereiner AA et al., 2016), while in HepG2 cell and liver NaHS can stimulate mitochondria bioenergetics by the sulfhydration of ATP synthase alpha subunit at Cys 244 and 294 (Zangh H et al., 202; Mòdis K et al., 2016).

1.8.3 H_2S and obesity

Obesity is a pathological condition characterized by an excessive accumulation and storage of fat in the body that leads to an increase in the adipose mass regionally, globally, or both. Obesity is defined as an increase in the body mass index (IBM, IBM of 30 Kg/m^2 or greater) and according to the World Health Organization (WHO), 20% of European and 30% of Americans are obese. Obesity is considered a risk factor for the development of T2DM, hypertension, dyslipidemia, and MetS, which in turn leads to cardiovascular disease. Adipose tissue is the largest endocrine organ in the human body involved in energy homeostasis. Adipose tissue stores the excess nutrients and ensures a supply of energy to all organs and tissue in conditions of nutrient deficiency (Reem T et al., 2019). In addition to the adipose tissue expanse, due to lipid accumulation, several factors occur in the pathogenesis of

obesity, including histological changes in adipose tissue, adipose tissue dysfunction, and pro-inflammatory or pro-diabetic adipocytokines (Comas F et al., 2020).

During the last decade, numerous emerging studies supported the importance of H_2S in the pathogenesis of obesity.

It has been demonstrated that administration of NaHS, as an exogenous source of H_2S, increased insulin sensitivity and improved glucose tolerance in mice fed with HFD (Cai J et al., 2018). In addition, the reduction of endogenous H_2S production, prevented the increase of fat mass induced by HDF, while the increase of endogenous H_2S production was able to stimulate fat mass accumulation in mice (Comas F et al., 2021).

In humans, the involvement of H_2S in the pathogenesis of obesity was first demonstrated by Whiteman et al., which showed a decrease in H_2S plasma in overweight individuals with altered glucose metabolism and non-obese individuals affected by T2DM (Whiteman M et al., 2010).

The first finding of H_2S production in adipose tissue was by Feng et al (Feng X et al., 2009), which demonstrated that H_2S is produced by CBS and CSE in rat adipocytes (Feng X et al., 2009). Afterward, it has been demonstrated that H_2S is produced in rat perivascular adipose tissue, and it is mainly produced by CSE (Feng X et al., 2009). In addition to CBS and CSE expression in adipose tissue, later studies also have demonstrated the presence of the 3MST (Katsouda A et al., 2018). In adipose tissue, H_2S is involved in the regulation of lipids, glucose, and mitochondrial metabolism.

The inhibition of the -CSE-derived H_2S in mice adipocyte affected lipid metabolism by the stimulation of lipolysis, and L-Cys or H_2S donors GYY4137 had opposite effects, suggesting that the increase or the decrease of H_2S production exerts opposite effects on lipolysis (Geng B et al., 2013). Thereafter, in animals fed with HFD, CSE inhibition by PAG increased the rate of lipolysis concerning chow mice, and only in HFD mice, the treatment with H_2S donor reduced lipolysis (Geng B et al., 2013). In contrast, it has been observed that the infusion of H_2S in adipose tissue in control or obese rat increased the glycerol and cAMP levels and PAG reduced glycerol increase only in the obese rat (Beltowsky J et al., 2017). Ding et al (Ding

Y et al., 2020) demonstrated that the persulfhydration of protein, in particular modified perilipin 1, by H_2S can be involved in the regulation of lipid metabolism and turn decrease the rate of lipolysis and promote adipocyte lipid accumulation (Ding Y et al., 2020). This has also been confirmed by Comas et al (Comas et al., 2021) the protein involved in the adipocyte metabolism required higher persulfidation levels, supporting the importance of H_2S in the pathophysiology of adipose tissue.

During the progression of obesity, the hypertrophy and the expansion of adipose tissue can result in ROS production that can lead to oxidative stress and a pro-inflammatory state. H_2S is also involved in oxidative stress and inflammatory response to obesity respectively through its antioxidant effects and in the control of the release of adipokines. In 3T3-L1 mouse cells in a high glucose environment following the CSE overexpression or NaHS treatment, a decrease in the secretion of pro-inflammatory cytokines was found secretion (Pan Z et al., 2014).

In experiments conducted on samples of human adipose tissue or isolated adipocyte Comas et al (Comas at al., 2021) reported that H_2S increased adipogenesis, the action of insulin, and the peroxisome proliferator-activated receptor γ (PPAR-γ). Conversely, the inhibition or gene silencing of H_2S production enzymes caused an alteration in adipocyte differentiation, cellular senescence causing inflammation (Comas et al., 2021). In addition, a reduction of the gene expression of CBS, CSE, and 3MST in both visceral and subcutaneous adipose tissue was observed in individuals affected by obesity (Comas et al., 2021). As described above obesity predisposes the human body to a pro-inflammatory state through the reduction of adiponectin levels and an increase of the production of pro-inflammatory mediators such as interleukin-6 and TNF-α. During the development of obesity, the adipose tissue macrophages showed their pro-inflammatory phenotype. In RAW264.7 macrophages or macrophages of mice, adipose tissue a reduction of H_2S levels, and an increase in mRNA and protein expression of CSE was observed (Velmurugan GV et al., 2015). A recent study demonstrated how H_2S regulates inflammation during the process of adipogenesis. In the 3T3-L1 cell line during the differentiation, the treatment with the H_2S donor GYY4137 abrogates the negative effects of inflammation (Comas F et al., 2019).

Nevertheless, the understanding of the molecular mechanism underlying the contribution of H_2S to obesity is not yet clear. Further investigation may help to clarify the mechanism and to develop novel therapeutic approaches.

1.9. Role of H_2S in the female urogenital system

1.9.1 H_2S and uterus

The uterus is a hollow organ characterized by a differentiated lining layer called endometrium, a muscular coat known as myometrium, and a serosal outer layer (Aguilar HN et al., 2010).

The uterus can produce a constant spontaneous contraction without hormonal or neural stimulation, so it's a myogenic organ (Efe OE et al., 2021). In non-pregnant conditions spontaneous contractions and relaxations play a pivotal role in the reproductive functions, for example, spontaneous uterine contractions help the sperms' journey to the tubes or contribute to the expulsion of menstrual debris (Bernardi M et al., 2017). In contrast abnormality in the spontaneous contraction can lead to the most common clinical disorders including dysmenorrhea and endometriosis (Harada T., 2013).

