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"Evaluation of vascular reactivity in physiological and pathological conditions"

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ABSTRACT

Gasotransmitters are small gaseous and biologically active molecules contributing to the control of vasodilation. Vasodilation is a physiological event necessary for human cardiovascular wellbeing. The major gasotransmitters involved in vascular relaxation are nitric oxide (NO) and hydrogen sulfide (H_2S). A wide range of evidence showed that NO and H_2S independently affect the vasculature, while other studies tried to address their cooperation in the control of vascular tone. However, the molecular mechanisms regulating NO and H_2S mutual interaction still need to be fully elucidated. The cystathionine beta-synthase (CBS) is an H_2S-producing enzyme, and its mutation leads to a severe high plasmatic level of homocysteine. This medical condition is strongly associated with endothelium dysfunction. L-serine is synthetized from L-cysteine by CBS activity during the H_2S production. On the other hand, L-serine is involved in the synthesis of sphingosine-1-phosphate (S1P), a well-known NO trigger. The first part of this thesis focused on the study of the vascular effect of L-serine compared to L-cysteine and to evaluate if the L-cysteine/H_2S signalling is associated to the L-serine/S1P/NO pathway in the vasculature through CBS activity. We demonstrated that L-cysteine and L-serine were able to induce vascular relaxation in an endothelium-dependent manner through the activation of the NO/S1P pathway. Then we demonstrated that L-cysteine was able to produce NO trough the involvement of CBS and to increase the level of S1P in aortic tissue. Finally, we demonstrated that CBS is expressed in the same endothelial cellular cavity of the endothelium NO-producing enzyme (eNOS). In summary, our results indicate that the CBS enzyme

plays a role in the control of vascular homeostasis not only as of the main endogenous source of H_2S but also by interfering with the S1P/NO signalling through the L-serine. These data suggest a mechanism of action that may in part explain the severe cardiovascular pathology observed in patients affected by CBS deletion.

The second part of this thesis focused on the study of the alteration of systemic vascular reactivity as a secondary outcome in airways allergic inflammatory diseases. It is well known that changing in the endothelium physiology leads to the initiation of CVD. However, the published clinical studies on the association among respiratory allergic diseases and CVD are controversial, and the literature is pauper of pre-clinical evidence on the effect of allergy on the systemic vasculature. We showed that a condition of systemic and pulmonary inflammation induced by an allergic stimulus impairs the systemic vasculature, alters the protein expression of the major enzymes involved in vascular functionality (i.e. eNOS, NADPH oxidase 1 and 4) and increases the expression of vascular pro-inflammatory genes. This evidence tried to unravel the correlation between allergic respiratory diseases and cardiovascular diseases.

1. PART I: INTRODUCTION

The term cardiovascular diseases (CVD) is commonly used to indicate a group of disorders of the heart and blood vessels. CVD are the first cause of death globally, where heart attacks and strokes account for the 85% of all CVD deaths and this frequently include people under 70 years of age [1]. CVD are a sub-class of pathologies belonging to the big family of non-communicable diseases, which comprise the all not directly transmissible diseases from one person to another [2]. However, the total economic loss due to CVD represents approximately half of the non-communicable diseases economic burden [3]. Thus, the research is extremely driven to find and understand the mechanisms involved in the development of CVD which are often unknown.

1.1 Endothelium dysregulation as "red flag" in CVD development

The response of the blood vessel to specific stimuli (vascular reactivity) can cause a reduction in the internal diameter (vasoconstriction) or relaxation of smooth muscle cells followed by an increase of the circumferential wall tension (vasodilation) [4].

The cardiovascular system runs across the entire body and alteration in vascular reactivity affects wall tension, resistance, tone, and blood flow, results in the development of cardiovascular [5], cognitive [6] and respiratory diseases [6]. Thus, the study of vascular reactivity represents an invaluable way to better understand vascular physiology and CVD.

The inner blood vessel layer is constituted by a cell monolayer indicate as the endothelium. The endothelium interfaces the blood and the vessel wall, and it covers the whole cardiovascular system from the heart to the smallest capillary.

The endothelium controls some important physiological and pathological mechanism such as permeability, metabolic activity, lipid transport, vascular tone and structure, immune response, tumour growth/metastasis, inflammation, angiogenesis and haemostasis [7]. Unbalancing in the control of all these roles "crowning" the endothelium as the progenitor of CVD such as atherosclerosis [8], hypertension [9], cardiomyopathy [10], myocardial infarction [11]. The hypothesis that the endothelium is not only a passive cell layer but actively influences the vasorelaxant response by the releasing of mediators (endothelium-derived relaxing factors-EDRF) begun in the 1980s when Furchgott and colleagues demonstrate that the nitric oxide (NO) was able to induce vasorelaxation, accounting this mediator as one of the EDRF [12]. The discovery of NO has also allowed evaluating the endothelial dysfunction. The term endothelium dysfunction is referred to the incapacity of the blood vessel to relax in response to stimulation by endothelium-dependent agonists such as acetylcholine, which induces a NO-mediated vasorelaxation. In animal models, vascular reactivity can be measured in isolated vessel segments using myography of vessel rings or vessel segments. In man, the endothelial function can be measured by using flow-mediated dilation (FMD) which is based on the response of brachial artery diameter to reflow after a period of vascular occlusion by a blood pressure cuff [13].

2. GASOTRASMITTERS AND VASCULAR REACTIVITY

The term "transmitter" indicates a variety of molecules of different sizes such as proteins, lipids, peptides, biogenic amines and amino acids, which can exert a wide range of biological properties. The Nobel Prizes Furchgott, Murad and Ignarro showed for the first time a small gaseous molecule, the NO, played a pivotal role in regulating biological functions in humans, introducing the new revolutionary concept of gasotransmitter[14]. Along with NO also the carbon monoxide (CO), mainly known as the enzymatic degradation product of the heme group in the blood cells, was recognized as a physiological gas-mediator in the human body [15]. Moreover, around the turn of the 20th century, the innovative investigation on the biological effects and therapeutic potential of hydrogen sulphide H_2S had begun[16]. This gaseous molecule is well-known for its peculiar smell of rotten eggs and toxicity. Nowadays, H_2S has been entirely identified as the third gasotransmitter [17] and it has many important regulatory roles in several physiological systems[16].

2.1 NO signalling in vascular tone

From L-arginine, NO is synthesized by three nitric oxide synthase (NOS) enzyme isoforms which are encoded by distinct genes: brain or neuronal NOS (nNOS; encoded by NOS1), inducible NOS (iNOS; encoded by NOS2), and endothelial NOS (eNOS; encoded by NOS3). eNOS, which is the dominant isoform in the endothelium and nNOS are constitutively expressed and highly regulated by transcriptional, post-transcriptional, and post-translational mechanisms, including phosphorylation, acetylation, protein-protein interaction, S-nitrosylation, and S-glutathionylation. On

the contrary, iNOS, the inducible isoform, is activated by pro-inflammatory and oxidative stress stimuli. Structurally, NOSs are oxidoreductase homodimer enzymes composed by (*i*) an amino-terminal oxygenase domain which presents sites for the substrate L-arginine, the cofactor tetrahydrobiopterin (BH_4), and a ferric haem cluster binding; (*ii*) a reductase domain with binding sites for the electron donors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). The amino and reductase domains are connected by a sequence that binds calcium (Ca^{2+})-complexed calmodulin. This complex is essential for the transfer of electrons that occurs from the flavins to the haem in the oxygenase domain of the other monomer trough NADPH, allowing oxygen (O_2) binding on the reduced haem iron (Fe^{2+}) and the conversion of L-arginine to HO-L-arginine, then NO and L-citrulline [18].

Conditions such as low bioavailability of L-arginine, which was found in hypertensive pregnant women [19], dramatical increased concentration of intracellular Ca^{2+} or BH_4 deficiency, which is the consequence of a rare inborn error of metabolism [20], result in NOS uncoupling, leading to the production of oxygen radical species (ROS). ROS can *per se* alter the vascular function or cause changes in vascular tone by altered NO bioavailability or signalling in a feedback loop [21].

Interestingly, the posttranslational modification, S-glutathionylation is demonstrated to be responsible for eNOS uncoupling [18], where eNOS-derived ROS has been shown to contribute to the development and progression of atherosclerosis [22] and hypertension [23]. Moreover, the activity/inactivity state of eNOS in the endothelium is regulated by

the interaction with caveolins [24], which are scaffolding proteins present in plasma membrane invaginations (caveolae), with caveolin-1 and caveolin-3 (Cav-1/3) isoforms mainly expressed in endothelial cells or cardiac myocytes, respectively [25].

The endothelium stimulation by acetylcholine, bradykinin, sphingosine-1-phosphate or vascular endothelial growth factor trigger a cascade of events which start with the increasing of Ca^{2+} cellular levels, followed by the destruction of the Cav-1-eNOS inhibitory interaction and the activation of the serine/threonine-protein kinase (AKT and PKC, respectively) which in turn, phosphorylates eNOS on Ser1177, which is the primary eNOS phosphorylation site, and phosphorylate eNOS on Thr495 which decreases enzyme activity [18]. Previously, studies demonstrated the inhibitory effect of Cav-1-eNOS biding by using Cav-1 chimeric peptide [26] and Cav-1 deficient mice (Cav-$1^{-/-}$) [27]. Moreover, eNOS activation has been associated with a protective effect against the development of atherosclerosis [28], and Cav-$1^{-/-}$ backcrossed with atherosclerosis-prone mice [apolipoprotein E–null ($ApoE^{-/-}$) mice] have a 70% to 80% of reduction in atherosclerotic lesion size compared to control $ApoE^{-/-}$ mice [29]. Consistent with this result are studies on endothelial cells cultured in the presence of hypercholesterolemia serum in which was demonstrated an increase in Cav-1 expression and a decrease in NO production [30].

Endothelial dysfunction plays an important role in the pathogenesis of CVD [5]. Interestingly, increase in Cav-1 expression, but no alteration in eNOS expression was also observed in the aorta of diabetic mice with impairment in endothelium dysfunction [31], suggesting Cav-1 expression perturbation contribute to endothelium dysfunction.

Other studies tried to investigate eNOS activation in endothelium dysfunction, and some authors reported an improvement of endothelial function in atherosclerotic animals after eNOS gene transfer [32]. Whereas, other evidence showed a reduction in L-citrulline formation, which is the eNOS metabolic product along with NO, but not in eNOS expression in endothelium dysfunction in Apoe$^{-/-}$ mice [33]. In support of this, alteration in vascular functionality but not in eNOS expression was found in an *ex vivo* model of myocardial infarction [34]. Additional studies provide to demonstrate how unbalancing between phospho-eNOS and eNOS is involved in vascular damage induced by hyperhomocysteinemia [35], which is a risk factor for CVD.

In light of these controversial findings, the role of eNOS in endothelium dysfunction needs further studies, and it is possible to speculate about the involvement of other factors that may compensate for eNOS activity in endothelium dysfunction. Moreover, a better understanding of eNOS/NO anomalies in various cardiovascular diseases may arise new perspectives for therapeutic interventions.

Vasorelaxation occurs when NO diffuses from endothelium to vascular smooth muscle cells and here NO binds its main downstream target: the heterodimeric enzyme, soluble guanylate cyclase (sGC). The NO-sGC binding leads to the formation of a second messenger cyclase guanosine 3′-5′-phosphate (cGMP) and the cGMP cellular levels are regulated by the phosphodiesterase enzymes (PDEs), which counteract vasorelaxation by the hydrolysis of phosphodiester bonds. Notably, sGC is sensitive to oxidation, which can interrupt NO signalling [18]. ROS production, NADPH and glutathione cells depletion cause an sGC oxidation which decreases the enzyme

activity and lower affinity to the NO [36]. Interestingly, different cardiovascular conditions, associated with impaired NO–sGC–cGMP-signalling, such as heart failure, result ameliorated in the presence of the sGC activator cinaciguat (BAY 58-2667) which increases sGC enzyme activity (NCT01064037) [37]. Moreover, the efficacy of cinaciguat to improve endothelium functionality by increasing sGC activity was also demonstrated *in vitro* model of vascular nitro-oxidative stress endothelium dysfunction [38], suggesting a potential role of cinaciguat in endothelium dysfunction treatment.

To summarize, NO is historically considered the major mediator involved in the control of vascular tone. However, there are still several key issues to be clarified on the eNOS/NOS role in endothelium dysfunction. As the research progresses, and with the discovery of other relevant vasodilating mediators, it is growing the idea that these mediators may affect NO signalling. Among these, H_2S represents the most eligible candidate that could affect the NO pathway or *vice versa*.

2.2 H_2S: endogenous biosynthesis

In mammalian tissue, H_2S is naturally synthesized by three enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST). These enzymes are constitutively present in a wide array of biological cells and tissues and their expression can be induced by several disease states [39]. H_2S is released from L-cysteine, a sulfur-containing amino acid, the L-cysteine oxidized dimer, by CSE and CBS in a pyridoxal-5′-phosphate (PLP) dependent reaction [40]. 3MST produces H_2S from 3-mercaptopyruvate (3-MP), which is generated

from L-cysteine and α-ketoglutarate (α-KG) by cysteine aminotransferase (CAT) in the presence of the cofactors thioredoxin and dihydrolipoic acid [41].

CBS is a homotetramer consisting of ~63 kDa monomers able to catalyse H_2S production. The reverse transsulfuration pathway consists in sub sequential reactions in which CBS; (*i*) catalyses the condensation of serine and homocysteine to give cystathionine [42]; (*ii*) links homocysteine with cysteine [43]; (*iii*) converts cysteine to serine and lanthionine. Yet, only the latter two reactions yield H_2S biosynthesis [39] (Figure 1).

In humans, the CBS RNA expression results enhanced in liver and pancreas compared to other organs and apparatus. However, CBS has been detected in many organs including the brain, kidney, liver, ileum, uterus, placenta, heart and blood (https://www.proteinatlas.org/ENSG00000160200-CBS/tissue). Regarding CBS localization in cells, it is mainly localized in vesicles and nucleoli (https://www.proteinatlas.org/ENSG00000160200-CBS/cell). PLP represents the most known and studied cofactor involved in CBS catalytic activity [44]. Each CBS subunit binds at the central catalytic core a single heme and PLP that are separated by 20 Å [45–47]. The presence of iron in the heme group affects the capacity of CBS to binding also NO and CO. Actually, in the ferrous state (Fe^{2+}) the heme group binds these latter two gases resulting in the downregulation of the activity of CBS [48,49]. Furthermore, the CBS complex regulation is not only mediated by PLP as a cofactor but also by other several activators. Indeed, the NH_2 terminal end of CBS contains the binding site of a heme cofactor group that further regulates the activity of CBS by functioning as a redox-

sensitive gas sensor [50]. However, up to date, this function is still poorly understood. At C-terminal end position, CBS is allosterically modulated and activated by adenosyl-methionine (AdoMet), an important product of the methionine cycle [51].

Like CBS, also the human CSE exists entirely as a tetramer in the PLP-bound states [52] and uses homocysteine as a substrate to produce H_2S, α-ketobutyrate, ammonia and homolanthionine [39] (Figure 1). CSE is highly expressed in the liver [40,53–55], however, CSE results to be the most relevant H_2S-producing enzyme in the cardiovascular system; in particular, it is abundant in the heart and vascular smooth muscle. Within the cells, CSE has been classically considered a cytoplasmatic enzyme [39] nevertheless, it has been demonstrated to translocate to mitochondria to support ATP production under stress conditions [56]. Yang et al, showed the calcium/calmodulin complex is also involved in CSE activation to produce H_2S. *In vitro*, they demonstrated that calmodulin binds CSE in the presence of high calcium concentration (2 mM) and this interaction is diminished in the presence of calcium chelator and by calmodulin antagonist [40]. Furthermore, the physiological concentration of calcium (Ca^{2+}; 100 nM) effectively activates CSE [39], while at this concentration Mikami and colleagues demonstrated calmodulin does not affect CSE activity, suggesting that H_2S production by CSE at the steady state low Ca^{2+} concentrations mainly depends on PLP and not on calmodulin [57]. Recently, it has been demonstrated that the inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by H_2S ameliorates heart failure [58]. This would further imply that for both CBS and CSE the cofactors which regulate these enzymes may play a crucial role in physiological and pathological conditions.

Lately, 3MST has been added along with CBS and CSE among the enzymes producing H_2S. However, unlike CBS and CSE, 3MST does not require PLP as a cofactor. 3-MST catalyses the conversion of 3-MP to pyruvate by degrading cysteine. In this pathway, cysteine is first converted by cysteine aminotransferase (CAT), also known as aspartate aminotransferase (AAT), to 3-MP via the incorporation of α-ketoglutarate into the reaction. 3-MST then forms a persulfide by transferring sulfur from 3-MP onto itself which in the presence of a reductant like thioredoxin produces pyruvate and H_2S. Dihydrolipoic acid has also been identified to associate with 3-MST to release H_2S [59–61] (Figure 1). The Genotype-Tissue Expression (GTEx) analysis reveals a ubiquitous tissue expression of 3MST in humans (https://www.genecards.org/cgi-bin/carddisp.pl?gene=MPST#expression). 3MST cellular localization is both cytoplasmatic and mitochondrial at the physiological condition. Thus, the 3MST/CAT pathway can produce H_2S both in the mitochondria and in the cytosol [62].

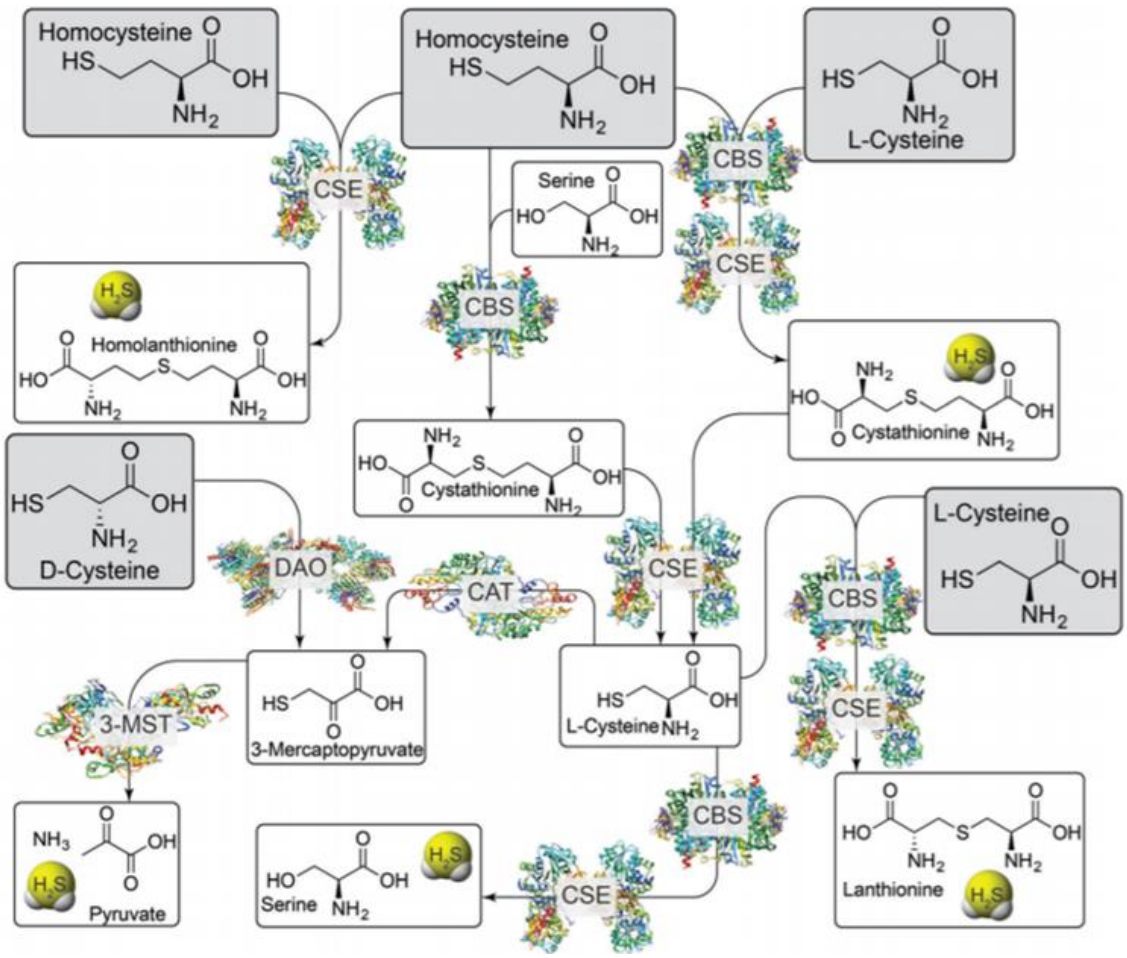

Figure 1 Hydrogen sulfide (H_2S) enzymatic pathway

Reproduced from Hartle and Pluth [63], with permission from the Royal Society of Chemistry.