Myometrial contraction during estrus, pregnancy, and parturitions is regulated by several events and different signaling molecules are involved. Despite the recent progress in the knowledge of the physiological basis of uterine contraction and relaxation at the cellular and molecular level, the mechanism of myometrium spontaneous contractility is still unclear (Wray S et al., 2001).

Several experimental evidence suggested that G-protein-coupled receptors, NO, CO, and H_2S, can act as a signaling molecule in uterine tissue, playing a pivotal role in the regulation of spontaneous contraction and relaxation in the uterus, in non-pregnant conditions (Mancardi D et al., 2009; Efe OE et al., 2021).

Sidhu et al (Sidhu R et al., 2001) for the first time demonstrated the involvement of the H_2S pathway in the uterus showing the effect of L-Cys and NaHS in rat uterine tissue in vitro. In particular, L-Cys and NaHS significantly increased spontaneous uterine contraction, and other related molecules such as D-Cys or D, L homocysteine had no effects (Sidhu R et al., 2001). Only later studies reported the

expression of CBS and CSE in human and rat uterine tissue, and that they can produce H_2S. In addition, CBS and CSE were detected in rats' intrauterine tissue, human placenta, and myometrium (Patel P et al., 2009). In another study, by using human myometrium biopsies it has been reported that in human laboring tissue CBS and CSE were localized in the smooth muscle cell and both mRNA and the expression of these enzymes were downregulated during the labor (You et al., 2011). Furthermore, You et al. demonstrated the ability of L-Cys in reducing the spontaneous uterine contraction in non-laboring and laboring tissue, while this effect was more pronounced in laboring myometrium strips (You et al., 2011). A high concentration of L-Cys (1mM) showed an opposite effect on myometrium strips; indeed L-Cys 1mM was able to increase the frequency of uterine spontaneous contraction and induced tonic contraction (You XJ et al., 2011).

In addition to L-Cys, GYY4137 a synthetic H_2S donor reduced both oxytocin-induced, and spontaneous contraction and the relaxant effect was mainly mediated by the interaction of H_2S with Ca^{2+} rather than K^+ channels in human pregnant myometrium tissue stimulated with oxytocin (Robinson H et al., 2012). In parallel, this relaxant effect of H_2S increased with the advancement of gestation, whereases it was abrogated during labor (Robinson H et al., 2012). These data suggested the importance of H_2S in the female urogenital tract and mainly in uterus physiology. In addition, H_2S can contribute to the transition state from quiescence to contractile state before labor, mainly through the action on Ca^{2+} channels.

In 2016 Mitidieri E et al. demonstrated that both CBS and CSE were expressed, and able to convert L-Cys into H_2S in mouse uterus (Mitidieri E et al., 2016). L-Cys or NaHS reduced spontaneous uterus contractility and the blockage of CBS and CSE reduced this latter effect even if a major role for CSE than CBS was observed. This data was strongly confirmed by using $CSE^{-/-}$ mice (Mitidieri E et al., 2016). Therefore, L-Cys/CSE/H_2S signaling modulates the mouse uterus motility opening different therapeutical approaches for the management of the uterus abnormal contractility disorders.

2. AIM I

DM is a common complex of metabolic chronic diseases, characterized by elevated levels of blood glucose, that affects millions of individuals worldwide and it continues to increase in numbers and significance. Nevertheless, despite the growing interest in the contribution of H_2S in complications associated with DM, in literature little is reported on the role of H_2S in uterine homeostasis in the non-pregnant condition in DM. Reproductive dysfunction (RD) is a common, but little-studied complication of DM. Diabetes can disrupt the physiological morphology of the myometrium, and it also can affect the normal uterus physiological functionality (Bazot M et al., 2000; Leyendecker G et al., 2004). Despite the intensification of insulin therapy, these problems have declined, but do persist (Sjöberg L et al., 2013). Poor contractility compared to non-diabetic subjects in uteri from diabetic patients has been observed (Al-Qahtani S et al., 2012). Interestingly, it has also been shown that H_2S modulates uterus contraction. Indeed, experiments conducted on $CSE^{-/-}$ mice reported that CSE derived-H_2S has a tocolytic effect (Mitidieri et al., 2016). Therefore, the study aimed to better clarify the role played by the H_2S pathway in changes driven by DM in the alteration of uterine contraction by using an animal model of T1DM i.e., non-obese diabetic mice (NOD).

2.1. MATERIALS AND METHODS I

2.1.1 Animals

Non-obese diabetic (NOD) mice, usually used as an animal model of T1DM, represent a strain with an elevated susceptibility in developing T1DM, showing changes with the evolution of pathology. The progression of diabetes and its clinical outcomes in these animals are like those in humans. More in detail, the early phase (4-10 weeks of age) is characterized by localization of inflammatory cells, including T cell and activated macrophages, around the pancreatic islet, inducing peri-insulitis; subsequently (12-30 weeks of age), T cell and activated macrophages infiltrate islet and initiate a progressive beta cell destruction, causing a drastic reduction in insulin plasma level (Makino S et al., 1980). NOD mice can be classified into three groups: NODI represent a mouse group in which diabetic state is not yet present; NODII mice have glycosuria and elevated glycemia; NODIII

group shows a severe pathology with higher levels of both glycosuria and glycemia (Brancaleone V et al., 2008). In previous experiments CD1 control mice showed a similar profile with NODI (Figure 6), so for this reason and according to data in the literature, the study was carried out by using NODIII mice and age-matched CD1 control (CTR) mice (Charles River, Italy).

Virgin female NOD and age-matched CD1 named CTR mice were housed in the animal care facility at the Department of Pharmacy, University of Naples, Italy, in a controlled environment temperature (21 ± 2°C) and humidity (60 ± 10%) and 12 h light/dark cycles. All animal care and experimental procedures were approved by the Italian Minister of Health according to International and National law and policies (EU Directive 2010/63/EU and DL 26/2014) for animal experiments. In our experimental condition, diabetes was assessed through the measurement of glycemia (monitored weekly) by applying a drop of blood to a chemically treated, disposable 'test-strip', which is then inserted into an electronic blood glucose meter. When glycemia values were higher than 500 mg/dL, mice were euthanized and classified as NODIII. Uteri were rapidly harvested to perform functional and molecular studies.

2.1.2 Organ bath studies

The uteri, harvested from both CTR and NODIII mice, were cleaned from the adherent tissue, dissected, and finally divided into two horns. Each horn was crosscut into two strips and mounted in an isolated organ bath containing oxygenated (95% O_2 and 5% CO_2) Krebs' solution [115.3 mM NaCl, 4.9 mM KCl, 1.46 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose (Carlo Erba, Milan, Italy)] at 37 °C (Mitidieri E et al 2016). Uteri horns were connected to an isometric transducer (7006, Ugo Basile, Comerio, Italy) and stretched until a resting tension of 0.3 g. After 30 minutes of equilibration, when the spontaneous contractility becomes homogeneous, the response to acetylcholine (Ach, 10 μM, Sigma, Milan, Italy), oxytocin (Oxy, 0.005 U/mL, Sigma, Milan, Italy), or prostaglandin F2α (PGF2α, 0.1 μM, Sigma, Milan, Italy) was evaluated.

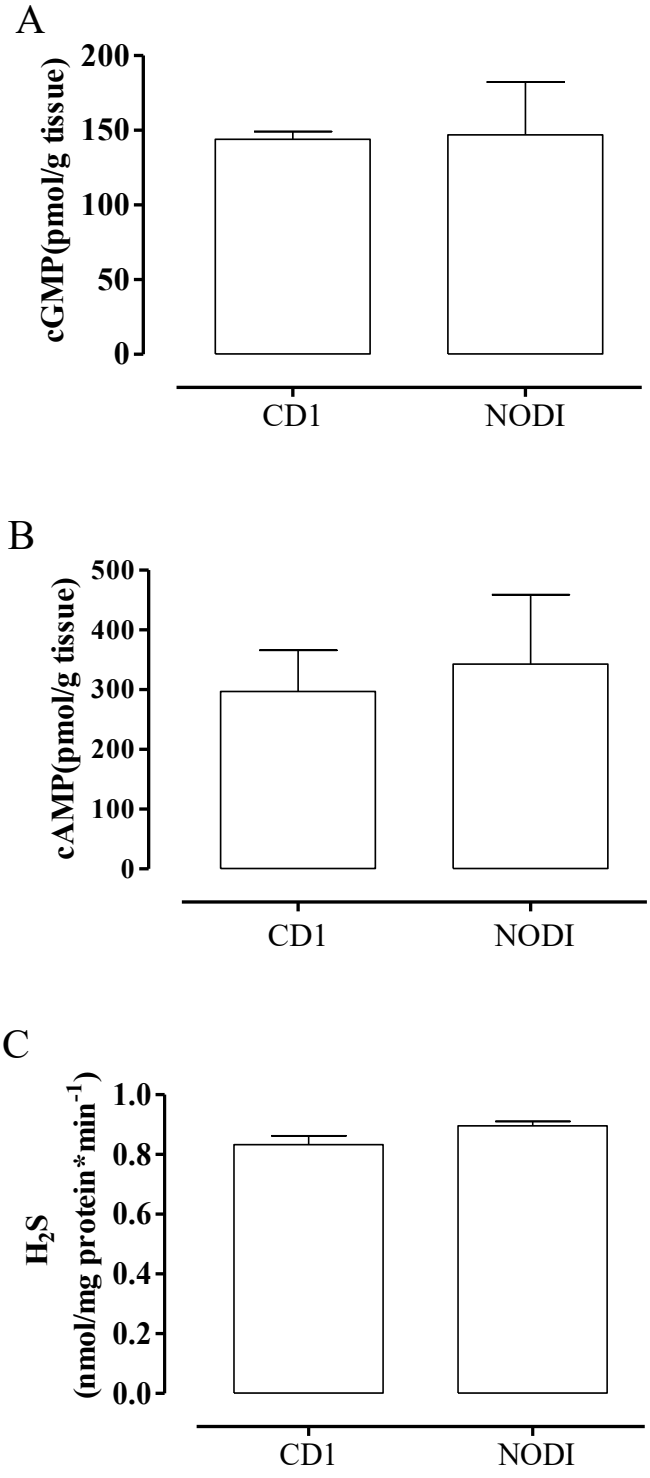

Figure 6. Level of cGMP, cAMP, and H_2S in uteri harvested from CD1 and NODI mice. The basal levels of (A) cGMP, (B) cAMP, and H_2S (C) were not significantly different between CD1 (control mice (CTR)) and NODI mice. NODI mice have normal levels of glucose.

In a different set of experiments, on stable spontaneous contractility, a concentration-response curve of L-Cys (100 nM–300 μM, Sigma, Milan, Italy) or NaHS (100 nM–300 μM, Sigma, Milan, Italy) was achieved. Data were calculated as frequency (peaks/min or % of spontaneous motility) or as force (g or dyne/mg tissue). Results were expressed as the mean ± SEM (n=6 mice) and analyzed by using analysis of variance (ANOVA) followed by Bonferroni post hoc test or unpaired Student's t-test as needed. $P < 0.05$ was considered significant.

2.1.3 H_2S determination

Samples of mice uteri, harvested from CTR and NODIII mice, were lysed in a modified potassium phosphate buffer (100 mM, pH 7.4, sodium orthovanadate 10mM, and protease inhibitors), and consequently, the protein concentration was determined by using Bradford assay (Bio-Rad Laboratories). Homogenates samples were added to a mixture of a reaction containing pyridoxal-5'-phosphate (2 mM, Sigma, Mila, Italy), L-Cys (10 mM, Sigma, Milan, Italy), or vehicle. The H_2S production measured in presence of vehicle corresponds to the basal values and considered the contribution of all three enzymes. The addition of L-Cys to the homogenates caused an increase in H_2S production derived from the activity of both CBS and CSE. In another set of experiments, the inhibitors of H_2S biosynthesis, PAG (10 mM, CSE inhibitor, Sigma, Milan, Italy), aminoxiacetic acid (AOAA, 1 mM, CBS inhibitor, Sigma, Milan, Italy), or a combination of both were added 4 minutes before the addition of L-Cys in uterus homogenates. The reaction was performed in sealed Eppendorf tubes and initiated by transferring tubes ice to a water bath at 37°C for 40 minutes. Next, trichloroacetic acid solution (TCA, 10% wt/vol, Sigma, Milan, Italy) was added to each sample followed by Zinc acetate (1% wt/vol, Sigma. Milan, Italy). Subsequently, N, N-dimethyl-p-phenylenediamine sulfate (20 mM, Sigma, Milan, Italy) in HCl (7.2 M) and $FeCl_3$ (30 mM, Sigma, Milan, Italy) in HCl (1.2 M) were added, and optical absorbance of the solutions was measured after 20 min at a wavelength of 668 nm. All samples were assayed in duplicate, and H_2S concentrations were calculated against a calibration curve of NaHS (3–250 μM). Data are reported as nmol per mg of protein per min. Results were expressed as mean ± SEM (n=6 mice, experiments performed

in the presence of inhibitor) or mean ± SEM (n=10 mice, experiments performed in the absence of inhibitors). Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. $P < 0.05$ was considered significant.