2.3 H_2S catabolism

H_2S is metabolized through three pathways: 1) oxidation, 2) methylation, and 3) reactions with metalloproteins or disulfide-containing proteins [64].

The oxidation reactions occur in the mitochondria. H_2S is rapidly metabolized to thiosulfate (S_2O^{3-}) which is further converted to sulphite (SO_3^{2-}) by thiosulfate cyanide sulfurtransferase (TST). Furthermore, SO_3^{2-} in turn, is sulfate (SO_4^{2-}) rapidly oxidized to SO_4^{2-} by sulfite oxidase (SO). S_2O^{3-}, SO_4^{2-} or free H_2S are excreted mainly in the urine [64,65](Figure 2).

Conversely to oxidation, the methylation reaction takes place in the cytosol. An unbalance in this detoxification mechanism has been demonstrated to be strongly involved in the development of gastrointestinal-related pathologies [66–69]. The thiol S-methyltransferase (TMST) is the enzyme which catalyses the following reaction:

RSH + S-adenosyl- L-methionine (SAM) →$RS\text{-}CH^{3+}$ S-adenosyl- L-homocysteine (SAH) (Figure 2). In which R may be one of a large number of diverse sulphur-containing groups or H_2S [69]. Interestingly, adenosine in SAH will be hydrolysed yielding to homocysteine. Homocysteine, as mentioned above, will enter in the H_2S biosynthesis. Up to date, alteration of homocysteine circulating levels in the human body is related to the development of certain pathologies including CVD [70]. This is discussed further below.

Finally, haemoglobin (Hb) actively participates in H_2S metabolism. Hb plays a central role not only in canonical O_2/CO_2 exchange but also in non-canonical NO [71] and CO

metabolism [72]. While H_2S can be scavenged by methemoglobin forming sulfhemoglobin [73]. Thus, Hb acts as common "sink" for H_2S, NO, and CO. This could affect their availability resulting in an altered biological response.

This demonstrates how not only the enzymatic formation of H_2S but also its effective removal and/or metabolism is likely to be of fundamental importance in physiological condition.

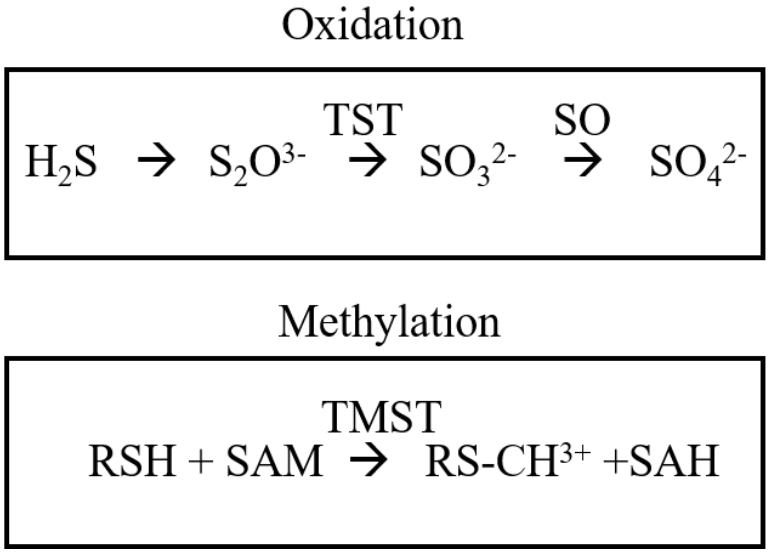

Figure 2 H_2S catabolism

Sulfite oxidase (SO), thiol S-methyltransferase (TSMT), thiosulfate: cyanide-sulfurtransferase (TST), S-adenosyl- L-methionine (SAM), S-adenosyl- L-homocysteine (SAH).

3. H_2S AND THE CARDIOVASCULAR SYSTEM

3.1 Plasmatic levels of H_2S

Up to date, experimental models have represented a valid and reliable source for understanding H_2S activity in the cardiovascular system. For long-time researchers have tried to measure the exact amount of H_2S in the human bloodstream. However, due to the lack of specific techniques, all the past findings only gave an approximation of H_2S blood concentration. In 2008 Whitfield and colleagues measured H_2S concentrations in plasma and whole blood from a variety of mammals in real-time using a polarographic H_2S electrode and they found that H_2S is undetectable at the resolution of the sensor (~15 nM), and when exogenous H_2S is added to blood, it is rapidly consumed [74].

Moreover, H_2S is soluble in water, and in plasma exists as 20% H_2S, 80% HS^- ion and a very negligible amount as S^{2-} at a pH of 7.4. H_2S, HS^-, S^{2-} are commonly termed as free sulfide but they can also exist in a bounded form as sulphates, sulfide, sulfonates and elementary sulphur [75]. Free sulfide or bounded sulfide can be detected by classically colourimetric methods, such as methylene blue test, absorbance-based techniques, and chromatography [76–81]. Recently, more sensitive fluorescent-based methods are contributing to correctly quantify the H_2S in the plasma. Karunya and colleagues measured the basal level of endogenous H_2S in the plasma of healthy volunteers by using the microfluid method and this level was found to lie in the range of 70 μM -125 μM [82].

To date given the limitations discussed above [75,83–85], the examination of circulating levels of H_2S in patients with diseases has been limited.

Few accepted studies on a cohort of 63 subjects affected by type 2 diabetes, a well-known CVD risk factor [86], demonstrated that diabetes is associated with lower circulating levels of H_2S [87]. Moreover, reduction of H_2S plasmatic level was also seen in overweight patients [88], suggesting adiposity could affect plasma H_2S levels.

Evidence about H_2S plasma levels and CVD in human were given by a clinical study in which the plasma-free H_2S levels were assessed in 193 subjects in which, after angiography, the presence of CVD was confirmed. Using a reversed-phase high-performance liquid chromatography (RP-HPLC) analytical method (NCT01407172.), Peter and co-workers showed that free-plasma H_2S levels in patients with peripheral artery disease (PAD) alone or coronary artery disease (CAD) alone were significantly greater than patients without vascular diseases. However, differences in plasma-free H_2S levels between patients with CAD alone versus PAD alone were not significant. Thus, plasma-free H_2S alone was not able to discriminate between different forms of vascular disease [89].

If on one hand, the literature is pauper of evidence of the determinant effect of CVD on human plasmatic H_2S level, on the other hand, it is well known how disturbances in the H_2S pathway are involved in the development of CVD.

3.2 Physiological effects of H_2S in cardiovascular system and its mechanism of action

The understanding of the role of H_2S in the cardiovascular system has grown enormously over the past twenty years. In 1997, Hosoki and colleagues demonstrated that CSE, but not CBS, is expressed in the rat thoracic aorta and portal vein. Moreover, they showed H_2S is produced in rat thoracic aorta and this production is significantly inhibited in a dose-dependent manner by D, L-propargylglycine (PAG), an irreversible inhibitor of CSE and by b-cyano-L-alanine, a competitive inhibitor of CSE. Interestingly, in Hosoki's research group studies sodium hydrosulfide (NaHS), a rapid releasing H_2S donor was able to relax pre-constricted rat thoracic aorta rings in a concentration-dependent manner [90]. However, the mechanism(s) of action behind this relaxant effect was unknown. A few years later, in 2001, Zhao and colleagues investigated the possible biological routes involved in the physiological effect of H_2S on the cardiovascular system. Firstly, they observed a transient decrease in mean arterial blood pressure of anaesthetized rats, this effect was mimicked by a bolus intravenous injection of a potassium ATP (KATP) channels opener (pinacidil). Hence, this hypotensive effect was antagonized by prior blockade of KATP channels with glibenclamide. To confirm the possible involvement of K^+ channels activities in the H_2S vasorelaxant effect, Zhao and colleagues evaluated H_2S induced vasorelaxation in presence of different concentrations of potassium chloride (KCl), which was used to constrict rat thoracic aorta rings before performing a concentration-response curve of H_2S. They demonstrated potassium conductance could influence vasorelaxation

mediated by H_2S. Furthermore, the presence of glibenclamide, but not 4-aminopyridine (4-AP), a voltage-dependent K^+ channels inhibitor, and not charybdotoxin or iberiotoxin (two selective KCa channel inhibitors) completely inhibited H_2S-induced vasorelaxation on rat thoracic aorta rings pre-constructed with phenylephrine. Despite these results, the exact mechanism of how KATP channels are activated by H_2S remains to be elucidated. Besides, Zhao and colleagues showed that the relaxant effect of H_2S was significantly reduced in endothelium-free aortic tissue, demonstrating the relevance of H_2S in the control of vascular tone in physiological condition [55].

Further studies on H_2S role in the vascular functionality were made possible by using mice lacking for the CSE enzyme. Endogenous H_2S levels in the aorta and heart of homozygous mutant male mice ($CSE^{-/-}$) were both decreased by about 80%. Moreover, CSE mutant mice developed age-dependent hypertension and the methacholine (a cholinergic agonist)-induced relaxation of mesenteric arteries was impaired in $CSE^{-/-}$ mice, demonstrating that H_2S displays properties characteristic of an EDRF formed in vascular endothelial cells [40]. Additionally, Mustafa et al. proved in mice mesenteric artery rings, that 70%-80% of acetylcholine-induced endothelium-dependent vasorelaxation was insensitive to NO and prostacyclin biosynthetic enzymes inhibition, which are the main EDRFs in large conduit arteries; this relaxant effect was reduced by approximately 60% in $CSE^{-/-}$ mice, suggesting that H_2S may play a role in the regulation of total peripheral resistance vessels acting as endothelial-derived hyperpolarizing factors (EDHFs) [91]. Where to EDHFs belong the non-NO and non-prostaglandin endothelium-dependent vasodilator mediators [92]. To support the

hypothesis that H_2S acts as EDHF, electrophysiological studies were performed on intact or endothelium-denuded mouse mesenteric arteries. NaHS and methacholine induced hyperpolarization and following vasodilation of smooth muscle cells, whereas PAG induced depolarization and subsequent contraction in intact rings; the effect of methacholine was not observed in endothelium-denuded rings or endothelium-intact rings isolated from $CSE^{-/-}$ mice. Similarly, the hyperpolarizing effect of L-cysteine, the H_2S bio precursor, and the depolarizing effect of CSE inhibitor were absent in $CSE^{-/-}$ mice vessel, confirming the H_2S pivotal role in vasodilation [93].

It is established that CSE represents the major enzyme involved in H_2S production in the cardiovascular system. Early studies on mRNA CBS isoforms confirmed CBS is abundant in the liver and brain and isoforms 3 and 4 were also detectable in the mouse heart [94]. Interestingly, a recent study carried out by Saha and colleagues demonstrated that CSB is expressed in human umbilical vein endothelial cells (HUVEC) and this expression was about 9-fold higher than CSE. This drove further studies on CBS role in endothelial cells. Authors evaluated H_2S production in HUVEC after incubation with AOAA and PAG, commonly used as pharmacological CBS [95] and CSE [96] inhibitors. Inhibiting H_2S production by blocking CBS with AOAA, caused a decrease in H_2S levels more pronounced than PAG. Moreover, silencing CBS by using shorts interfering RNA (siRNA), which induce a post-transcriptional gene silencing, affected proliferation, migration, and vessel formation [97]. As aforementioned, along with CBS and CSE also 3MST enzyme is expressed in the cardiovascular system. Specifically, both 3-MST and CAT are localized in the vascular endothelium [98] and smooth muscle

cells [99]. Coletta and colleagues examined that the 3-MST/H_2S system plays physiological regulatory roles in endothelial cells. They demonstrated that the exogenous 3-MP which is the 3-MST substrate, provoked a concentration-dependent decrease in microcirculatory pressure blood flow (perfusion pressure) in isolated and perfused rat mesenteric vascular bed and the vasorelaxant effect of 3-MP on the mesenteric tone was mimicked by NaHS. Moreover, *in vivo* administration of 3-MP increased circulating H_2S level [100]. More recently, Mitidieri and co-workers thoroughly investigated the contribution of the 3-MST/ H_2S pathway in the mouse aorta. Initially, by using cell-free assays and an H_2S -selective fluorescent probe, they demonstrated the ability of 3-MP to act as an H_2S donor in a concentration-dependent manner. Next, functional studies, using mouse aortic rings, showed exogenous 3-MP relaxed aortic rings *in vitro* in an endothelium-dependent manner, this relaxant effect was significantly reduced by the presence of glibenclamide, while NO inhibitors did not affect 3-MP-induced relaxant effect, suggesting 3-MP may act independently from NO. Finally, to exclude the enzymatic involvement in 3-MP-induced relaxant effect these experiments were repeated by using thoracic aortic rings harvested from 3-MST$^{-/-}$ mice. Interestingly, 3-MP caused concentration-dependent relaxation in vascular tissue lacking 3-MST. To support the hypothesis that exogenously 3-MP acts as H_2S donor in a non-enzymatic way, H_2S levels were measured in 3-MST$^{-/-}$ and wild type mice (WT) aortic tissue homogenates. Not surprisingly, the 3-MP addition caused a significant increase of H_2S generation in wild type aorta mice homogenates. Interestingly, in homogenates from 3-MST$^{-/-}$ mice, a significant increase in H_2S

production was still observed compared to the basal condition that reached approximately 80% of that seen in WT mice [101].

In conclusion, as research in H_2S area progresses and more data are becoming available, it seems clear that H_2S, through its enzymes CBS, CSE and 3-MST, plays a pivotal role in the physiology of in the cardiovascular system. Moreover, studies are also providing evidence that H_2S signalling is involved in the pathogenesis of several diseases.

3.3 CBS mutation and alteration in vascular functionality

H_2S release by CBS is possible thanks to the involvement of a thiol-containing amino acid: homocysteine. Homocysteine enters in the methionine cycle as a metabolic product but also enters in the transsulfuration pathway as CBS/CSE substrate in the H_2S biosynthesis. Moreover, the homocysteine re-methylation is driven by the involvement of 5-methlyterathydrofolate (MTHFR). Thus, homocysteine links three irreversible routes; folate, methionine and transsulfuration pathways [70] (Figure 3).

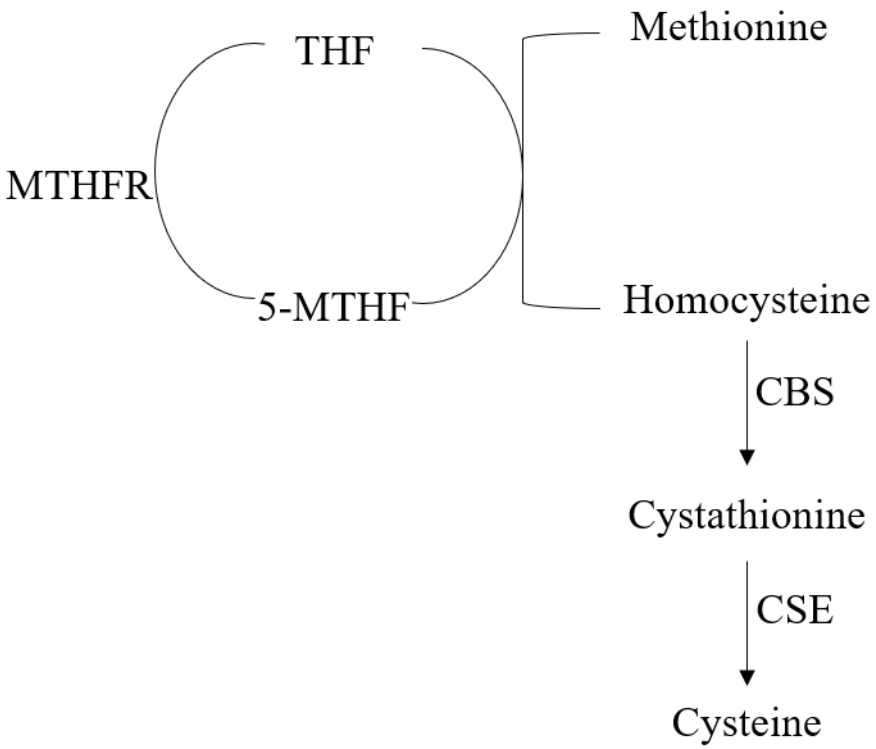

Figure 3 Homocysteine as branch-point of folate, methionine and transsulfuration pathways

Tetrahydrofolate (THF), methylenetetrahydrofolate reductase (MTHFR), 5-methylenetetrahydrofolate (5-MTHF), cystathionine beta synthase (CBS), cystathionine gamma-lyase (CSE).

Deficiency in folate intake and genetic mutation in the MTHFR and CBS genes can lead to reduced function or inactivation of these enzymes resulting in mild or severe elevated plasmatic levels of homocysteine [102], this condition is indicated as hyperhomocysteinemia. Typically, physiological homocysteine levels in the bloodstream are <15 μmol/L. A level among 15 and 30 μmol/L is considered mildly elevated, between 30 and 60 μmol/L is considered moderately elevated, and >60 μmol/L is considered severely elevated. Individuals with rare homocystinuria typically have levels of >100 μmol/L [103].

In humans, a common genetic variant in the MTHFR gene is a 677C>T polymorphism (NM_005957.4:c.665C>T, rs1801133) where the homozygous variant leads to a mild hyperhomocysteinemia, while the 1298A>C polymorphism (NM_005957.4:c.1286A>C, rs1801131) does not cause increased homocysteine levels in heterozygous or homozygous individuals [104]. On the other hand, more than 150 mutations have been identified in the CBS gene. As a result, homocysteine not only severely increases in the blood but it is also excreted in urine causing hyperhomocisteinuria [105]. This rare condition occurs in approximately 1 of 100,000 births [106].

The correlation between high plasmatic homocysteine levels and several systemic diseases has been extensively reported [70,107]. However, it is still open the debate if homocysteine could trigger *per se* the release of mediators involved in diseases development or if higher homocysteine levels are a passive consequence of MTHFR and CBS genes mutation.