2.1.4 Western blot analysis

Frozen mouse uteri, harvested from CTR and NODIII mice, were lysed in modified RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, o.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1% Igepal) (Roche Applied Science, Italy) and protein inhibitor cocktail (Sigma-Aldrich, USA. Protein concentration was determined by using Bradford assay and albumin (BSA)was used as standard (Sigma-Aldrich, USA). Denatured proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Membranes were blocked by incubation in phosphate buffer solution (PBS) containing 01% v/v Tween 20 and 5% non - fat dried milk for 1 h at room temperature, followed by overnight incubation at 4°C with the following antibodies: mouse monoclonal antibody for CSE (1:1000; Abnova, Milan, Italy), rabbit polyclonal for CBS (1:1000; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti–3MST (1:500; Novus Biologicals, Cambridge). Membranes were washed in PBS containing 0.1% v/v Tween 20 and then with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. Subsequently, membranes were washed and developed using Chemidoc imaging system (Biorad, Mila, Italy) The target protein band intensity was normalized over the intensity of the housekeeping protein β–actin (1:3000, Sigma-Aldrich, Milan, Italy) expression, as referential protein. Data were calculated as optical density (OD)*mm2. Results were expressed as mean ± SEM (n=8 mice) and analyzed by unpaired Student's t-test. $P <0.05$ was considered significant.

2.1.5 cGMP and cAMP measurement

Samples of horn uterus of both CTR and NODIII mice were dropped into 5–10 vol (mL buffer/g tissue) of TCA (5%) and homogenized by using a polytron-type homogenizer. Next, samples were centrifuged at 1500g for 10 min and cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP)

were measured in supernatants as described in the manufacture's protocol of cGMP and cAMP EIA Kit (Cayman, Vinci Biochem, Vinci, Italy). All samples were assayed in duplicate, and the concentrations of cyclic nucleotide were calculated against a calibration curve of standard cGMP or cAMP. Data were calculated as pmol/g tissue. Results were expressed as mean ± SEM (n=6 mice) and analyzed by unpaired Student's t-test. $P < 0.05$ was considered significant.

2.2 RESULTS I

2.2.1 Diabetes reduces spontaneous uterine motility and contractility

Uteri harvested from NODIII mice showed a strong reduction in uterine spontaneous motility compared to CTR mice as reported in typical tracers (Figure 7). The frequency (expressed as peak/min) of spontaneous motility was significantly reduced in NODIII mice compared to CTR mice (Figure 7A; ***$p<0.001$). The contraction force of spontaneous contraction in NODIII and CTR mice was evaluated in the same experiments. The force of contraction (expressed as gram) was significantly reduced in the uterus of NODIII mice compared to CTR mice (Figure 7B; **$p<0.01$). In addition, the contraction force was also evaluated considering the weight of the uterus, since it was observed that the weight of the horn uterus from NODIII mice was significantly reduced in NOD III mice (Figure 8; *$p<0.05$). The contraction force (expressed as dyne/mg tissue) was significantly lower in NODIII mice compared to CTR mice (Figure 7C; ***$p<0.001$).

2.2.2 Isolated uteri of NODIII mice display an impaired response to different contracting agents

The contractile response to different contracting agents, including Ach, Oxy, and PGF2α was evaluated in uteri harvested from CTR and NODIII mice.

The typical tracers of uterus response to Ach, Oxy, and PGF2α are shown in figure 9A, D, and G.