In early observations, McCully and colleagues examined different postmortem tissues harvested from subjects passed away for cardiovascular events presenting homocystinuria due to CBS mutation. Histology studies showed severe changes in big and small arterial vessels in several organs concluding that an elevated accumulation of homocysteine or its derivatives, due to alteration of its catabolism, could lead to arterial damage-causing hyperhomocysteinuria symptoms [108].

A growing body of clinical studies reported the connection between hyperhomocysteinemia and CVD suggesting routine screening and treatment for elevated homocysteine to prevent CVD [108–112]. On the contrary, Faeh and colleagues argued regarding the emerging worry about hyperhomocysteinemia as CVD risk factors. They demonstrated no efficacy of lowering homocysteine concentration correlated with reduced incidence of CVD [106]. Thus, further studies are needed to unravel the putative role of hyperhomocysteine in CVD development.

In a scenario of MTHFR mutation, the transsulfuration pathway represents the alternative way to catabolize the excess of homocysteine. Herein, the transsulfuration multiple reactions ended in the release of H_2S. Thus, it is legitimate to ask about the role of H_2S in hyperhomocysteinemia.

As a potential CVD risk factor, hyperhomocysteinemia is also associated with thrombotic events [113–115]. However, if it seems clear the correlation between hyperhomocysteinemia and platelet activation, the detailed biological mechanism(s) are still poorly understood. In a previous study, it has been demonstrated that NaHS

concentrations (up to 10 mM) inhibit platelet aggregation induced by different stimuli and without toxic effects on platelet viability harvested from healthy volunteers [116]. Furthermore, d'Emmanuele di Villa Bianca and colleagues demonstrated (*i*) that the increased effect in platelet aggregation induced by L-cysteine and NaHS in healthy platelets results boosted in patients with MTHFR mutation (MTHFR++ carrier) and the presence of CBS/CSE inhibitors reverted this effect to healthy platelet percentage of aggregation; (*ii*) MTHFR++ carrier platelet H_2S production induced by L-cysteine is significantly higher compared to platelet form healthy donors; (*iii*) H_2S plasmatic levels are significantly higher in MTHFR++ patients compared to healthy volunteers; (*iv*) the acid arachidonic signalling is involved in the enhanced H_2S pro-clotting effect in MTHFR++ platelet [117]. However, the literature is lacking in studies on the H_2S role in platelet aggregation in patients with CBS mutation.

The suggestion that CBS is strongly implicated in the human health was provided by the genome-wide association study (GWAS) of 2,710 participants from the Framingham Heart Study (FHS) and of 2,100 participants from the Vitamin Intervention for Stroke Prevention (VISP), in which the CBS [MIM 613381] gene *locus* reached the predetermined genome-wide significance threshold after methionine loading test, which is a method to assess hyperhomocysteinemia, indicating the direct correlation between CBS and hyperhomocysteinemia [118]. However, apart from this study, the literature is poor in the genomic, genetic and epigenetic investigation on CBS mutation in CVD.

Moreover, hyperhomocysteinemia is associated with endothelial dysfunction [119,120], where platelet activation is the result of impairment in endothelium functionality [121].

Evidence on the possible mechanisms behind vascular damage induced by elevated homocysteine levels, suggesting hyperhomocysteinemia enhanced oxidative stress [122], inflammation of vascular endothelial cells, and reduced the production and bioavailability of NO by the endothelium [123].

Researches tried to investigate the mechanism underlying the endothelial dysfunction in an experimental model of hyperhomocysteinemia. Homozygous mice lacking for the CBS genes ($^{-/-}$) are infertile, have a high neonatal mortality rate and the surviving mice present severe homocysteine plasmatic levels. To overcome the issue of neonatal lethality, CBS heterozygous mice ($^{+/-}$) are currently available. These mice have a normal mean lifespan and ∼50% lower in CBS activity versus wild-type littermates, resulting in a slight increase in plasmatic homocysteine levels which provides a good starting point for assessing the effect of mild hyperhomocysteinemia [124]. The previous study demonstrated that vasorelaxation to acetylcholine was significantly impaired in $CBS^{-/-}$ mice compared with $CBS^{-/+}$. However, the activity of eNOS was significantly reduced in endothelial cells from both $CBS^{-/-}$ and $CBS^{-/+}$ mice, suggesting that perturbation in NO signalling could lead to endothelial dysfunction in hyperhomocysteinemia [125]. Nevertheless, this study did not explain why $CBS^{+/-}$ mice present a normal response to acetylcholine also if the eNOS activity is compromised. It is possible to speculate that, in CBS $^{+/-}$ mice, H_2S produced by the not deleted CBS

enzyme may exert its relaxant effects. Moreover, a recent study demonstrated that exogenous H_2S supplementation and triple gene therapy (CBS/CSE/3MST) increase eNOS availability in $CBS^{+/-}$ mice, suggesting a protective effect of H_2S in hyperhomocysteinemia [126].

It is interesting to note that genetic mutations in CBS, leads to an alteration in vascular functionality *via* perturbation of the NO signalling [125,126]. This possible interaction between NO and H_2S in hyperhomocysteinemia requires further investigations. However, this strongly convinced the researchers to also address the interplay between NO and H_2S in physiological conditions.

4. INTERPLAY BETWEEN H_2S AND NO

The interaction between H_2S and NO has always attracted strong interest in researchers, mainly because these two gases present many similarities in their pathways. They are both synthesized from amino acid (L-arginine and homocysteine, cysteine, cystathionine, respectively) which are enzymatic substrates [nitric oxide synthase (NOS) family, CBS/CSE/3MST respectively], they modulate the redox signalling and generate biological active intermediates as low-molecular-weight thiol (RSH) to form nitrosothiols from NO and persulfides from H_2S. These molecules trigger a cascade of second messengers thanks to the binding to transition metal ions present in proteins, (e.g. iron in soluble guanylate cyclase or copper in cytochrome c oxidase) [127] (Figure 4). However, despite a growing interest this potential "cross-talk" remains to be fully clarified [16,127–130].

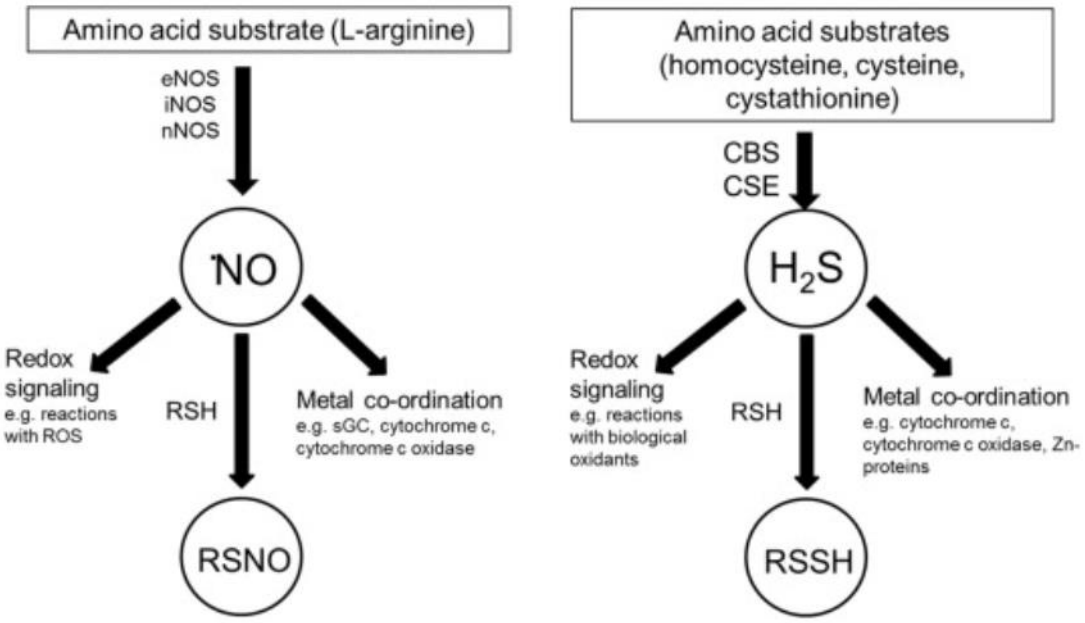

Figure 4 Similarities between H_2S and NO

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4.1 H_2S and NO in the cardiovascular system

In elegant work, Coletta et al. demonstrated that H_2S and NO do not act as independent pathways, but they mutually cooperate in the physiological control of vascular tone and in promoting angiogenesis. They reported that the intracellular second messenger cGMP resulted increased by both NO and H_2S in rat thoracic aorta [131]. The mutual action on cGMP is the result of the H_2S inhibitory effect of phosphodiesterase type 5 (PDE5) which decreased cGMP degradation [132] whereas NO migration from endothelial cells to muscular cells activated soluble guanylate cyclase (sGC) to produce cGMP and consequential vasorelaxation [18]. Additionally, they showed a shift to the right in relaxation-response curves induced by NaHS vascular rings from $eNOS^{-/-}$ mice indicating that the vasorelaxant action of H_2S requires, at least in part, the presence of endogenously produced NO. Moreover, the Akt kinase phosphorylation by H_2S stimulated angiogenesis and as a result of this, the eNOS is activated [133]. Evidence showed that H_2S upregulate the silent information regulator1 (Sirt1) suggesting the role of this gas in age-associated diseases [134,135]. Sirt1 increases vascular endothelial growth factor (VEGF) level, and cGMP concentration [136]. Moreover, Sirt-1 induces vasorelaxation by eNOS activation [137] and mice knockout for endothelial Sirt1 present vascular defects [138]. Thus, the augmented H_2S effects on Sirt-1 could promote the NO release through cGMP, and therefore promoting vasorelaxation. However, the mechanisms underlying the H_2S/Sirt1/NO signalling are still to clarify.

Additional studies on the cross-talk between NO and H_2S in the control of vascular physiology showed that: the potent relaxant effect induced by exogenous NO donor,

such as sodium nitroprusside (SNP), is strongly reduced in aortic tissue pre-treated with H_2S [93]; NO and H_2S interact to generate nitrosothiol, a biological active intermediate with potent vasodilatory activity [139,140]; and CSE inhibition significantly reduces NO-induced vasorelaxation [141]. However, there are issues to address on H_2S/NO crosstalk in the vascular physiology. While mice knock out for eNOS are hypertensive and present an altered endothelium functionality [13], confirming the relevance of NO as EDRF factor, this role is not so well defined for H_2S. Indeed, studies on CSE knockout mice, showed a hypertensive phenotype and impaired vascular response to methacholine [142], while other authors reported that $CSE^{-/-}$ mice are normotensive [143]. This could be explained by the compensatory effect of CBS and 3MST, which are expressed in the vasculature and this, unfortunately, does not allow a thorough study of NO/H_2S crosstalk in this experimental model. Also, this compensatory hypothesis fails for mice lacking for CBS which present a severe impairment in endothelium functionality [119].

In conclusion, it is unquestionable that H_2S and NO independently play a critical role in the regulation of the cardiovascular system. Although accumulating evidence suggests that the eNOS/sGC/cGMP/PKG pathway could not be completely self-sufficient in the cardiovascular system, requiring the H_2S involvement. However, the natural precise underlying mechanism remains unclear, and a better understanding of H_2S/NO crosstalk could open promising avenues in the development of new pharmacological tools [144] as ZYZ-803.

ZYZ-803 is a novel hybrid donor of H_2S and NO which exerts a powerful protective effect on the cardiovascular system, strengthening the NO and H_2S cooperation hypothesis in the control of vascular tone.

In physiological condition, ZYZ-803 relaxes aortic rings, and this vasorelaxation is abolished by L-NAME and/or PAG, indicating that ZYZ-803 produces H_2S and NO through the stimulation of both CSE and endothelial NOS. Moreover, ZYZ-803 exerts its activity by opening the KATP channel through the cGMP pathway and exerts an effective, stable, and durable vasorelaxation compared to the classically H_2S donors used [145]. In a mouse model of hindlimb ischemia, oral administration of ZYZ803 elevated NO and H_2S levels and more effectively promoted angiogenesis when compared with either its H_2S or NO-generating compounds alone, through the SIRT1/VEGF/cGMP pathway supporting the mutual influence of NO and H_2S

ZYZ-803 results from the coupling of an H_2S donor (S-propargyl-cysteine, SPRC) and NO donor (furoxan). It has been demonstrated that with ZYZ-803 the release of H_2S and NO is significantly slower compared to SPRC and/or furoxan [136]. This positive data could move ZYZ-803 from preclinical to clinical studies in the treatment of cardiovascular diseases.

5. AIM

During H_2S production from L-cysteine by CBS activity, another metabolic product is generated: the L-serine. L-serine, a nonessential amino acid, is involved in the synthesis of sphingosine-1-phosphate (S1P). S1P is one of the most important products in the ceramide pathways and it plays an essential role in the pathogenesis of several cardiovascular diseases as a well-known NO trigger. The first part of this thesis aimed to investigate the vascular effect of L-serine compared to L-cysteine and to evaluate if the L-cysteine/H_2S signaling is associated with the L-serine/S1P/NO pathway in the vasculature, through CBS activity.

6. MATERIAL AND METHODS

6.1 Animals

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, ARRIVE guidelines, and the Basel declaration including the 3R concept). Male CD1 mice (18–22 g, Charles River, Calco, Italy) 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 provided with free access to standard rodent chow and water. All animals were allowed to acclimate at least 5 days before experiments and were subjected to a 12-hr light-dark schedule. Experiments were conducted during the light phase. Mice were randomly used for *in vitro* experiments. All effort was taken to minimize the number of animals used and their suffering.

6.2 Organ bath assays

CD1 mice were culled by enflurane overdose, and the thoracic aorta was rapidly harvested and placed in cold Krebs solution (NaCl 118 mM, KCl 4.7 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, and glucose 10.1 mM; Carlo Erba Reagents S.r.l., Milan, Italy). The aorta was cleaned from adherent connective and fat tissue and cut in rings (1–1.5 mm) and mounted in isolated organ baths filled with oxygenated (95% O_2 and 5% CO_2) Krebs solution and constantly maintained at 37°C. The rings were connected to an isometric transducer (FORT25, World Precision Instruments, 2Biological Instruments, Besozzo VA, Italy), and changes in tension were

continuously recorded with a computerized system (Power Lab 8/35, 2Biological Instruments). Initially, the rings were stretched to a resting tension of 1 g. After 30 min of stabilization at this tension, in which the bathing solution was periodically changed, the tension was reset, rings were first challenged with phenylephrine (Phe, 1 μM; Sigma-Aldrich, Milan, Italy) until the response was reproducible and this was repeated in each set of experiments. The integrity of the endothelium was assessed by a cumulative concentration-response curve to acetylcholine (Ach, 0.01–30 μM, Sigma-Aldrich) performed on rings pre-contracted by Phe [101]. In another set of experiments, a cumulative concentration-response curve of ACh (0.01–30 μM) was performed on denuded aortic rings pre-contracted by Phe to confirm the effective mechanically endothelium removal. To assess the contribution of the endothelium in exogenous L-serine effect, a cumulative concentration-response curve to L-serine (0.1–300 μM, Sigma-Aldrich) was performed on intact or endothelium-free rings. The involvement of H_2S in L-serine effect was evaluated by using a range of H_2S pathway inhibitors; aminooxy acetic acid (AOAA, CBS inhibitor, 1 mM; Sigma-Aldrich), D, L-propargylglycine (PAG, CSE inhibitor 10 mM; Sigma-Aldrich), or glibenclamide (10 μM; ATP-dependent potassium [KATP] channel inhibitor, Sigma-Aldrich). A concentration-effect curve of L-serine (0.1–300 μM) was performed in the presence of these inhibitors. In parallel, a concentration-response curve of L-cysteine (0.1–300 μM, Sigma- Aldrich) was performed on intact aortic rings.

To investigate the contribution of NO in the mechanism(s) underlying L-serine- or L-cysteine-induced relaxation, a pharmacological modulation was used. To examine the

role of eNOS, a concentration-response curve of L-serine (0.1-300 μM) or L-cysteine (0.1-300 μM) was carried out in presence of L-NG-nitro-arginine methyl ester (L-NAME, an inhibitor of NOS, 100 μM, 20 min; Sigma-Aldrich). In parallel, a concentration-response curve of L-serine (0.1-300 μM) was performed in the presence of wortmannin (irreversible inhibitor of PI3K, 0.01 μM, 20 min; Sigma-Aldrich). Subsequently, to evaluate the participation of the sphingosine-1-phosphate (S1P) pathway, the aortic rings were incubated with SCH202676 (inhibitor of the binding to GPCR, 1 μM, 40 min; Sigma-Aldrich) or W146 (S1P1 receptor antagonist, 0.1 μM, 20 min; Sigma-Aldrich) before the challenge with L-serine (0.1–300 μM) or L-cysteine (0.1–300 μM). Myriocin (SPT inhibitor, 0.3 mg·kg−1, i.p.) was administered to mice 24 hr before the aorta was collected [146], and thereafter, the tissues were challenged with L-serine (0.1–300 μM) or L-cysteine (0.1–300 μM). Data, expressed as mean ± standard error of the mean (SEM) (n = 6), are reported as % of relaxation for Phe-induced tone.

6.3 NOx determination

Mouse thoracic aorta was incubated with L-serine (1–100 μM) or vehicle for 15 min. In parallel, tissues were incubated for 20 min with L-NAME (100 μM) before the challenge with L-serine (100 μM). In another set of experiments, mouse aortic tissues were incubated with L-cysteine (10 μM to 1 mM) or vehicle for 30 min. The effect of L-cysteine (1 mM) was evaluated in the presence of L-NAME (100 μM), AOAA (1 mM), or PAG (10 mM) for 20 min. The reaction was stopped by dropping the tissues into liquid nitrogen, and samples were stored at −80°C. Next, tissues were

homogenized in modified RIPA buffer (50-mM Tris-HCl pH 8.0, 150-mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1-mM EDTA, 1% Igepal; Roche Applied Science, Monza, Italy) and protease inhibitor cocktail (Sigma-Aldrich) and then incubated in a microplate with cadmium (Sigma-Aldrich; 50 mg per well) for 1 hr to convert the inorganic anion nitrate (NO^{3-}) to nitrite (NO^{2-}) [147]. After centrifugation at 8000× g, total NOx content was determined using a fluorometric method by Promega Glomax explorer (Madison, WI) and calculated against a standard curve of sodium nitrite ($NaNO_2$, 50–2.000 nM; Sigma-Aldrich). Each independent experiment was performed in duplicate. Data, expressed as mean ± SEM (n = 6), are reported as $nmol \cdot mg^{-1}$ of protein.

6.4 S1P determination

Mouse aorta was incubated with L-serine (1–100 μM) for 15 min, L-cysteine (10–100 μM and 1 mM) for 30 min, or vehicle at 37°C. The reaction was stopped in liquid nitrogen, and samples were stored at −80°C. Samples were homogenized in the following buffer: Tris–HCl (20 mM, pH 7.4); glycerol (20%); β-mercaptoethanol (1 mM); EDTA (1 mM); Na orthovanadate (1 mM); NaF (15 mM); PMSF (1 mM); protease inhibitor cocktail 1%; deoxypyridoxine (0.5 mM); and β-glycerophosphate (40 mM). After total protein concentration evaluation, the homogenates were diluted to obtain a protein concentration of 1.5 μg·μl−1. S1P content was measured in homogenates as described in the manufacturer's protocol of S1P ELISA Kit (Echelon Biosciences Inc., Salt Lake City, UT) by using ThermoFisher multiscan Go (ThermoFisher Scientific) with plate absorbance at 450 nm. All samples were assayed

in duplicate, and S1P concentrations were calculated against a calibration curve of standard S1P (0.03–4 μM). Each independent experiment was performed in duplicate. Data, expressed as mean ± SEM (n = 8), are reported as nmol·mg^{-1} of protein.