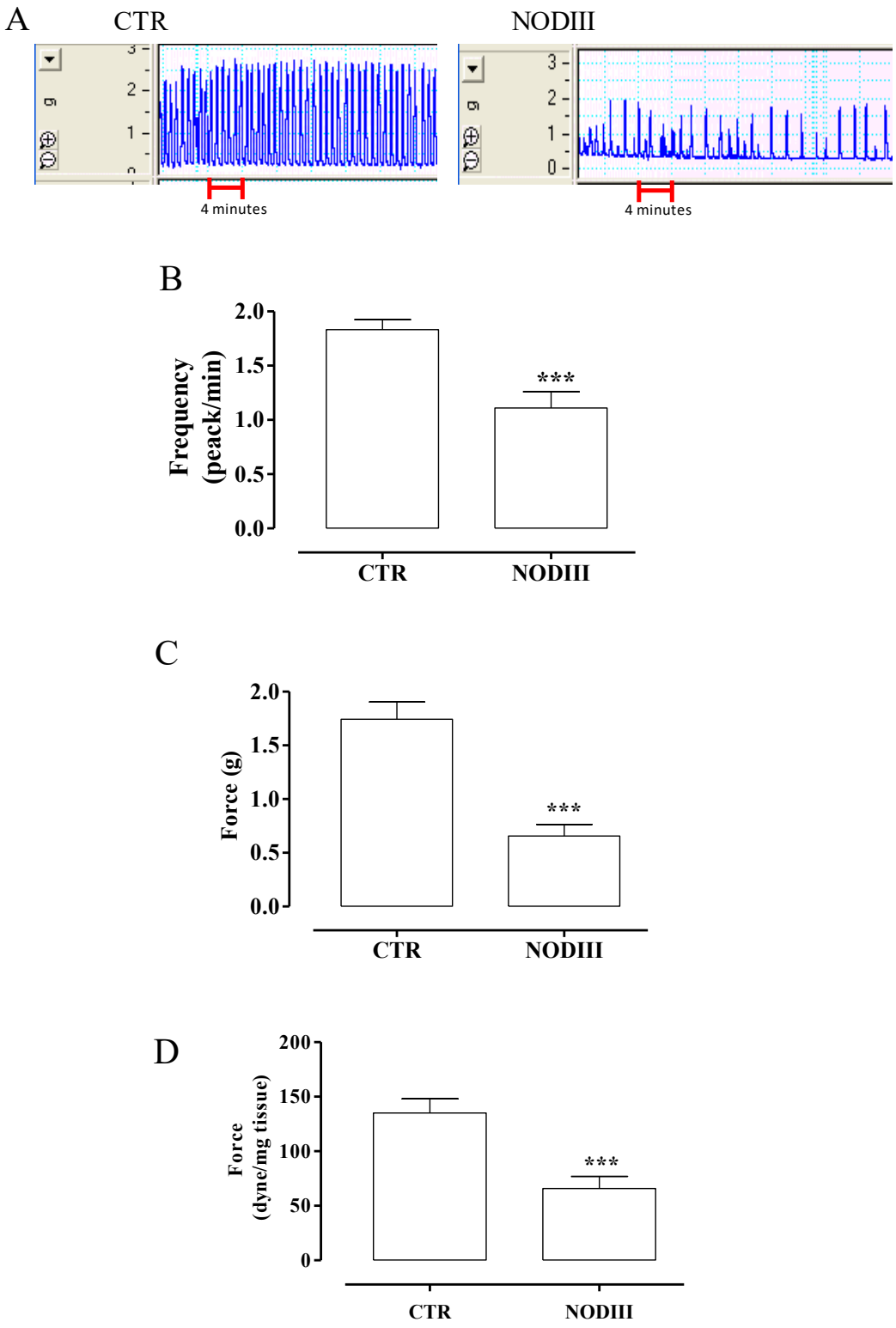

Figure 7. The basal response of uteri harvested from NODIII mice is significantly reduced compared to CTR mice. Data are reported as frequency, i.e., peak/min (B; ***p <0.001); contraction force, expressed in grams (C; ***p <0.01); or as dyne/mg tissue (D; ***p <0.01).

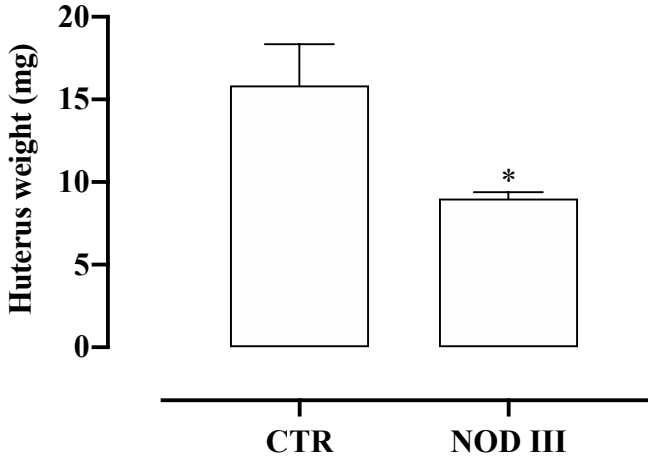

Figure 8. Horn uterus weight harvested from CTR and NODIII mice. The horn uterus weight is significantly reduced in NOD III mice by about 30% (*p<0.05). Data are reported in milligram.

The contractile response to Ach, expressed either as g or as dyne/mg tissue, was significantly reduced in NODIII mice (Figure 9B, C respectively; **p<0.01).
Likewise, the contraction induced by Oxy was significantly reduced in NODIII mice compared to CTR (Figure 9E, F; *p<0.05; **p<0.01). PGF2α-induced contraction in the uterus was significantly lower in NODIII compared to CTR mice (Figure 9H, I; *p<0.05)

2.2.3 The content of cGMP and cAMP is higher in uteri harvested from NODIII mice

To better define the possible mechanism involved in the reduced motility of the diabetic uterus harvested from NODIII mice, the content of cyclic nucleotides, i.e. cGMP (Figure 10A) and cAMP (Figure 10B) was measured.
The content of both, cGMP and cAMP were significantly increased in the uterus of NODIII mice compared with CTR mice (*p<0.05). The levels of both cyclic nucleotides were increased 2-fold over the control value.

2.2.4 Diabetes can interfere with the H_2S pathway

To assess the role of H_2S, a concentration-response curve to L-Cys (10nM-300μM), an endogenous source of H_2S, was performed in horn uteri harvested from NODIII and CTR mice. The tocolytic effect induced by L-Cys was significantly reduced in NODIII compared to CTR mice (Figure 11A; ***p<0.001). No significant difference was found between NOD III and CTR mice in the tocolytic effect of NaHS (10nM-300μM), an exogenous source of H_2S (Figure 11B). Thereafter the H_2S levels in tissue homogenates of NODIII and CTR mice were detected. The basal amount of H_2S was significantly increased in diabetic uteri compared to CTR (Figure 11C; ***p<0.001). Thus, the enzymatic ability of CBS and CSE was evaluated by adding L-Cys to homogenized tissue samples in the presence or absence of the inhibitors. The amount of H_2S produced in presence of L-Cys was significantly higher when compared to the respective basal (Figure 11C; ***p<0.001 and °°°p<0.001, respectively).

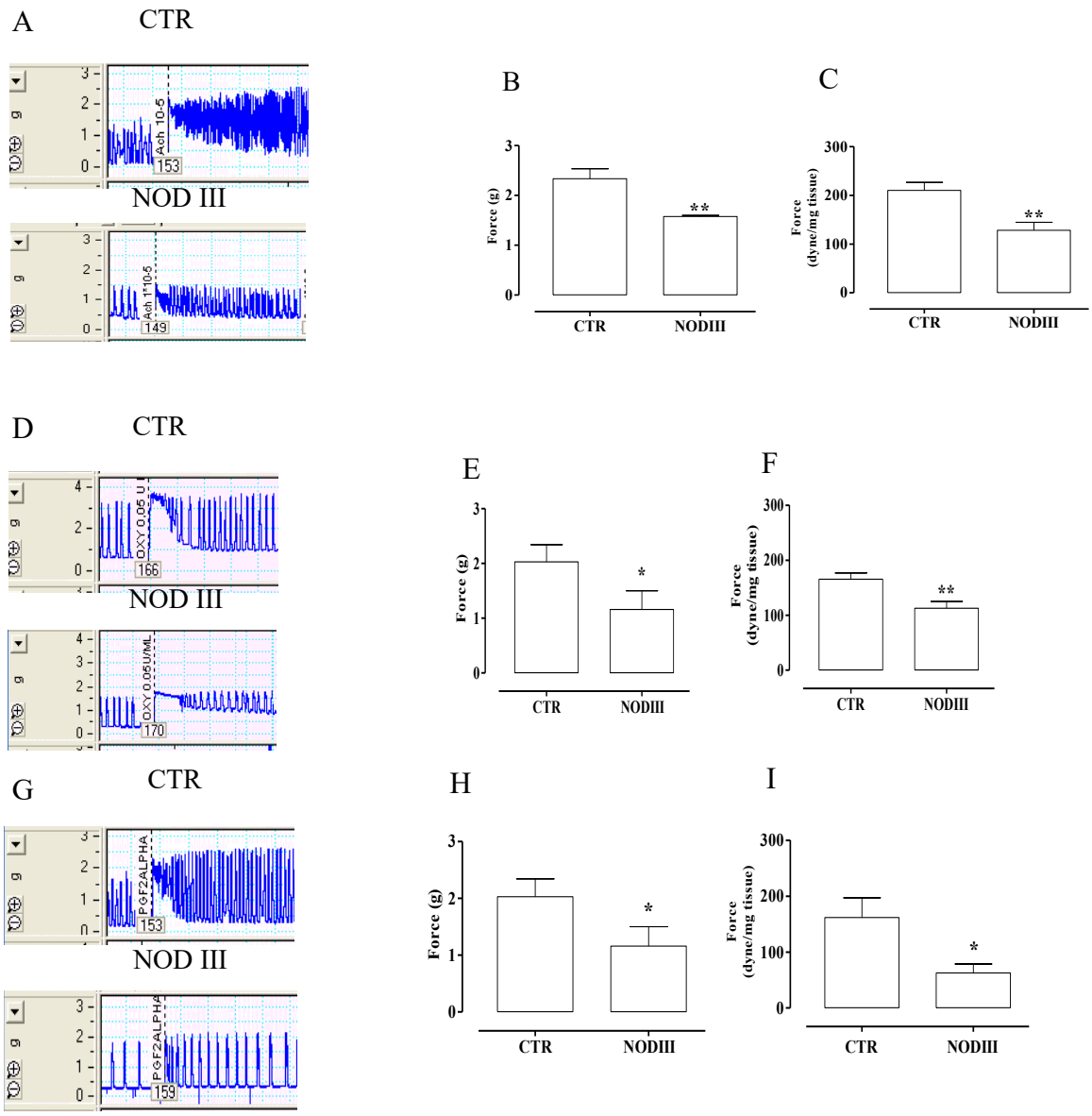

Figure 9. Uterine response to contracting agents in NODIII and CTR mice. Typical tracer of the contractile response to Ach (A), Oxy (D), and PGF2α (G). The contractile response to Ach (10μM), reported as gram (B) or dyne /mg tissue (C) is significantly reduced in NODIII mice compared to CTR mice (**p<0.01), The contractile response to Oxy (0.05 U/ml), reported as gram (E) or dyne /mg tissue (F), is significantly reduced in NODIII mice compared to CTR mice (*p<0.05; **p<0.01, respectively). (G). The contractile response to PGF2α (0.1 μM) reported as gram (H) or dyne /mg tissue (I) is significantly reduced in NODIII mice compared to CTR mice (*p<0.05).

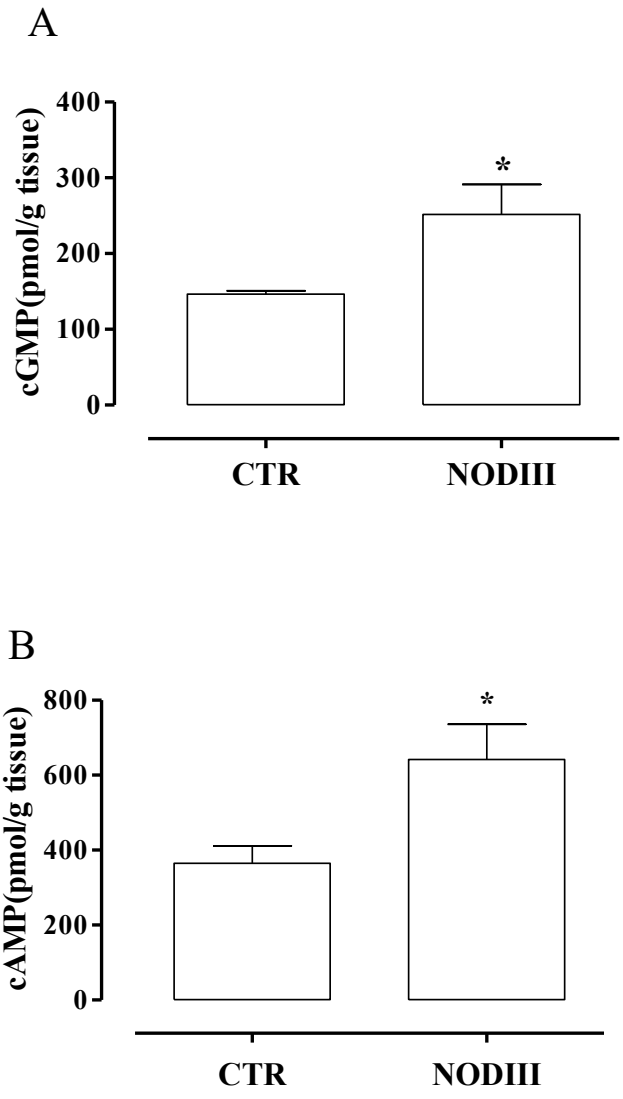

Figure 10. Changes in cAMP and cGMP content in uteri harvested from CTR and NODIII mice. The uterus content of cGMP (A) or cAMP (B) is significantly increased in NODIII mice compared to CTR mice (*$p<0.05$).

Finally, to determine whether the changes in H_2S content were related to an alteration in the expression of H_2S producing enzymes, the expression of CBS, CSE and 3MST was evaluated in homogenates of uteri harvested from CTR and NODIII mice. Western blot analysis showed that the expression of 3MST was significantly increased in uteri harvested from NODIII mice (Figure 12B; $^{**}p<0.01$). Conversely, CBS and CSE expression were significantly lower in NODIII mice compared to CTR mice (Figure 12C, D; $^{*}p<0.05$).