6.5 Immunohistochemistry and immunofluorescence of CBS expression in aorta

Mouse aortic tissues (n = 5) were fixed overnight in 4% buffered formalin and paraffin-embedded. Sections of 7 μm in thickness were used for CBS detection by immunohistochemistry or for CBS and caveolin-1 detection by immunofluorescence. For immunohistochemistry, deparaffinized and rehydrated slides were unmasked using unmasking reagent (Vector Laboratories, Burlingame, CA) and treated with 0.3% H_2O_2 for 12 min before the incubation in serum-free Protein Block (DakoCytomation, Denmark) for 30 min. Sections were then incubated with a polyclonal antibody to CBS sc-67154, Santa Cruz Biotechnology, Dallas, TX; 1:50 dilution), in 1% blocking reagent/0.3% Triton X-100 (MP Biomedicals, Eschwege, Germany), overnight at 4°C. On the following day, slides were incubated with biotinylated anti-rabbit IgG (Jackson ImmunoResearch, Cambridge, UK; 1:500 dilution) for 30 min. Slides were then washed and incubated with streptavidin-HRP (1:200; Sigma-Aldrich) for 15 min. Following washing with PBS, positivity was detected with 3,3′- diaminobenzidine substrate (DAB Vector Laboratories), and the nuclei were counterstained with haematoxylin and eosin (Vector Laboratories). Then, sections were dehydrated through a graded ethanol series and mounted in Entellan (Merck). Images were observed using a microscope Leica DM RB (Leica Microsystems, Wetzlar, Germany) and acquired

using Leica Application Suite V 4.1.0 software. The presence of brown granules in endothelial cells and smooth muscle cells was defined as a positive signal. For negative controls, sections were processed as described above without primary antibody. For co-localization of CBS and caveolin-1 in mouse aorta, combined direct and indirect double-staining immunofluorescence procedure was applied. The processed slides were prepared as described above and were incubated with a mixture of two primary antibodies: polyclonal antibody to CBS (sc-67154, Santa Cruz Biotechnology; 1:50 dilution) and caveolin-1 (sc-53564 AF594, Santa Cruz Biotechnology; 1:50 dilution) Alexa-Fluor-594- conjugated, in casein plus Triton X-100 0.3% for 3 hr. After three washings with PBS, the CBS antibody was detected by incubation with fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, 1:250 dilution) for 30 min. Slides were mounted, and nuclei were counterstained with DAPI (Vector laboratories). The omission of the primary antibody was used as negative controls. Images were observed using a fluorescence microscope Leica DM RB and acquired using Leica Application Suite V 4.1.0 software.

6.6 Data and statistical analysis

Data were expressed as mean ± SEM of n observations, where n represents the number of animals. Statistical evaluation was performed by one-way or two-way ANOVA using GraphPad InStat (Graphpad Software Inc., San Diego, CA) followed by Dunnett or Bonferroni post hoc test for multiple comparisons. Post hoc tests were run only when F achieved $p < 0.05$ and there was no significant variance inhomogeneity. A p value <0.05 was used to define statistically significant differences between mean values.

7. RESULTS

7.1 L-serine relaxes mouse thoracic aorta

L- serine (0.1–300 μM) causes vasorelaxation on Phe-induced contraction aortic rings in an endothelium-dependent manner (Figure 5A). To study the possible mechanisms behind the L-serine relaxant effect, H_2S, NO and S1P pathway inhibitors were used as pharmacological modulators. Hampering the H_2S biosynthesis by CBS or CSE inhibition and blocking KATP channels, which have a role in H_2S-induced relaxation, did not modify the relaxant effects of L-serine (Figure 5B), excluding H_2S contribution. The inhibition of NOS by L-NAME significantly reduced the L-serine relaxant effect, suggesting the influence of NO. Moreover, to investigate the NO signalling in the L-serine effect, aortic rings were incubated with wortmannin, an inhibitor of eNOS phosphorylation. Interestingly, this pre-treatment significantly abrogated L-serine relaxant effect supporting the involvement of NO (Figure 5C, D). In a separate set of experiments, the evaluation of the SPT/S1P pathway involvement as possible by blocking sphingolipids biosynthesis at different steps. The SPT inhibition with myriocin and the blockade of S1P to GPCRs or its receptor S1P1 by SCH202676 and W146 respectively, markedly reduced L-serine induced relaxant effect pointing out the possible link between S1P signalling, along with NO, in the vascular L-serine effect (Figure 6).

7.2 L-cysteine relaxes mouse thoracic aorta

As aforementioned, L-cysteine leads to H_2S and L-serine production. On this basis, the relaxant effect of L-cysteine (0.1–300 μM) on intact artic rings was evaluated in the

presence of the same inhibitors used for L-serine. Notably, L-NAME, myriocin, SCH-202676, and W146 markedly reduced the L-cysteine vasorelaxant effect (Figure 7). Thus, indicating NO and S1P pathways are entailed also in L-cysteine-induced relaxation.

7.3 Expression of CBS and its co-localization with caveolin-1 in the mouse aorta

Immunohistochemistry experiments on mouse aortic tissue revealed that the CBS enzyme is localized on endothelium (Figure 7B), confirming previously published data [97]. We were able to confirm the presence of CBS also in smooth muscle cells (Figure 7B). In addition, immunofluorescence analysis showed CBS and CAV-1 localization in the endothelium (Figure 7D, E), with co-localization of CBS and caveolin-1 (Figure 7F, G).

7.4 NOx measurement

To confirm the involvement of NO in L-serine and L-cysteine vascular effect, *in vitro* incubation of aortic tissue with L-serine (1–100 μM) or L-cysteine (10, 100 μM, and 1 mM) followed by NOx levels measurement demonstrated that both L-serine and L-cysteine incubation significantly increased NOx level in aortic tissue compared to the vehicle. Furthermore, the NOx tissue levels after incubation with L-serine at 100 μM and L-cysteine at 1 mM are reverted to the vehicle level in the presence of L-NAME (Figure 8A). Moreover, AOAA (CBS inhibitor) but not PAG (CSE inhibitor) were also able to reduce the NOx levels triggered by L-cysteine (1mM) (Figure 8B).

7.5 S1P measurement

The further step was to address the contribution of L-serine (1–100 μM), and L-cysteine (10, 100 μM, and 1 mM) to S1P production in the thoracic aorta. Incubation with L-serine and L-cysteine significantly increased the tissue S1P levels at the highest concentration of 100 μM and 1 mM compared to the vehicle, respectively (Figure 9).

8. FIGURES

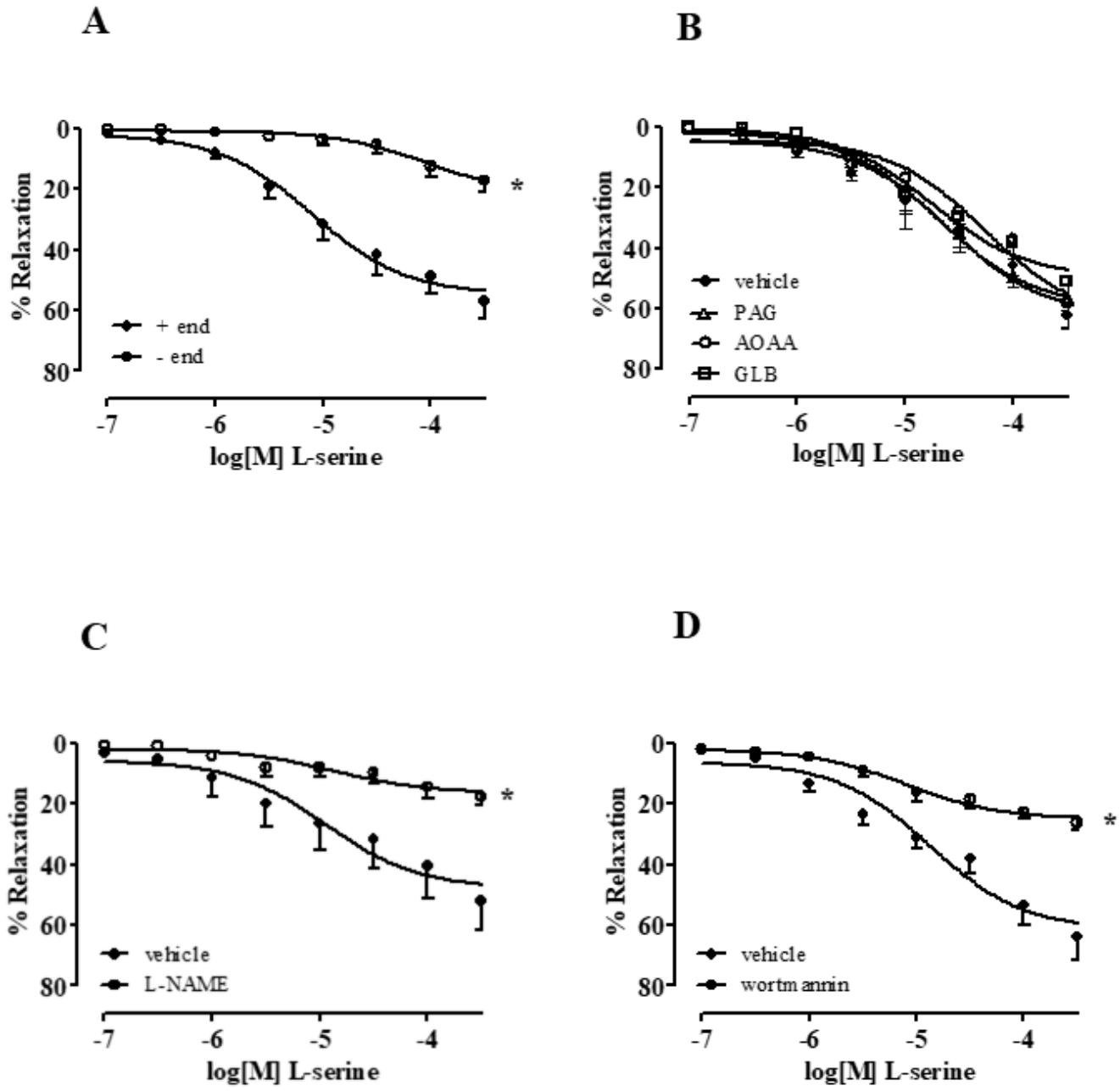

Figure 5 L-serine effects on mouse thoracic aorta

(A) L-serine (0.1–300 μM) relaxed phenylephrine-induced contraction (1 μM) aortic rings in an endothelium-dependent manner. **(B)** PAG (10 mM, CSE inhibitor), AOAA (1 mM, CBS inhibitor), and glibenclamide (GLB, 10 μM, KATP-channels inhibitor) did not affect the relaxant effects of L-serine. **(C)** L-NAME (100 μM, NOS inhibitor) or **(D)** wortmannin (0.01 μM, an inhibitor of PI3K) significantly reduced the relaxant effects of L-serine. Values shown are means ± SEM; n = 6 mice. *$p < 0.05$, significantly different from the vehicle; two-way ANOVA with Bonferroni's post hoc test.

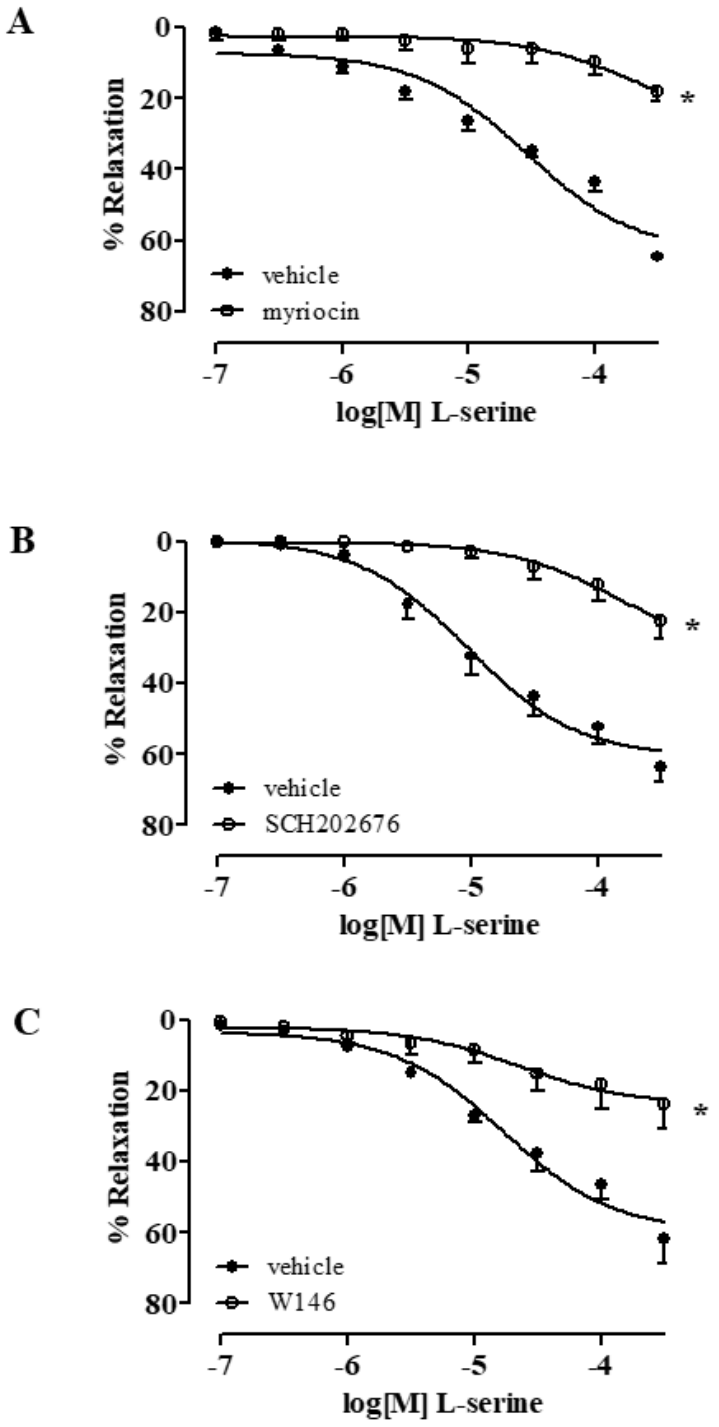

Figure 6 L-serine effects on mouse thoracic aorta

(A) Myriocin (SPT inhibitor, 0.3 mg·kg−1 i.p. was administered 24 hr before aorta was harvested), **(B)** SCH202676 (1 μM; inhibitor of GPCR) or **(C)** W146 (0.1 μM; antagonist of S1P1 receptor) reduced the relaxant effects of L-serine (0.1–300 μM) in aortic rings precontracted with phenylephrine (1 μM). Values shown are means ± SEM; n = 6 mice. *$p < 0.05$, significantly different from the vehicle; two-way ANOVA with Bonferroni's post hoc test.

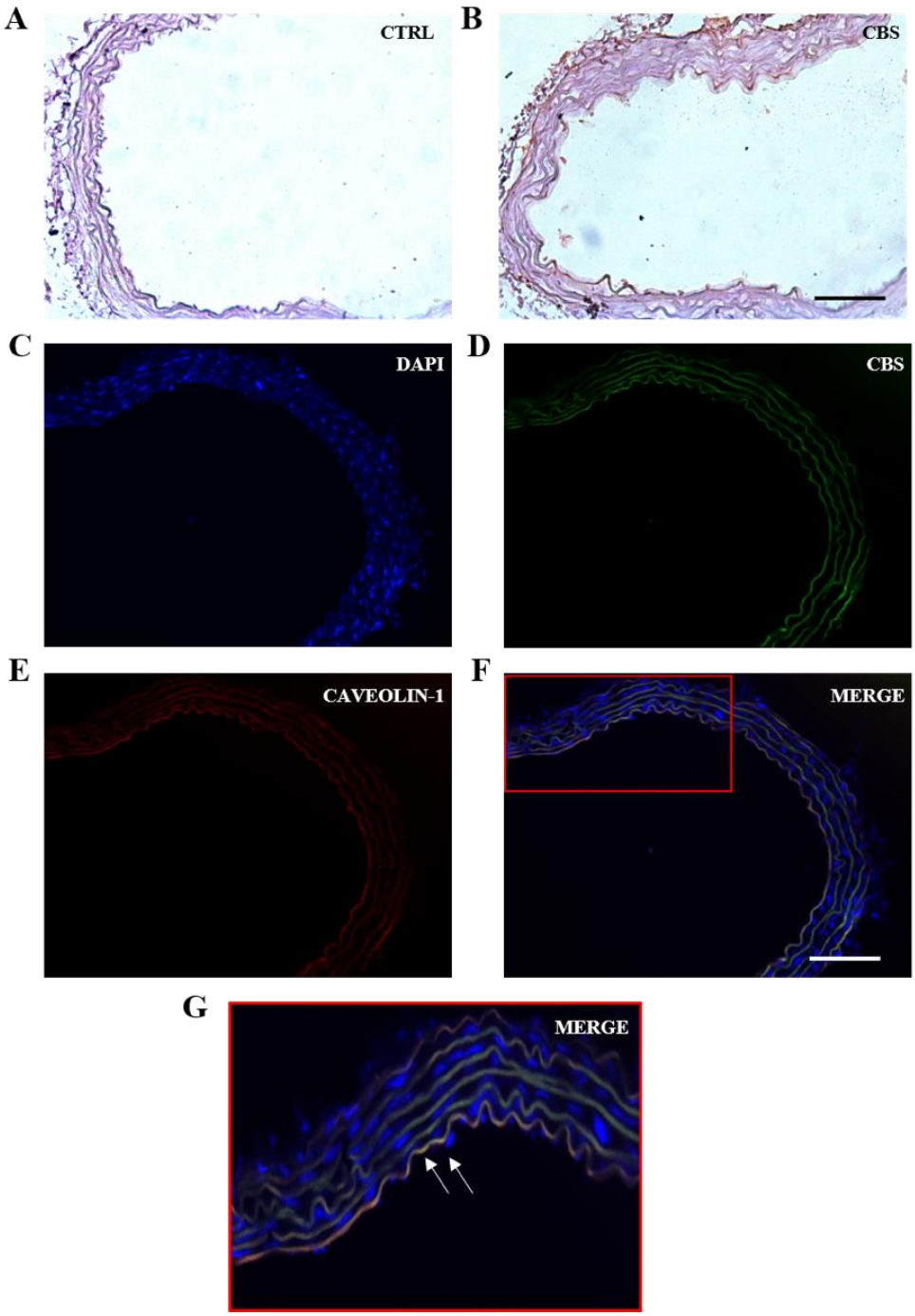

Figure 7 CBS expression in smooth muscle cells and Caveolin-1 co-localization

Immunohistochemical (IHC) analysis of CBS expression in mouse aorta. **(A)** Aorta negative control. **(B)** Representative IHC staining for CBS expression. CBS protein was strongly expressed in endothelial cells and the smooth muscle cells **(B**, brown granules**)**. Original magnification ×200. Scale bar: 100 μm. Immunofluorescence detection of caveolin-1 and CBS in mouse aorta **(D–G)**. Panel **(G)** is the image at further magnification ×20 of the panel **(F)**. Areas of co-localization (merge) of caveolin-1 and CBS in aortic endothelium results in yellow **(F**, arrows in **G)**. Cell nuclei (blue) were counterstained with DAPI **(C)**. Original magnification, ×200. Scale bar: 100 μm.

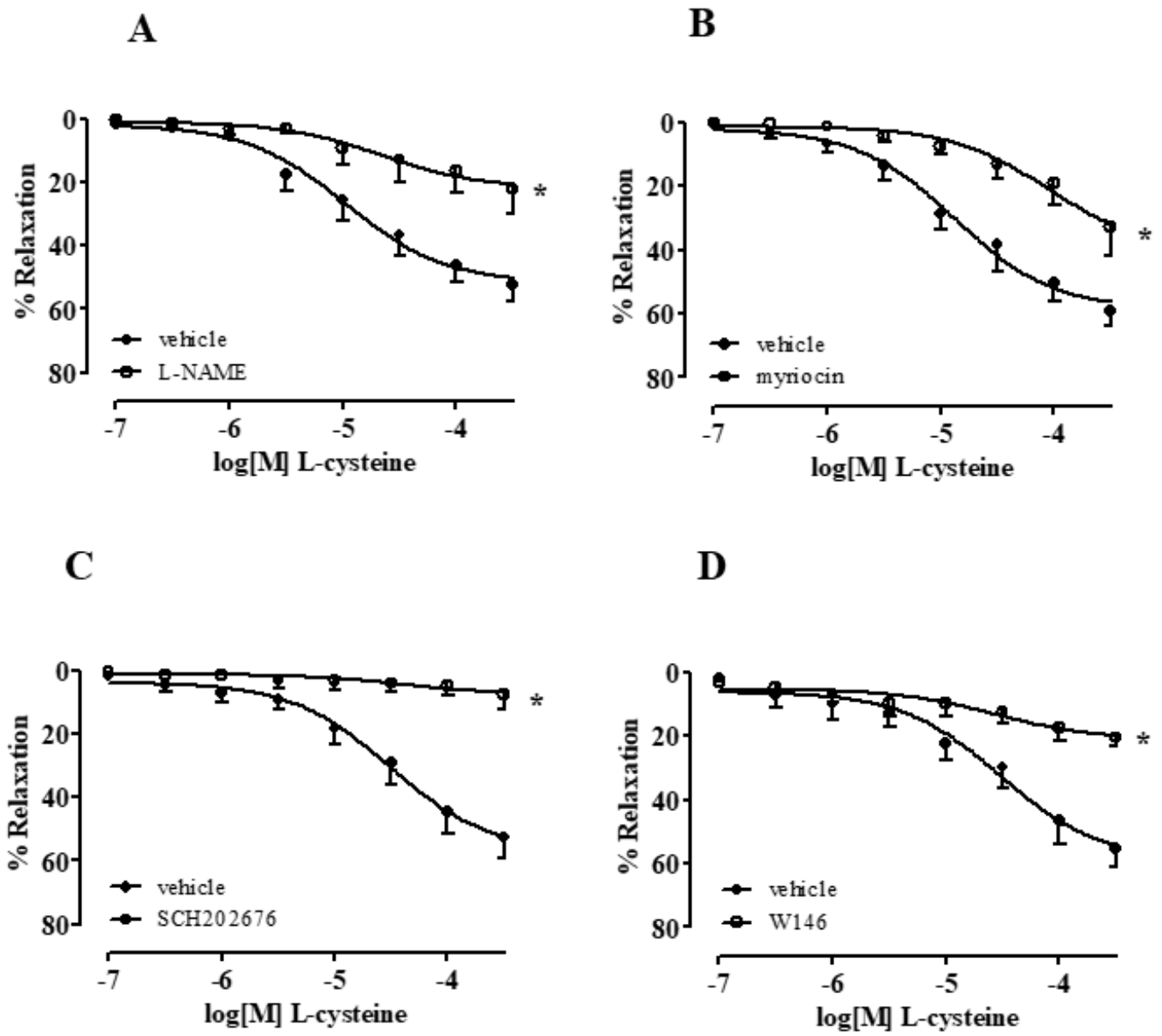

Figure 8 L-cysteine effects on mouse thoracic aorta

(A) L-NAME (100 μM, NOS inhibitor), **(B)** myriocin (SPT inhibitor, 0.3 $mg \cdot kg^{-1}$ i.p. administered 24 hr before aorta explantation), **(C)** SCH202676 (1 μM; inhibitor of GPCR) or **(D)** W146 (0.1 μM; antagonist of S1P1 receptor) significantly reduced L-cysteine (0.1–300 μM) relaxant effect in aortic rings pre-contracted with phenylephrine (1 μM). Values shown are means ± SEM; n = 6 mice. *$p < 0.05$, significantly different from the vehicle; two-way ANOVA with Bonferroni's post hoc test.

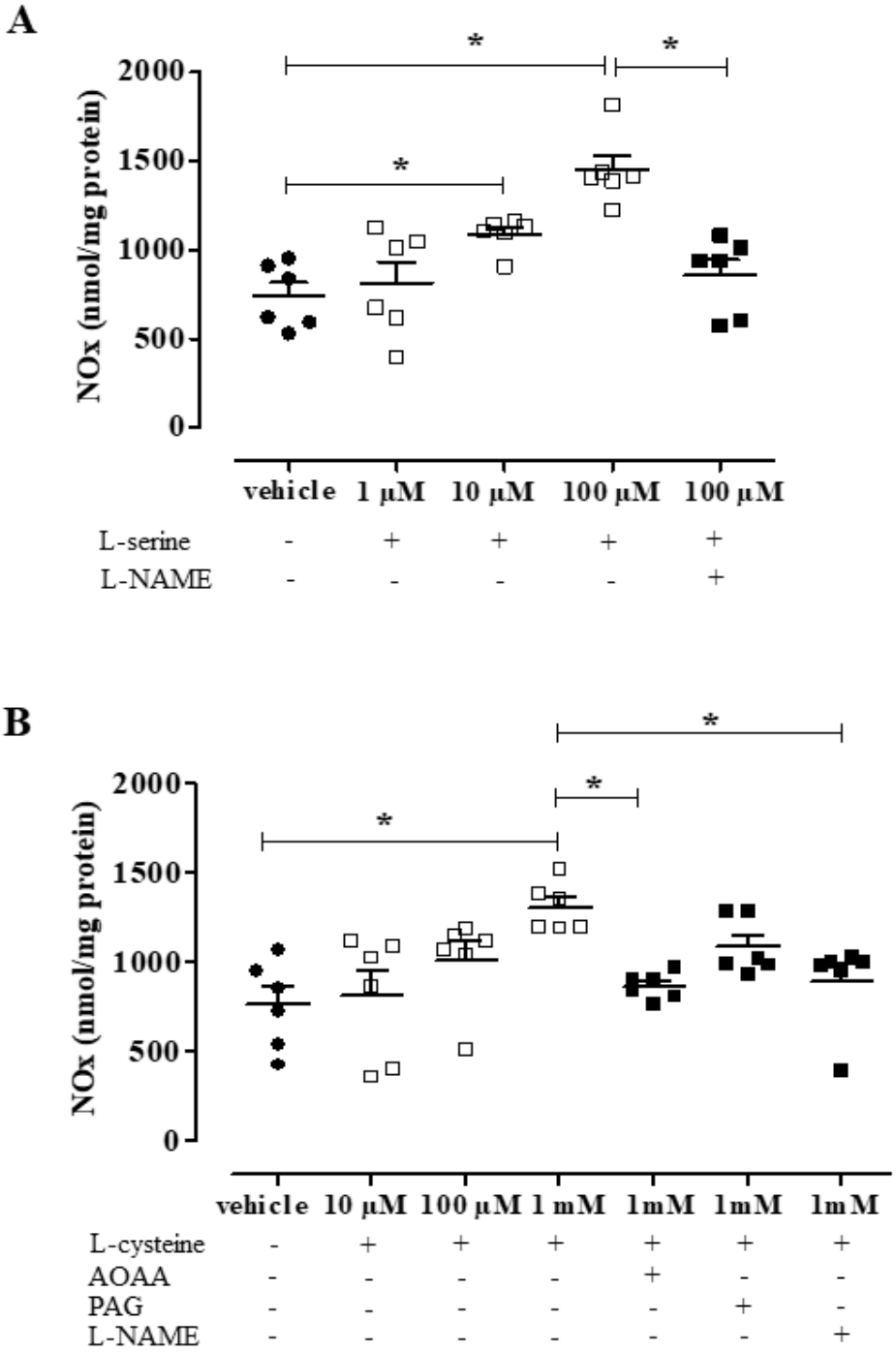

Figure 9 NOx measurement in mouse thoracic aorta in the presence of L-serine or L-cysteine

(A) L-serine 10 and 100 µM significantly increased NOx production compared with vehicle. L-NAME (100 µM, NOS inhibitor) reverted the L-serine (100 µM) increase in NOx production. **(B)** L-cysteine (1 mM) significantly increased NOx production compared with the vehicle; AOAA (1 mM, CBS inhibitor) or L-NAME (100 µM, NOS inhibitor) but not PAG (10 mM, CSE inhibitor) significantly reduced L-cysteine-increased NOx production. Values shown are means ± SEM; n = 6 mice. *$p < 0.05$, significantly different as indicated; one-way ANOVA with Bonferroni's post hoc test.

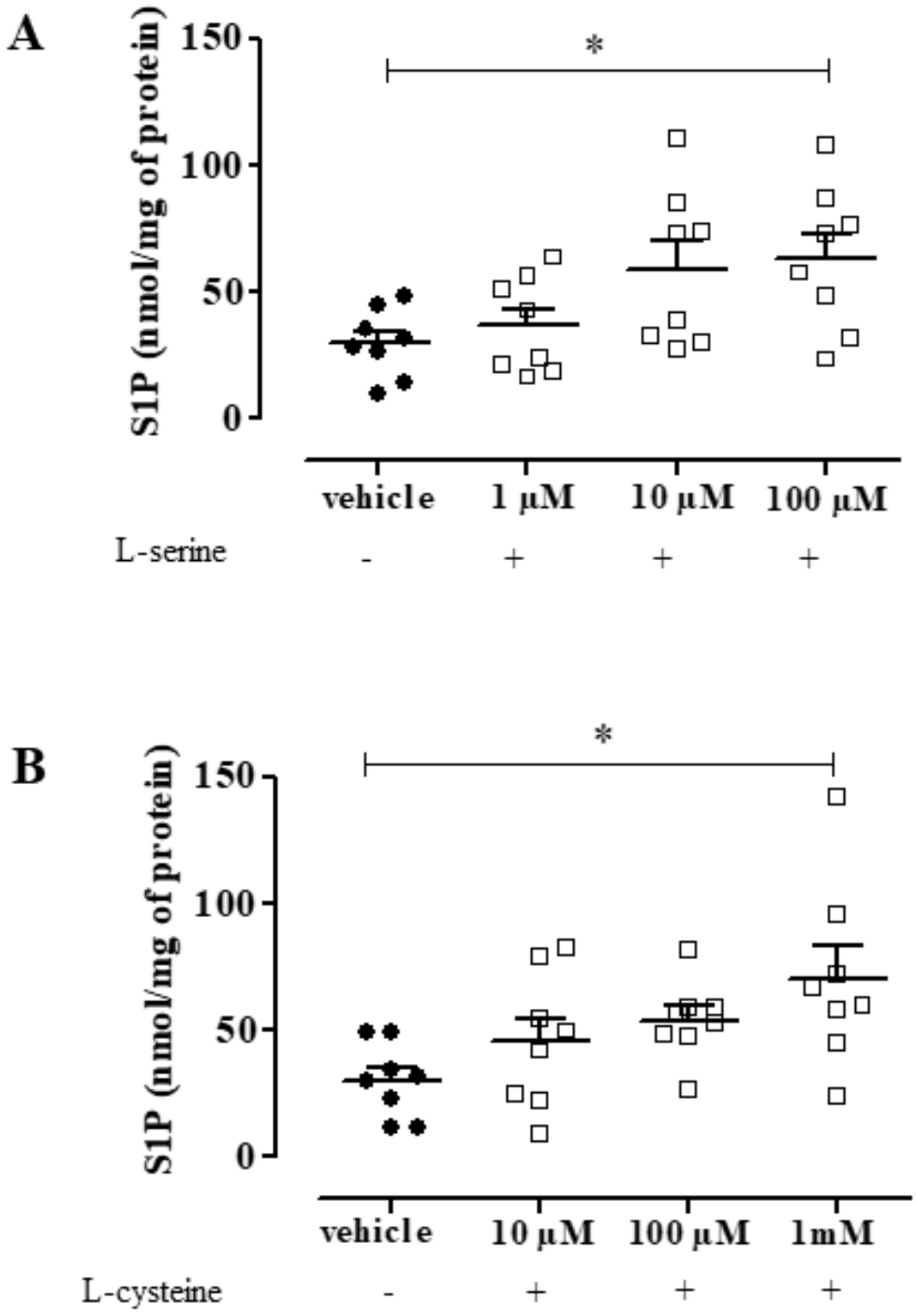

Figure 10 S1P levels in mouse thoracic aorta in presence of L-serine or L-cysteine

(A) L-serine (100 µM) caused an increase in S1P production compared with vehicle. **(B)** L-cysteine (1 mM) caused an increase in S1P production compared with vehicle. Values shown are means ± SEM; n = 8 mice. *$p < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's post hoc test.

9. DISCUSSION

The hypothesis regarding potential cooperation between H_2S and NO has always been at the centre of many studies [131]. These two gases independently play a pivotal role in the cardiovascular system in physiological conditions. eNOS, located in endothelial caveolae, synthesizes NO in a Ca^{2+} calmodulin-dependent reaction in the vasculature, and NO induces vasorelaxation through the sGC/cGMP signalling [18]. H_2S is synthesized from L-cysteine across CBS, CSE, and the sequential action of CAT and 3-MST enzymes [148].

During the past few years, evidence emphasized the role of CSE in H_2S and NO interplay in health. It has been shown that CSE, as eNOS, is activated by Ca^{2+} and its expression and activity in H_2S production is enhanced by NO [149]. In parallel, the vasodilatory response to NO is reduced in mice aorta rings when CSE is inhibited, silenced or absent [40]. This emphasis on CSE could be explained because CSE is classically considered the predominant enzyme involved in H_2S production in the cardiovascular system [150].

CBS is also expressed in endothelial cells and previous studies demonstrated that silencing CBS affects endothelial cells proliferation [97]. Unfortunately, thorough studies on CBS in physiological vascular function are hampered by the lack of mice model knockout for the CBS gene. CBS homozygous ($^{-/-}$) animals have a high mortality rate and the surviving animals present sever plasmatic levels of homocysteine. This latter condition reflects what happens in human [124]. Actually, hyperhomocysteinemia leads to endothelium dysfunction *via* NO-signalling dysregulation, supporting that CBS, like

CSE, plays a pivotal role in the control of vascular tone [123]. However, the role of CBS in healthy vasculature and its interplay with NO still need to be fully addressed.

Using L-cysteine as a precursor, CBS is the only of the three enzymes involved in the H_2S signalling to release beside H_2S also another cytosolic metabolite: L-serine [148].

L-serine, with palmitoyl CoA, takes part in the generation of sphingolipids. S1P belongs to the sphingolipids family and it represents the major sphingolipid regulator in the cardiovascular system [151]. It has been demonstrated S1P activates eNOS/NO signalling trough endothelial S1PR1 receptor [152], which were found in the caveolin-enriched microdomains [153].

Furthers studies showed that L-serine *per se* promotes endothelium-dependent vasodilation in rat resistance arterioles and this effect is greater in chronic L-NAME-treated hypertensive rats [154]. This evidence raises the hypothesis that L-serine could interact with the endothelium and in a hypertension scenario, in which the NO system is compromised, L-serine could activate compensatory response which explains its hypotensive effects. This is consistent with the observation that, other than NO, EDHF contributes to flow-mediated vasodilatation in vivo at microcirculation level. In line with these findings, other authors showed that *in vitro* L-serine exerts protective effects in human umbilical vein endothelial cells by releasing NO, suggesting that L-serine could have beneficial effects on endothelium homeostasis interacting with NO signalling [155]. However, at best of our knowledge, there are no reports on the direct

effects of L-serine on the large vessel such as the aorta, and the mechanisms underlying are still to clarify.

Here, we reported for the first time the mice aorta vascular effect of exogenous L-serine in physiological condition. L-serine relaxes mouse thoracic aorta in a concentration-dependent manner and the absence of endothelium significantly modifies this relaxant effect.

To investigate the mechanisms behind L-serine action on isolated aorta vessel, a pharmacological approach was used. In presence of AOAA and PAG, which are commonly pharmacological tools for CBS [95] and CSE [96] inhibition, and blocking KATP channels by glibenclamide [55], the relaxant effects of L-serine were not affected. Thus, the H_2S is not involved in the L-serine-induced relaxant effect. Pre-treatment with L-NAME (NOS inhibitor) or wortmannin (inhibitor of eNOS phosphorylation), reduced L-serine relaxant effect in intact aortic rings, demonstrating the contribution of the eNOS pathway.

As aforementioned, L-serine enters in the sphingolipids biosynthesis. Hence, we evaluated the L-serine vascular effects in presence of different sphingolipids biosynthetic route inhibitors: (*i*) myriocin, an SPT inhibitor, this enzyme catalyses the decarboxylation reaction between L-serine and palmitoyl-CoA and this reaction represents the first and rate-limiting step of the *de novo* biosynthesis of sphingolipids; (*ii*) SCH-202676, a non-selective inhibitor of GPCRs (it has been shown sphingolipids modulate multiple physiological functions through GPCRs) [156]; (*iii*) W146, which is a S1P1 receptor antagonist, where S1P1 belongs to the vast family of S1P receptors and

it represents the endothelial S1P target causing vasodilation via NO pathway [152]. The involvement of S1P signalling was confirmed by the finding that myriocin and SCH-202676 inhibited L-serine-induced relaxation. Moreover, the reduction in L-serine relaxant effect in the presence of W146 demonstrated the influence of the S1P/NO pathway.

Immunohistochemical study showed that CBS is expressed on the vascular endothelium corroborating previous findings [157], and in addition, we demonstrated that CBS co-localized in the endothelium with Cav-1. It is interesting to note that the activity of eNOS is depending on the binding to Cav-1 in endothelial caveolae [26], and S1PR1 resides in caveolin-enriched microdomains and is critically involved in endothelial cells barrier enhancement [153].

As above mentioned, L-cysteine is a CBS substrate in H_2S and L-serine production. Previous studies demonstrated that L-cysteine induced mouse thoracic aorta vasodilation in a concentration- and endothelium-dependent manner [158]. Here we addressed the L-serine/S1P/NO pathway role in the L-cysteine vasodilatory effects. Firstly, in these experiments, we show the treatment with L-NAME impairs the L-cysteine relaxant effect in thoracic aorta rings. Surprisingly, the inhibition of S1P signalling by myriocin, SCH-202676 and W146 significantly reduces L-cysteine vasodilating effects. To establish NO and S1P participation in the H_2S pathway, the aortic tissue was incubated with L-serine or L-cysteine followed by NO and S1P levels measurement. Both the amino acids, at the highest concentration, were able to enhance the NO tissue level; this increase is significantly reverted in the presence of L-NAME.

Moreover, L-cysteine induced-NO production was reduced by the AOAA inhibitor but not by PAG inhibitor; this evidence not only confirms the involvement of eNOS/NO signalling in L-serine and L-cysteine vascular effects but also suggest an impact of H_2S pathway on NO production. In parallel, also S1P tissue levels are increased after L-serine or L-cysteine incubation in the aortic tissue confirming both the aminoacids lead to S1P production.