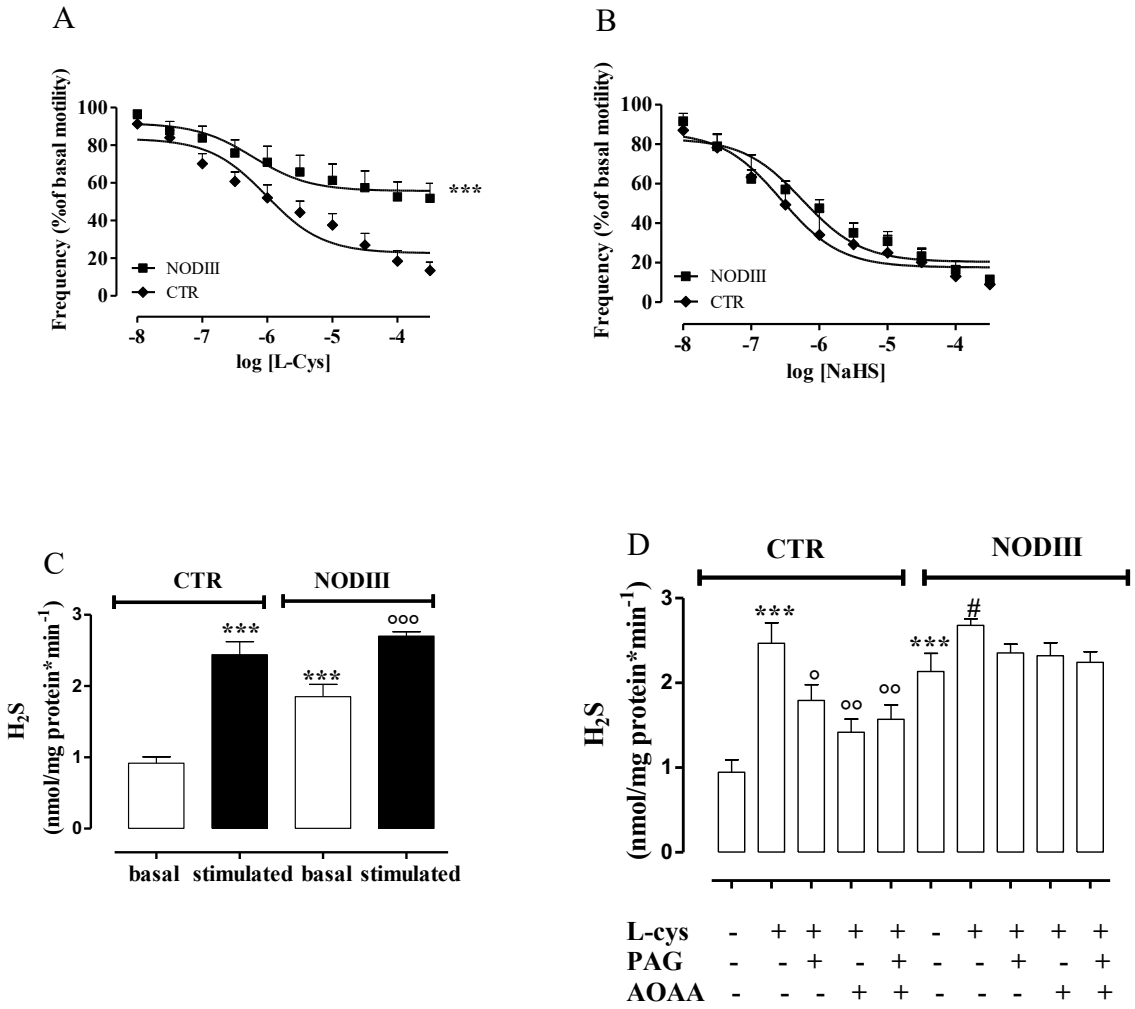

Figure 11. Changes in H_2S pathway in uteri of CTR and NODIII mice. The tocolytic effect of L-Cys (10nM-300μM) is significantly reduced in NODIII mice compared to CTR mice (A) (***p<0.001). The tocolytic effect of NaHS (10nM-300μM) does not differ between NODIII and CTR mice (B). The basal amount of H_2S is significantly increased in NOD III mice compared to CTR mice (***p<0.001). H_2S production induced by L-Cys is significantly increased in CTR mice (***p<0.001) or in NODIII mice (°°°p<0.001) compared to the respective basal values (C). L-Cys significantly increases H_2S production in both CTR and NODIII mice uterus (*** $p < 0.001$ and $^{\#}$ $p < 0.05$, respectively). PAG (10 mM, CSE inhibitor), AOAA (1 mM, CBS inhibitor), or their combination were added before L-Cys (10 mM) challenge in uterus homogenates. The incubation with inhibitors significantly reduced the increase in H_2S production induced by L-Cys in CTR mice (°p<0.05 and °° p<0.01) but not in NODIII mice (D).