In conclusion, CBS could represent a possible knot between L-cystine/H_2S and S1P/NO signalling trough L-serine. Moreover, CBS, eNOS and S1PR1 are localized in the same endothelial cellular compartment and they are the main players in three routes which have a critical role in the control of vascular tone. This could drive the research toward future studies on CBS/eNOS/S1P improving the understanding in the crosstalk between NO and H_2S in physiological condition but also explaining for some extent the severe impairment in vascular functionality observed in patients affected by CBS deletion (98). Moreover, demonstrating that the L-serine/S1P/NO pathway additionally contributes to the L-cysteine/CBS/H_2S vasorelaxant effect may propose future genomic, genetic and epigenetic studies on the investigation of S1P pathway-related genes in CBS mutated patients, suggesting a new therapeutic target for cardiovascular events in hyperhomocysteinemia.

10. PART II: INTRODUCTION

Allergic respiratory diseases are a group of pathologies involving the lower and higher airways in the respiratory tract. Allergic rhinitis, rhino-conjunctivitis and allergic asthma belong to this family [160]. These pathologies can coexist in the same subjects who present a hypersensitive immune response to several environmental stimuli indicated as "allergens". The most common allergens are animal dander, house dust mite, pollen (i.e. ragweed) and grass [161]. When a subject incurs in these allergens the activation of the immune system induces the releasing of immunoglobulins E (IgE) which are antibodies able to trigger the production from mast cells of a wide range of mediators including histamine [162]. Histamine has ubiquitin receptors widespread on different zones of the body [163] and the binding to these receptors result in the manifestation of classical allergic reaction symptoms such as itchiness, swelling, running nose, watery eyes, coughing, wheezing, trouble breathing, hives, rashes, and mucus production [164]. The immune system of an allergic subject reacts every time it will meet the allergens. This could happen several times during the year causing impairment in the daily life activities, academic and work performances [165], in severe cases or not properly treated, the allergic reaction could lead to a total airway occlusion and anaphylaxis [166]. Moreover, mediators released by the immune system easily boost inflammation [167], resulting in a plethora of mediators which in concert increasing pathology severity.

Asthma (allergic and not) which represents the most common respiratory disease [168], has a substantial direct and indirect monetary cost. Direct costs include the costs of

medication, medical bills, and documented episodes of health service utilization such as clinic visits and hospital admissions. Indirect costs include the adverse economic impact of the disease on an individual, family, and society (Global Initiative for Asthma (GINA) http://www.ginasthma.org).

Respiratory diseases and CVD share some important features: high incidence, high socio-economic impact, chronic inflammation and uncontrolled immune response [169]. In addition, previous studies demonstrated that patients affected by chronic inflammatory diseases are more inclined to CVD development [170]. On these bases, researchers tried to address the question if subjects suffering from allergic respiratory diseases are prone to CVD.

Early studies reported controversial data about the correlation between lung functionality and CVD development. Schroeder and colleagues, evaluated the forced expiratory volume in 1 s (FEV_1) and 10-year incidence of coronary heart disease among 14,480 participants in the Atherosclerosis Risk in Communities (ARIC) study and they found a strong association between lung function and incident coronary heart disease (CHD) among women and a weaker association among men, both in the full cohort and in never smokers [171]. Other authors reported a significant association in all-male cohorts [172]. While other evidence reported significant correlations between lung function and cardiovascular disease or CHD mortality with no difference in sex gender [173–176]. Recently, a study which analysed 4,019 participants from the Wuhan-Zhuhai cohort, with a follow-up of 3 years, demonstrated that reduced lung function was cross-sectionally and longitudinally associated with increased cardiovascular risk in Chinese

general population [177]. It is important to underly that in these studies data are adjusted excluding smokers because smoking is a well-known CVD risk and lung functionality detrimental factor. Moreover, these data are based on lung function measured by FEV_1. FEV1 only measures how much air a person can exhale; it does not give any kind of information about the biological mechanisms behind possible poor lung function.

Uncontrolled inflammation is the first cause of lung functionality impairment [178]. Asthma and COPD are the most common respiratory chronic inflammation-mediated diseases which lead not only to lung tissue remodelling but also an alteration in the pulmonary vascular bed [179]. Lung inflammation leads to a decrease in endothelium function and an increase in vessel permeability in patients affected resulting in an increase in vessel permeability followed by release of inflammatory mediators from the lung the system through the bloodstream [180,181]. Systemic effects are not only present during the progression of lower airway pathologies such as asthma or COPD but are also evident in high airway allergic diseases such as rhinitis [182]. Previous evidence for systemic endothelial dysfunction is greater in COPD [183–186] and asthma [6,187–189], compared to other upper airway respiratory diseases [190], but this correlation is strongly debatable due to the controversial published clinical studies.

A prospective cohort study of 446,346 Taiwanese adults reported a correlation between active asthma and cardiovascular consequence [191]. Another large prospective study on the aetiology of atherosclerotic-related diseases analysed the ARIC study and demonstrated an association with increased carotid artery intima-media thickness (IMT) [192] and incidence of CHD and stroke [193] in women with late-onset asthma, but

not in those with child-onset asthma. Similar studies have also reached the conclusion that late-onset asthma inferred a greater risk of CVD [194,195]. Other studies confirmed that both asthma and allergy were independently associated with an increased risk of CHD [196–199] and that asthma led to augmented vascular inflammation [200] and that patients with allergic rhinitis and asthma or chronic rhinosinusitis showed an increase of carotid IMT [201,202]. On the contrary, a biracial study on 13,501 participants investigating the association of self-reported, doctor-diagnosed asthma and CVD in adults followed over 14 years, showed that asthma and duration of asthma was not an independent risk factor for CHD [203]. A large retrospective, population-based, matched cohort study displayed lower incidence of acute myocardial infarction and cerebrovascular disease in patients affected by allergic rhinitis [204]. Similarly, another study found a reduced risk of developing acute ischemic stroke in 61,899 Taiwanese patients affected by rhinitis [205]. In summary, clinical and epidemiological data so far are not resolved.

10.1 Local and systemic endothelium in airway allergic inflammation

In the pulmonary vasculature, the role of endothelium is critical for the gas exchange. Moreover, several inflammatory cells can reach the lung tissue through the endothelium by a mechanism indicate as trans endothelial migration (TEM) in respiratory disease [6]. TEM play also a crucial systemic role. Actually, the reverse TEM of blood cells such as leukocytes contributes to the dissemination of systemic inflammation and to secondary organ damage [206]. In addition, the airway of patients with asthma is also characterized by microvascular hyperpermeability [207], supporting

the hypothesis that airway inflammation could lead to systemic inflammation via proinflammatory mediators release from the lung.

Inflammation and endothelium dysfunction are linked very firmly [208]. Previous investigators have reported an attenuated endothelium-dependent relaxation response in pulmonary arterial rings obtained from patients with chronic COPD compared with individuals without lung disease [209,210]. Other evidence has also revealed that asthma is accompanied by pulmonary endothelial dysfunction [6,211], and nasal allergen triggers the increase in expression of vascular adhesion molecules in bronchial mucosa [212,213]. In contrast, the literature is limited in pre-clinical studies investigating the association between allergic respiratory diseases and systemic endothelial dysfunction. Thus, a connection between these two remains speculative.

Evidence on systemic vascular dysfunction in allergic respiratory diseases induced by ragweed was provided by Hazarika and colleagues. The authors showed that the airway allergen exposure did not alter the constrictor response to phenylephrine, while the NO-mediated relaxant effect to acetylcholine (0.1-1 μM) resulted significantly reduced [214]. However, this study did not clarify the exact mechanism responsible for their findings.

In respiratory diseases, reactive oxygen species (ROS) play a major role in airway inflammation. Moreover, inflammatory cells (such as activated eosinophils, neutrophils, monocytes, and macrophages) can release ROS [215]. Interestingly, patients with allergic rhinitis and allergic asthma showed a marked oxidant/antioxidant systemic imbalance [216]. Al-Harbi and colleagues, demonstrated an increase of ROS-

producing enzyme expression in the aorta of rats sensitized and challenged with ovalbumin, which is one of the major allergens which causes IgE-mediated hypersensitivity [217], suggesting that the ROS produced during the allergic response could impair systemic vascular endothelium. However, the author did not show data about the effective impairment of vasorelaxation in this experimental model [218].

It is important to highlight that these studies did not report data about the role of NO in their experimental model. NO has several roles in the airways. It is produced by the endothelium in the vasculature, but it is also considered an endogenous modulator of airway functions from bronchoprotective to pro-inflammatory [219]. Moreover, in a state of inflammation NO is also involved in ROS production [220] and the detrimental pulmonary inducible NO enzyme is overexpressed in asthma [221]. Thus, a better understanding of the role of the NO pathway in systemic vasculature in the allergic respiratory experimental model could help to unmask the possible connection between allergic respiratory diseases and systemic endothelial dysfunction.

11. AIM

The major goal of the second part of my thesis is to investigate the mechanism(s) underlying potential endothelium dysfunction in a murine model of allergic pulmonary inflammation induced by ovalbumin.

12. EXPERIMENTAL STRATEGY

12.1 Choice of animal model

Because of our interest is the study of vascular reactivity in a murine model which mimic major features of human allergic respiratory disease, all studies were performed in 6-week-old male Balb/cOlaHsd (Balb/c) mice. Balb/c represents a common strain used for the study of allergic/pulmonary diseases because they display some key features of human allergic respiratory disease such as airway responsiveness and bronchial inflammation along with a hyperproduction of Th2 cytokines, in a 7-day exposure protocol [222]. Along with Balb/c also C57BL/6 mice are a prototype mouse strain for studies of respiratory allergy diseases. However, the rationale for employing the Balb/c instead of the C57BL/6 strain in our study was because the C57BL/6 strain displayed a mixed phenotype Th1/Th2 in a 7-day exposure protocol [222]. The Th1/Th2 response well reflects features of severe and chronic asthma. Thus, C57BL/6 mice are more suitable for chronic respiratory diseases study, while Balb/c mice are suitable for our acute respiratory disease model [222]. Moreover, in order to exclude any hormonal effects in our experimental model, only male mice were considered.

12.2 Choice of ovalbumin model of allergic respiratory diseases

No single model can sufficiently replicate the spatial and temporal recruitment of the several cell types involved in the different phases of clinical respiratory allergic diseases. The rationale for using ovalbumin was because this model represents the "classical" model of respiratory allergy and it is described in over 2000 literature references [223].

Two injections of ovalbumin with adjuvant, 7 to 14 days apart, usually induce a strong Th2-biased immune response with high levels of total and antigen-specific IgE, also in a short-term experimental plan. Ovalbumin is versatile and successful for intranasal challenge inhalation [223]. Thus, as this project aimed to study the effect of respiratory allergy on secondary organ damage in a short-term model, the use of ovalbumin to induce a respiratory allergic disease represents a reliable method.

12.3 Experimental design

The study design takes into consideration investigations at the following levels:

i) *Ex vivo* studies: wire myography studies to examine vascular reactivity of isolated thoracic aorta of naïve, ovalbumin sensitized and challenged and control mice.

ii) Molecular biology assays (ELISAs, RT-PCR, Western Blot) in ovalbumin sensitized/challenged mice as well in control mice to determine systemic and vascular changes in a condition of allergy.

13. MATERIAL AND METHODS

13.1 Animals

Balb/cOlaHsd male mice (5 weeks of age) were purchased from Envigo (UK) and kept in-house (Central Research Facility, University of Glasgow). Animals were maintained on a 12/12-hour light/dark cycle with free access to food and water. All the procedures were performed in accordance with local ethical and UK Home Office regulations.

13.2 Allergic sensitization and challenge with OVA

After seven days from their arrival, indicated as Day 0, mice were sensitized for the first time to ovalbumin (OVA, fraction V; Sigma-Aldrich, Poole, UK) allergen in presence or absence of Th2-driven adjuvant alum (2% Alhydrogel, Brenntag Biosector, Fredriksund, Denmark). The animals were divided into three treatment groups; Group 1 and Group 2 were sensitized intraperitoneally (i.p.) with 100 µL/mouse of a suspension of 100 µg OVA dissolved in 100 µl of sterile phosphate buffer solution (PBS, Life Technologies, Paisley, UK) and sterile alum (2% Alhydrogel); Group 3 was sensitized intraperitoneally (i.p.) with 100 µL/mouse of a suspension of 100 µg OVA dissolved in 100 µl of sterile PBS. The sensitization was repeated for all the groups 14 days apart from the first administration (Day 14). On Day 14, mice were anaesthetized with isoflurane, and 40 µl/mouse of a suspension of 10 µg of OVA dissolved in 40 µl of sterile PBS were administered intranasally (i.n.) alternating the administration per nostril in Group 1, while the Group 2 and Group 3 received i.n. 40 µl/mouse of sterile PBS alternating the administration per nostril. On Days 25, 26 and 27 the three groups were anaesthetized with isoflurane and 40 µl/mouse of a suspension of 10 µg of OVA

dissolved in 40 µl of sterile PBS was administered i.n. alternating the administration per nostril in Group 1. Group 2 and Group 3 received i.n. administration of 40 µl/mouse of sterile PBS. Mice were humanely culled on Day 28 (10 weeks old) using CO_2 and blood, lungs and thoracic aorta were collected.

The Group 1 indicated as OVA/ALUM+OVA represented the basic model of respiratory allergy, the Group 2 indicated as OVA/ALUM+PBS was the control of Group 1, and the Group 3 indicates as OVA+PBS was used to assess the possible effect of alum on vascular reactivity (Figure 11).

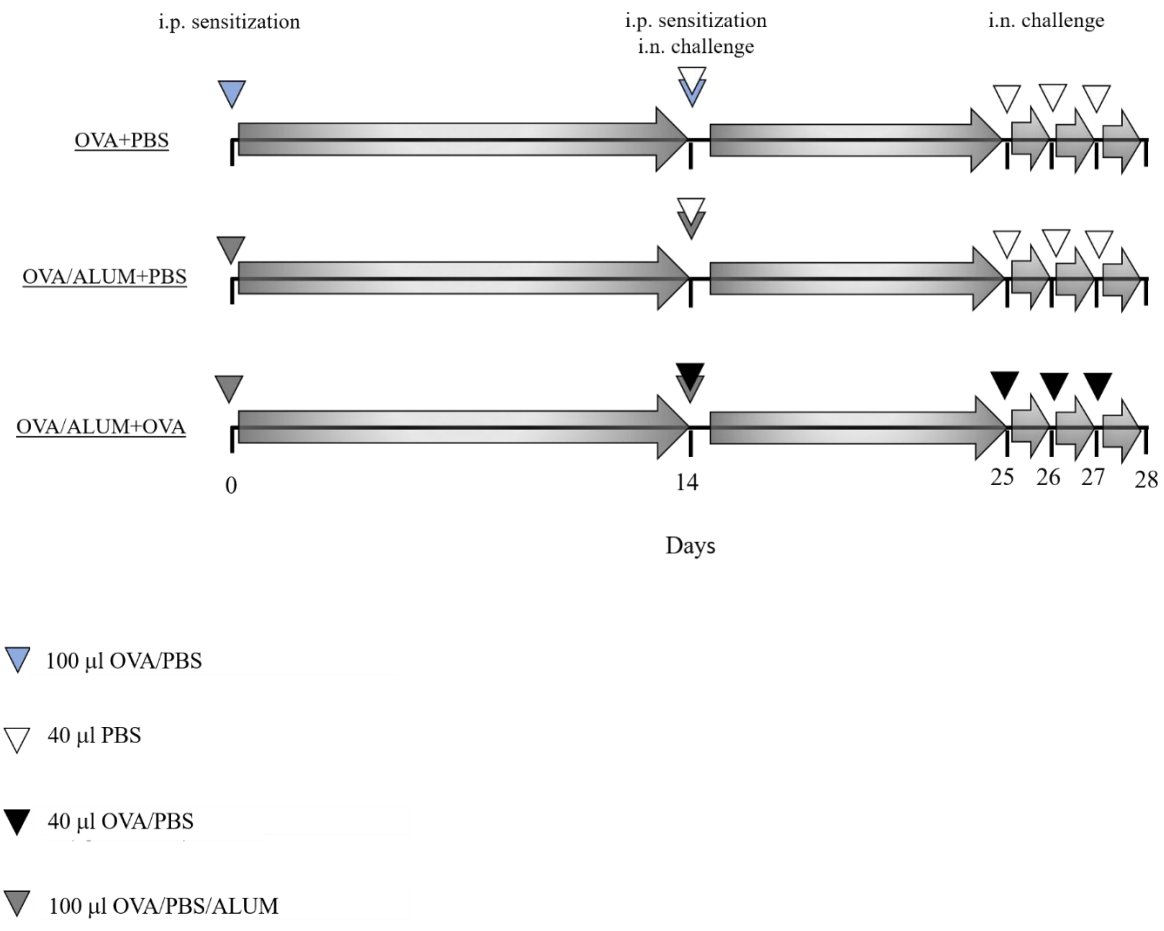

Figure 11 Allergic sensitization and challenge scheme

13.3 Modified allergic sensitization and challenge with OVA

After seven days from their arrival, indicated as Day 0, mice were sensitized for the first time to ovalbumin (OVA, fraction V; Sigma-Aldrich) allergen in presence or absence of Th2 -driven adjuvant, alum (2% Alhydrogel, Brenntag Biosector). The animals were divided into two treatment groups; Group 1 was sensitized intraperitoneally (i.p.) with 100 µL/mouse of a suspension of 100 µg OVA dissolved in 100 µl of sterile phosphate buffer solution (PBS, Life Technologies, Paisley, UK) and sterile alum (2% Alhydrogel), Group 2 was sensitized intraperitoneally (i.p.) with 100 µL/mouse of 100 µl of sterile PBS mixed with sterile alum (2% Alhydrogel).

The sensitization was repeated for all the groups 14 days apart from the first administration (Day 14). On Day 14, mice were also anaesthetized with isoflurane, and 40 µl/mouse of a suspension of 100 µg of OVA dissolved in 40 µl of sterile PBS was administered intranasally (i.n.) alternating the administration per nostril only in group 1, while group 2 received i.n. 40 µl/mouse of PBS alternating the administration per nostril.

On Days 21, 22, 23, 24, 25, 26 and 27 both of groups were anaesthetized with isoflurane and 40 µl/mouse of a suspension of 100 µg of OVA dissolved in 40 µl of sterile PBS was administered i.n. alternating the administration per nostril in Group 1. Group 2 received i.n. administration of 40 µl/mouse of sterile PBS alternating the administration per nostril. Mice were humanely culled on Day 28 (10 weeks old) using CO_2 and blood, lungs and thoracic aorta were collected.

Group 1 indicated as OVA/ALUM+OVA represented the model of respiratory allergy while the Group 2 indicated as PBS/ALUM+PBS represented our control (Figure 12).

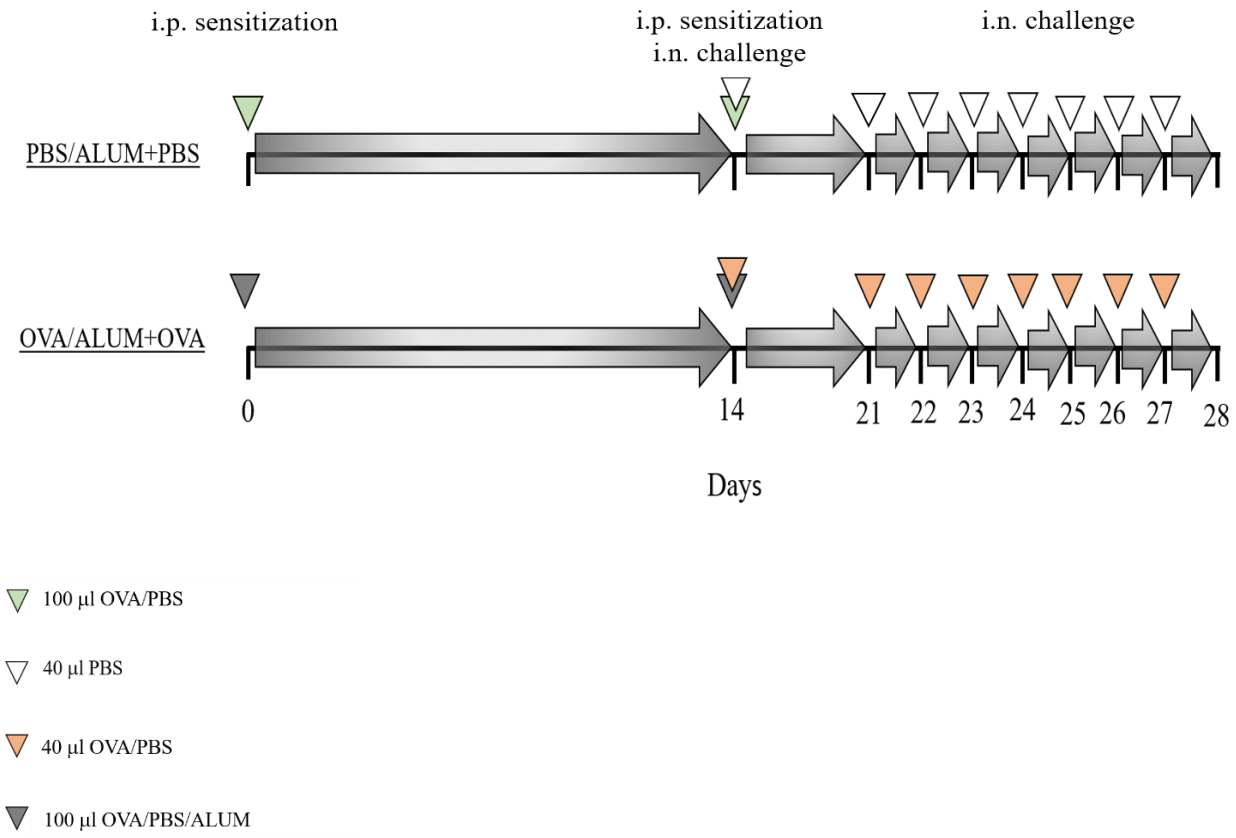

Figure 12 Modified allergic sensitization and challenge scheme

13.4 Lung histology preparation

The left lung lobe was carefully and rapidly harvested and put in plastic bijoux with 5 ml of 10% neutral buffered formalin (Sigma-Aldrich) for at least 24 h at room temperature before the evaluation of morphology with haematoxylin and eosin (H&E) staining. The lobe was then placed in the appropriate embedding cassette, processed and embedded in paraffin blocks. Successively, the tissues were cut in sections (5 μm thick) by using a microtome, the "flakes" were placed in a water bath at about 40-45ºC and then mounted onto slides and air dried for 30 minutes and then baked in the 45-50ºC oven overnight. Then the sections were deparaffinized with xylene (Sigma-Aldrich).

13.5 H&E staining

Lung sections (3 for each slide) were stained in haematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) solutions and then dehydrated in increasing concentration of ethanol (ThermoFisher Scientific, Waltham, MA USA).

At the end of the process, the slides were mounted, and the images were taken at 10x or 20x magnification in brightfield by using EVOS M7000 (ThermoFisher Scientific).

13.6 Total IgE assay

The blood was collected directly from the heart into not heparinised vials and kept in ice until clotting formation. The vials were centrifuged at 15000g for 15 min 4°C to obtain serum which was transferred in clean vials and stored at -80°C until analysis. Total IgE content was measured in serum as described in the manufacturer's protocol of Mouse IgE ELISA Kit (BD Biosciences, New Jersey, US).

13.7 Isolated aorta rings preparation

The thoracic aortas were isolated and rapidly placed in cold Physiological Saline Solution (PSS) with the formulation (NaCl 58.45 mM, KCl 74,557 mM, $CaCl_2$ 110.99 mM, $MgSO_4$ $7H_2O$ 246.498 mM, KH_2PO4 136.09 mM, $NaHCO_3$ 84.01 mM, EDTA 380 Mm and D-glucose 180.16 mM, pH adjusted to 7.4 at 37°C). The connective tissue and fat were removed, and the aorta was very carefully cut in 2 mm long rings. Rings were suspended between 2 mounting posts of a DMT 610M 4 channel myograph system (DMT-USA International, Atlanta, GA) between an isometric force transducer and length positioning support post and bathed in PSS warmed to 37°C and gassed continuously with a gas mixture of 95%O_2 + 5%CO_2. The rings were allowed to equilibrate for 30 min before to impose them a resting tension of 10 mN. After equilibration in PSS at resting tension for 30 min the pharmacological assessment started. Changes in tension were continuously recorded with a computerized system (LabChart, ADInstruments, Sydney AU). Following equilibration, the rings were stimulated with 62.5 mM K^+PSS solution until the maximum rings response (plateau) to test viability. The rings which did not reach a steady-state stress generation greater than 1 mN were not used for subsequent analysis. The K^+PSS response was used to normalize contractile data.

13.8 Vasoconstrictor responses

After stimulation with K^+PSS, the rings were rinsed with 37°C PSS for three times and rested for 30/45 min at basal tension before examining the contractility response. Cumulative dose-response curves for the α-adrenergic agonist phenylephrine (Phe,

0.001–30 μM) were performed, and the tissues were washed until the force returned to passive tension level. Data expressed as mean ± SEM are reported as % of contraction with respect to K^+PSS-induced tone.

13.9 Vasodilator responses

To assess the relaxation response 1 μM PE pre-constricted tissues were challenged with acetylcholine (Ach 0.01–30 μM) and sodium nitroprusside (SNP) (0.01 nM–30 μM) Data, expressed as mean ± SEM, are reported as % of relaxation with respect to Phe-induced tone.

13.10 Real-time PCR

Aortas were dissected and clean from the perivascular fat and placed in 1.5 mL RNA-free vials with 500 μL of RNAlater™ Stabilization Solution (ThermoFisher Scientific) and kept at 4°C for 24h. After 24h the solution was discarded, and the tissues were stored at -80°C until analysis. Frozen aortas were allowed to defrost and put in 2 mL vials with medium size metallic beads and 500 μL of TRIzol. Then the tissues were homogenate by using a homogenizer and after checking the tissue was destroyed the vials were left 5 min at room temperature (RT) on the benchtop and then was added 100 μl of chloroform. The vials were vortexed 15 sec, left 2-3 min at RT on the benchtop and centrifuged at 12000g 15 min 4°C. 300 μl of the aqueous phase, which contains the RNA, were transferred into the new tube**.** Then the same amount of 100% alcohol was added and mixed well. After this, the total RNA was extracted using the Direct-zol™ RNA Miniprep Plus kit (Zymo Research, Irvine, CA). RNA purity and quantity were measured by Nano-Drop spectrophotometry (ThermoFisher Scientific).

Samples required 260/280 ratios > 1.5 for inclusion. Reverse transcription was performed using 400 ng of RNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed on the 7900HT instrument (Applied Biosystems) using commercially available TaqMan assays for Ccl4, Ccr3, and Ccr5. Other cytokines, chemokines, growth factors, and other immune response genes were analysed with 96 TaqMan® Gene Expression Assays (ThermoFisher Scientific). TATA-box binding protein (Tbp) was selected as a constitutive gene for all real-time PCR experiments due to the high stability of expression across all tissues examined.

13.11 Cytokine detection assays

Serum concentrations of Eotaxin, fibroblast growth factor-basic, granulocyte-macrophage colony-stimulating factor, IFN-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, Il-9, IL-10, IL-12 (p40), IL-12 (p70) IL-13, IL-17A, KC, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) and tumour necrosis factor-α were assessed using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay according to the manufacturer's instructions (Biorad) and analysed using a Bio-Rad Luminex 100 Plate Reader (Hemel Hempstead, United Kingdom).

13.12 Western Blotting

Four aorta samples were analysed from PBS/ALUM+PBS and OVA/ALUM+OVA, proteins were isolated from tissues lysed in Pierce RIPA buffer (ThermoFisher

Scientific) and suspended in buffer containing EDTA (20 mM), NaCl (140 mM), Tris (100 mM), 5% SDS and Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Protein concentration was measured using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Electrophoretic separation was performed using 30 µg of protein loaded on 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Inc. Hercules, CA). Proteins were then transferred to 0.45 µm nitrocellulose membrane and nonspecific binding sites were blocked by incubation with 5% Bovine serum albumin (BSA, Sigma Aldrich) in Tris-buffered saline (TBS) solution for 1 hour at room temperature (RT) and washed with TBS containing Tween20 (0.01%). Subsequently, membranes were incubated overnight (4°C) with primary antibodies: Anti-eNOS/NOS Type III (BD Biosciences 610297, 1:1000), Anti-NOX1 (Sigma Aldrich SAB2501686, 1:1000), Anti-NOX4 (Novus Biologicals NB110-58849, 1:500). After washing, membranes were incubated with corresponding IRDye 800CW/680RD secondary antibodies (LI-COR Biosciences, Lincoln, USA) for 1 hour at RT. The fluorescent signal was detected using Odyssey Fc reader (LI-COR Biosciences) and data quantified using Biorad Quantity One® software.

13.13 Statistical methods

Data are expressed as means ± SEM. The student t-test was used to compare two groups. Statistical evaluation for analysis of vascular function studies was performed by two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, and t-tests were used for the analysis of mRNA/protein expression data. All these

statistical tests were performed GraphPad Prism (version 7). A p value <0.05 was used to define statistically significant differences between mean values

14. RESULTS I

We performed a preliminary investigation to establish the basal IgE level and the normal vascular responses to a group of vasoactive compounds in the thoracic aorta isolated from naïve Balb/c mouse, to consequently design experimental protocols. In these set of experiments, we examined the vascular response to the vasoconstrictor α1-adrenergic receptor agonist, phenylephrine (Phe), which is a potent and efficacious agent. Phe (0.001–30 μM) caused vasoconstriction in a concentration-dependent manner (Figure 13). The maximal constriction response was reached at a concentration of 10 μM and the submaximal concentration of 1 μM was used as a constrictor agent before the assessment of relaxant agents as acetylcholine (Ach) and sodium nitroprusside (SNP).

We next performed Ach cumulative response curve (0.01–30 μM) on 1 μM Phe pre-constricted rings. Ach relaxes thoracic aorta rings of naïve Balb/c mice in a concentration-response fashion, indicating our experimental strain model has good responsiveness to NO trigger drug (Figure 14). Moreover, we also tested the aorta response to the NO exogenous donor SNP. SNP (0.01 nM-30 μM) efficaciously relaxes precontracted rings indicating no alteration in the endothelium-independent response (Figure 15). Finally, we measured the basal IgE serum levels of Balb/c mice in order to investigate the environment effect on IgE production in this allergic sensitive mouse strain (Figure 16).

15. FIGURES I

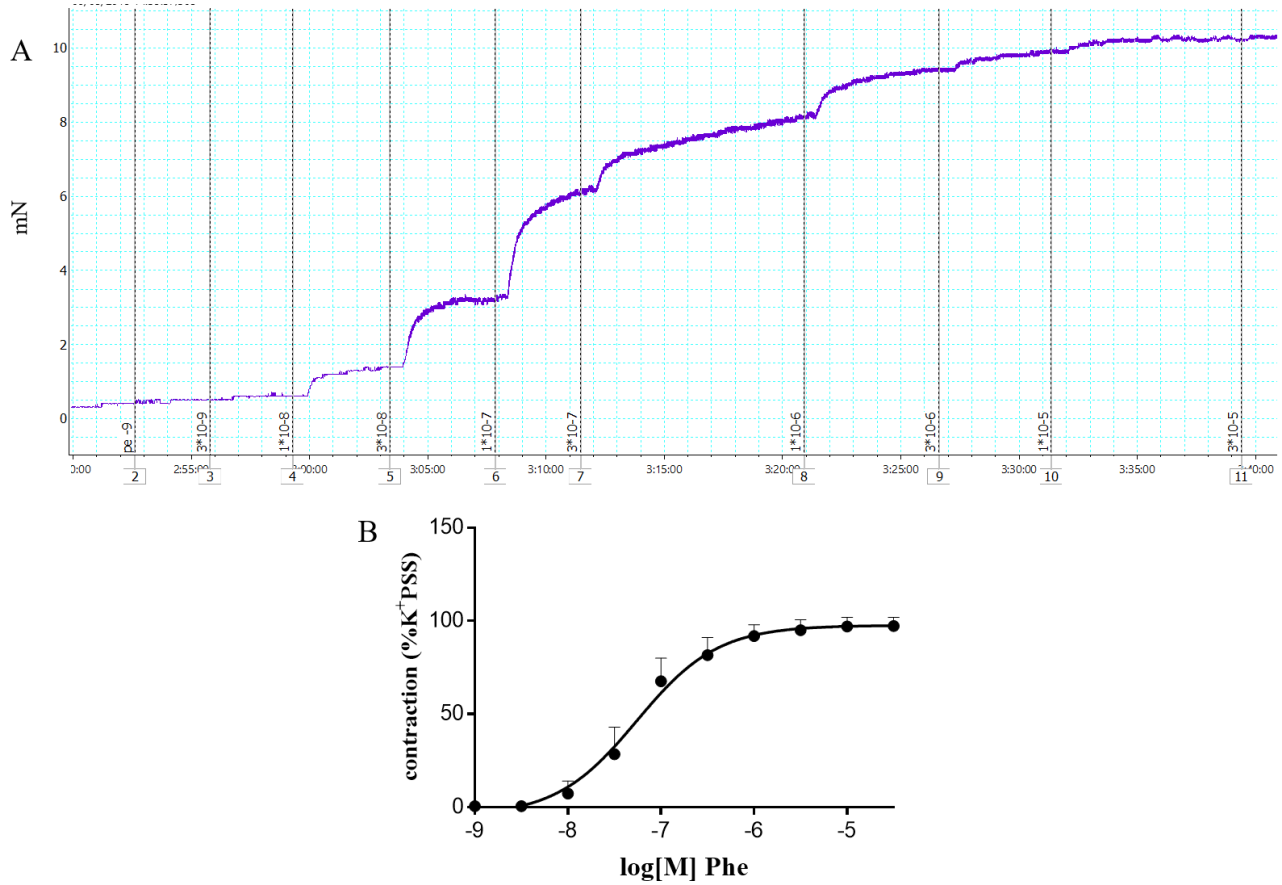

Figure 13 Vascular response**11**

(A) Representative original tracing of a dose-response curve of phenylephrine on naïve Balb/c mouse aorta ring, the X-axis represents the time while the Y-axis represents the tension (in mN). **(B)** Cumulative dose-response of thoracic aorta rings from naïve Balb/c mice to phenylephrine (Phe). Data points represent mean ± SEM, n = 6.

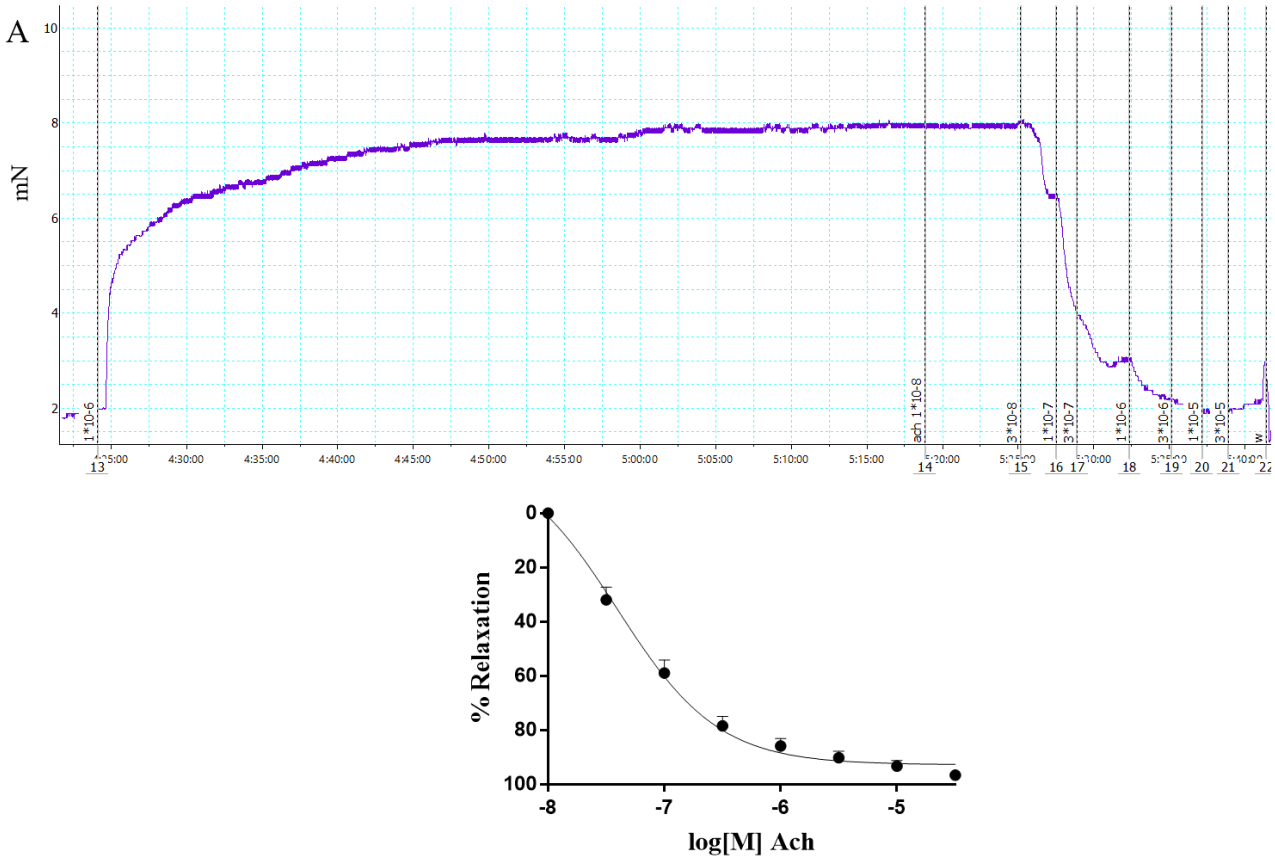

Figure 14 Vascular response

(A) Representative original tracing of a dose-response curve of acetylcholine (Ach) on phenylephrine-induced contraction naïve Balb/c mouse aorta ring, the X-axis represents the time while the Y-axis represents the tension (in mN). **(B)** Cumulative dose-response of thoracic aorta rings from naïve Balb/c mice to Ach. Data points represent mean ± SEM, n = 6.