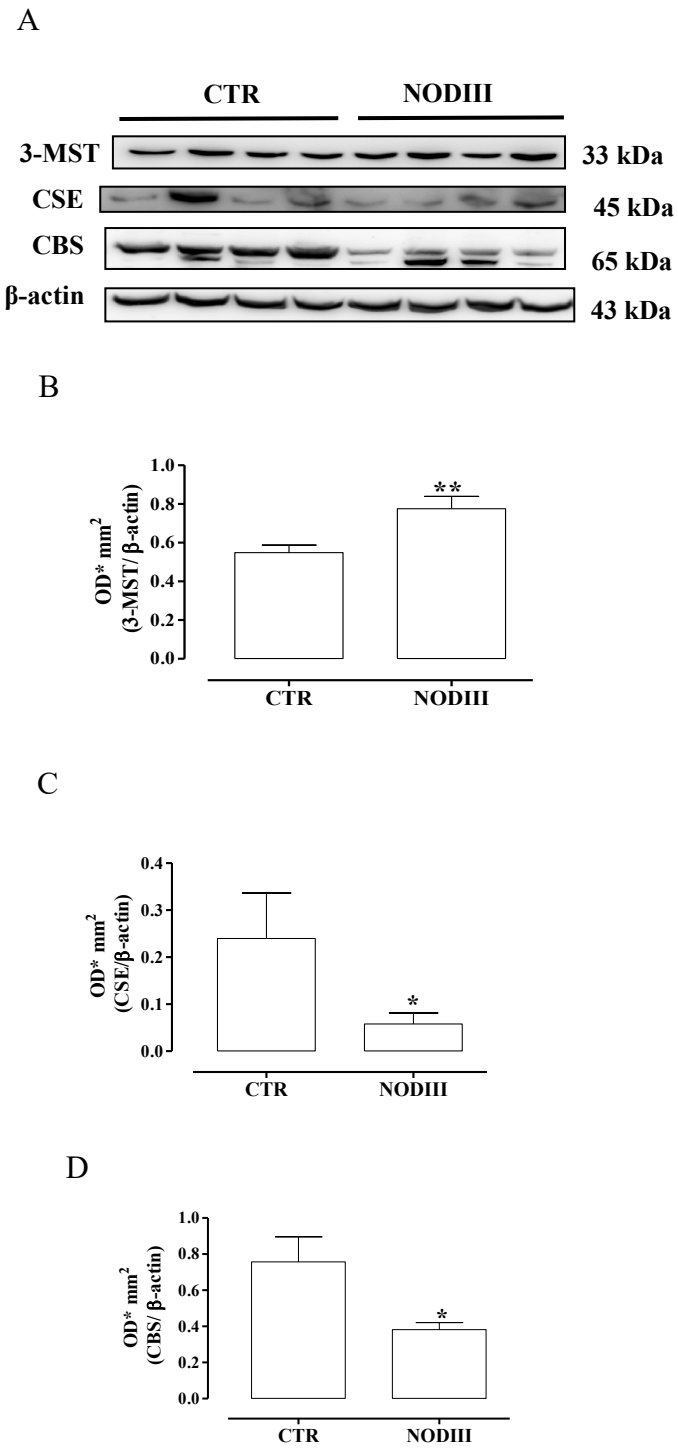

Figure 12. Expression of 3-MST, CSE, and CBS in uteri of CTR and NODIII mice. Representative western blot for 3-MST, CBS, and CSE (A). Expression of 3-MST is significantly higher in NODIII mice uteri vs CTR mice (** $p < 0.01$) (B). CSE expression is significantly reduced in NODIII mice vs CTR mice (* $p < 0.05$) (C). CBS expression is significantly reduced in NODIII mice vs. CTR mice (* $p < 0.05$) (D).

2.3 DISCUSSION I

DM, including T1DM and T2DM, is one of the most common chronic disorders worldwide affecting about 10% of the adult population in the world (American Diabetes Association., 2018). Higher levels of blood glucose or long-term hyperglycemia result in microvascular and macrovascular complications, with detrimental effects in many organs and systems, including the reproductive system (Orasanu G et al., 2009; Guan Q et al., 2018). Reproductive disorders (RD) can manifest early on in puberty or later and the spectrum of RD that occurs in DM includes delayed puberty and menarche, menstrual cycle abnormalities, subfertility, adverse pregnancy outcomes, and potentially early menopause (Thong EP et al., 2020). Today the improvement of diabetes therapy, such as insulin therapy, or the new therapeutic strategy, ameliorates some of these problems. However, alteration of uterine functionality in diabetic women is still under investigation, particularly in non-pregnant conditions. Thus, we have investigated uterus functionality in diabetes in non-pregnant conditions, aiming at the contribution of H_2S signaling. To address this issue, we used a well-established model of T1DM i.e., NOD mice which show the pathophysiological characteristics of long-term DM in humans (Favarro RR et al., 2010). In terms of high levels of glycemia, hypoinsulinemia and uterine morphology female NODIII mice replicate the long-term diabetes abnormalities in women with T1DM (Pichette J et al., 2016). In line with the literature, here we have demonstrated that isolated uterus harvested from NODIII mice revealed a reduction in spontaneous motility expressed as the force of contraction or peak of frequency. To better clarify the mechanism(s) involved in changes in uterus motility in diabetic conditions, we stimulated the uterus with endogenous uterotonic agents such as Ach, Oxy, and PGF2α. An impairment in the contractile response to these agents was observed in isolated uteri harvested from NODIII mice and this finding is in line with clinical evidence reported in the literature, where women affected by DM require a higher dose of Oxy to induce labor (Reinl EL et al., 2017). In this scenario, the role of the CSE-derived H_2S pathway in the maintenance of uterine tone in health status has been demonstrated. (Mitidieri E et a., 2016). The H_2S levels were higher in the uterus harvested from NODIII mice compared to control mice, and such increase was well correlated to

the increase in both cGMP and cAMP levels in the uterus of NODIII mice. This finding could be explained by the fact that H_2S is an endogenous inhibitor of PDEs activity (Bucci M et al., 2010), elevating the levels of cyclic nucleotides. Therefore, in uteri of NODIII mice the increase of H_2S levels, by elevating cGMP and cAMP, can contribute to the switch to a relaxing tone. Regarding the enzyme involved in H_2S biosynthesis, the expression of CSE and CBS resulted reduced in uteri harvested from NODIII mice, whereas 3MST expression was significantly increased. Thus, these data can suggest a major role for 3MST-derived H_2S in the diabetic uterus. To better clarify these data, we used a pharmacological modulation in isolated uterus harvested from CTR and NODIII mice in vitro experiment. The increase of H_2S production followed the incubation with L-Cys, the endogenous substrate of CBS and CSE, was higher in CTR mice compared to NODIII mice, and the treatment with CSE or CBS inhibitor cause a reduction in H_2S production. Conversely, in NODIII mice the treatment with CSE or CBS inhibitor did not affect the H_2S levels. These additional results can support the major role of 3MST, which is also suggested by western blot analysis. In parallel functional studies in isolated uterus demonstrated that the L-Cys relaxing effect is impaired in NODIII mice, confirming a minor role for H_2S derived from CSE and CBS. This data suggests that in the uterus of NODIII mice a re-arrangement of the H_2S pathway occurs with an increase of 3MST-derived H_2S that consecutively increase cGMP and cAMP levels. On the other hand, the effect of NaHS on isolated uteri had a similar profile in both NODIII and CTR mice suggesting that the dysregulation of H_2S signaling caused a tolerance to exogenous H_2S response.

In conclusion in the uterus of NODIII mice the increased level of H_2S 3MST-derived is involved in the shift toward a relaxing tone and, in turn leads to a reduction in spontaneous endogenous contraction and hypo-contractility in response to Oxy or PGF2α, that are commonly used in clinical practice to induce labor. A better understanding of the role played by H_2S could be useful for new therapeutic targets and the development of new therapeutic strategies.

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