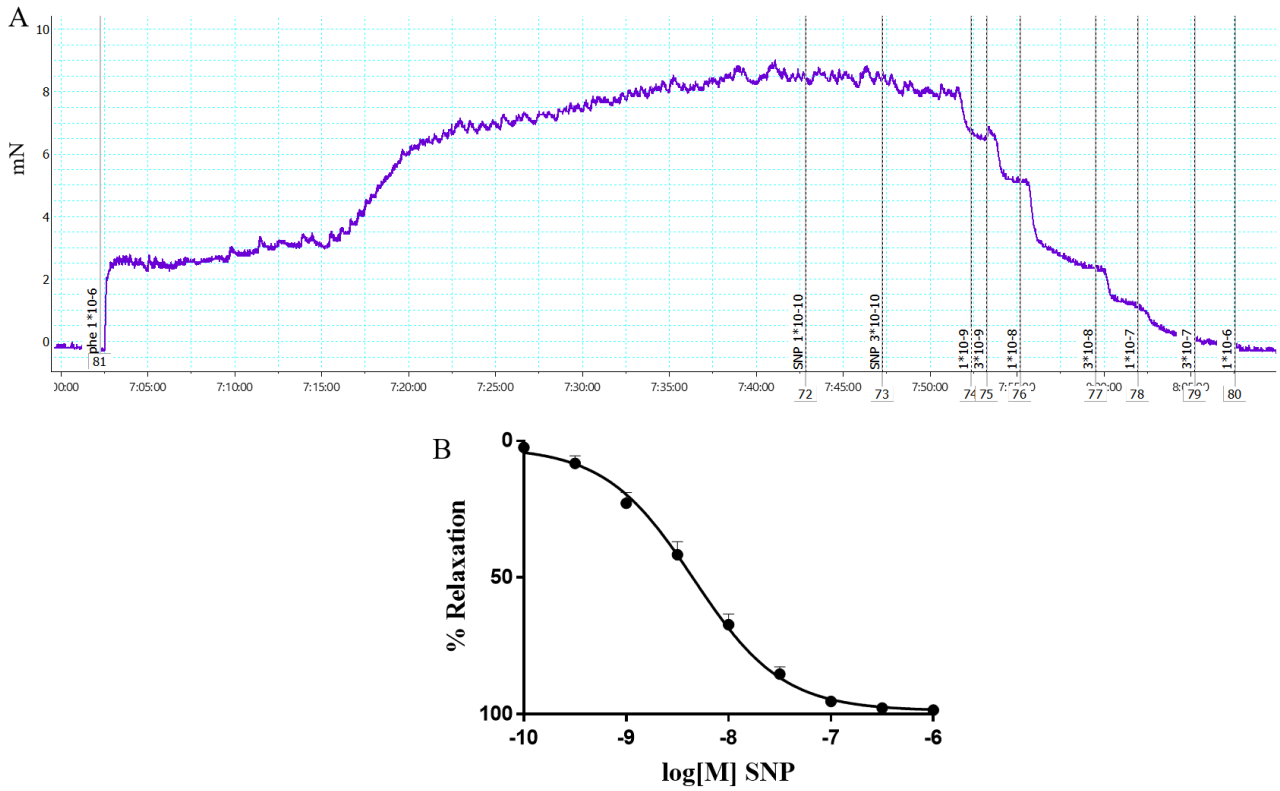

Figure 15 Vascular response

(A) Representative original tracing of a dose-response curve of sodium nitroprusside (SNP) on phenylephrine-induced contraction naïve Balb/c mouse aorta ring, the X-axis represents the time while the Y-axis represents the tension (in mN). **(B)** Cumulative dose-response of thoracic aorta rings from naïve Balb/c mice to SNP. Data points represent mean ± SEM, n = 6.

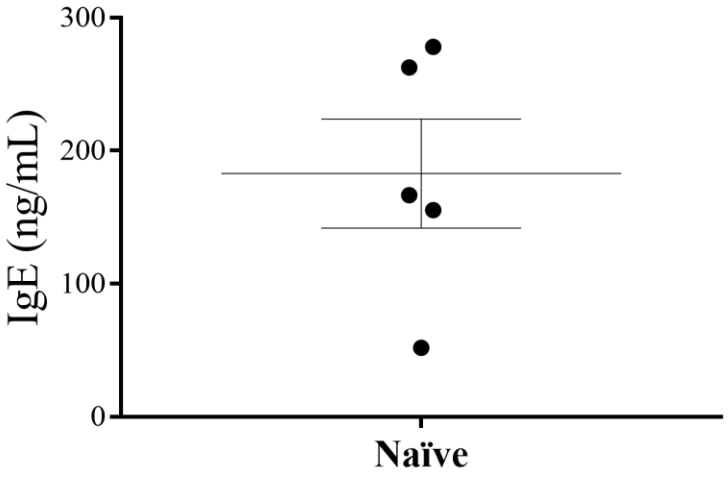

Figure 16 Serum IgE levels

Concentration is shown as nanograms (ng) of IgE per millilitre (ml) serum. Data are presented as mean ± SEM, n = 5.

16. CONCLUSION I

Circulating IgE is the most common and reliable marker used to assess the presence of an allergic response [224]. Moreover, Balb/c mice are a very sensitive strain to different air allergens. The air pollution particulate can significant increases plasmatic IgE levels [225]. Thus, these small set of experiments were undertaken to characterize the naïve Balb/c mouse serum IgE levels to understand the basal level of these immunoglobulins in our experimental model and its response to the environment where they are kept. Furthermore, we addressed the aorta response to pharmacological tools commonly used for the vascular reactivity study [226]. Balb/c aorta effectively contracts to phenylephrine, an agonist of α1-adrenergic receptors. Moreover, the aorta was well responsive to Ach, a cholinergic agonist well-known for its ability to activate NOS in endothelial cells, and to SNP, which breaks down in solution to release nitric oxide [227], resulting in an endothelium dependent and independent relaxation of tissues previously contracted with phenylephrine.

17. RESULTS II

In these preliminary experiments, we evaluated the systemic vascular effect of a respiratory allergic disease induced by ovalbumin.

17.1 IgE and lung morphology

IgE serum levels are commonly measured as proof of good sensitization. The group OVA/ALUM+OVA presented circulating IgE level significantly higher compared to the group OVA+PBS, but there was no difference with the group OVA/ALUM+PBS, suggesting that the presence of alum as an adjuvant and the systemic OVA were necessary to induce an allergic response (Figure 17). Lung morphology also demonstrates the effectiveness of sensitization (Figure 18). Indeed, the H&E clearly showed a normal morphological structure in OVA+PBS and OVA/ALUM+PBS groups, but morphological changes which include; alveolar septal thickening and inflammatory cell infiltration, were observed in the lung of the OVA/ALUM+OVA group (Figure 18) indicating the importance of allergen nasal installation to reproduce a model of pulmonary allergic disease.

17.2 Vascular response to vasoconstrictor and vasorelaxant substances

The aorta rings from OVA+PBS, OVA/ALUM+PBS and OVA/ALUM+OVA displayed a dose-dependent contractile response to phenylephrine (Phe, 0.001–30 μM). However, there was no difference between all the three groups (Figure 19A).

Aortic rings from all the groups were pre-contracted with a submaximal dose of 1 μM Phe (Figure 19B) and then a concentration-response curve of acetylcholine (Ach, 0.01–30 μM) (Figure 19C) was performed to evaluate endothelium-dependent relaxation

response. Subsequently, 1 μM Phe precontracted rings were exposed to SNP (0.01 nM–30 μM) (Figure 19D) to assess the endothelium-independent relaxation response. All the three groups did not show any difference in vasorelaxation induced neither by Ach nor SNP.

18. FIGURES II

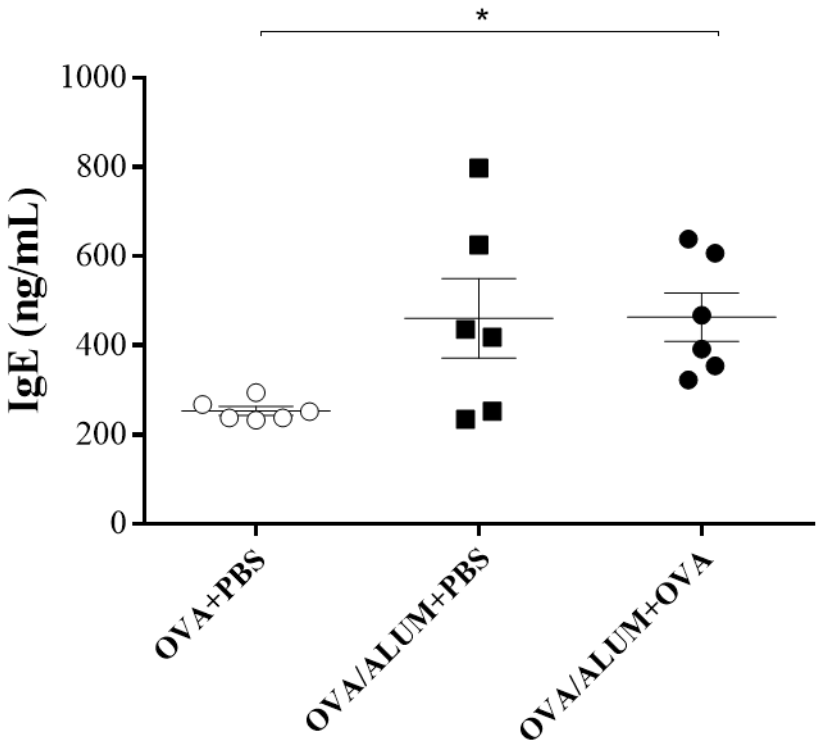

Figure 17 Serum IgE levels

Concentration is shown as nanograms (ng) of IgE per millilitre (ml) serum. Data are presented as mean ± SEM, n = 6, * $p < 0.05$.

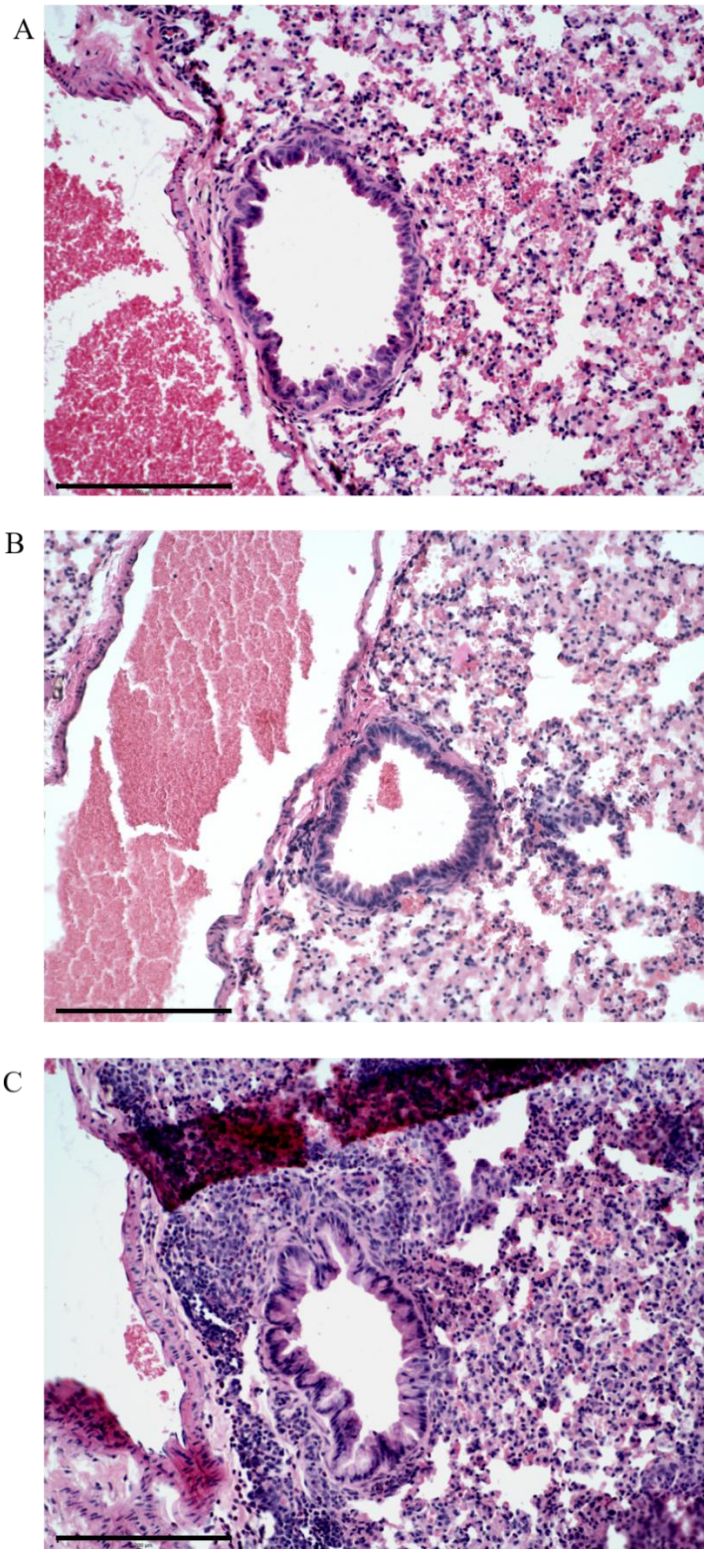

Figure 18 Lung morphology

(A) Lung morphology of the OVA+PBS group, **(B)** Lung morphology of the OVA/ALUM+PBS group, **(C)** Lung morphology of the OVA/+ALUM group, which presents peribronchiolar inflammation and inflammatory cell infiltration (scale bar = 200 μm).

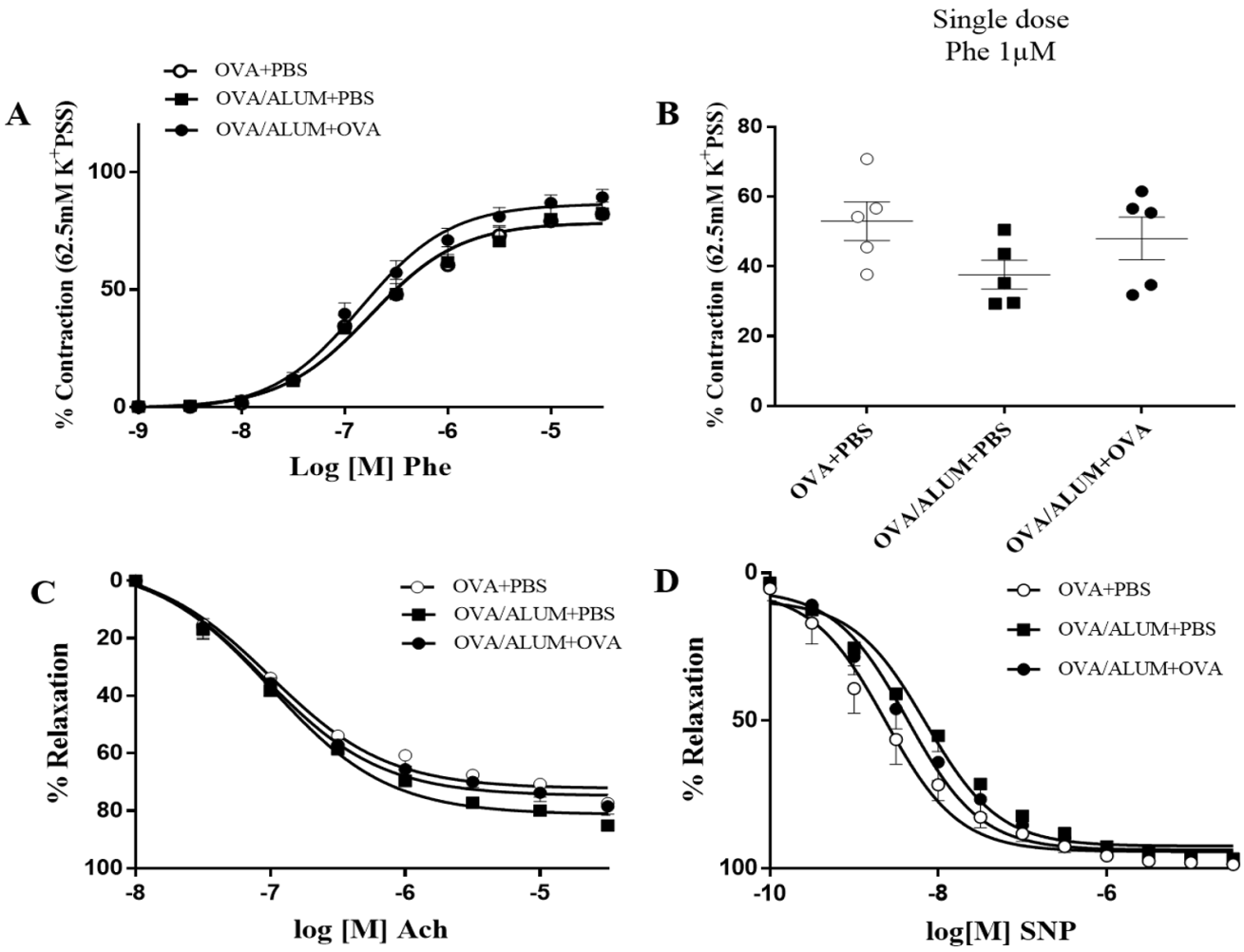

Figure 19 Vascular response

(A) Cumulative dose-response of thoracic aorta rings from all three groups mice to phenylephrine (Phe). **(B)** Percentage of contraction of 1 µM Phe response from all three groups. **(C, D)** Cumulative dose-response of thoracic aorta rings from all three groups mice to acetylcholine (Ach) and sodium nitroprusside (SNP) Data points represent mean ± SEM, n = 6.

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19. CONCLUSION II

IgE levels are the first hallmark considered for the evaluation of allergic response. It is well known the exposure to several allergens dramatically increases IgE levels in subjects affected by respiratory diseases compared to healthy subjects [228]. Conversely, in this pilot study, we showed no difference in serum IgE between mice belong to OVA/ALUM+PBS (our control group) and OVA/ALUM+OVA mice. Thus, the control group did not perfectly reflect what we aimed because the systemic sensitisation, with the allergen and the adjuvant, triggers the IgE production in the same fashion of the OVA/ALUM+OVA group. While was expected the observed difference in lung morphology between OVA/ALUM+OVA and control group.

We aimed to assess if the model of allergic disease induced by ovalbumin could represent a reliable experimental model also for the study of comorbidity between allergic respiratory disease and aorta endothelial dysfunction. However, in these set of experiments, we displayed no difference in the aorta response neither to constrictor agent such as Phe nor to vasorelaxant agents as Ach and SNP in all three groups. Thus, was not possible to appreciate if the ovalbumin sensitization and challenge have a direct effect on the contractile or relaxant capacity of the thoracic aorta.

In conclusion, this experimental model used for these set of experiments did not represent a reliable model for further study in the evaluation of systemic endothelium dysfunction in an allergic respiratory disease context.

20. RESULTS III

The previous results showed the impossibility to discriminate the vascular effect of allergy between allergic and control group. Therefore, in the following set of experiments, the control group was sensitized with alum but without allergen ovalbumin. This closely reflects a health condition of low circulating allergic mediators. Moreover, studies suggested a relationship between airborne pollen concentrations and daily cardiovascular and respiratory-disease mortality [229]. Thus, in the second set of experiments, we modified the ovalbumin concentration in the sensitization and challenge route, while we used no allergen in the control group.

20.1 Serum IgE

Serum IgE levels were dramatically increased in OVA/ALUM+OVA compared to PBS/ALUM+PBS (control) group indicating the effectiveness of sensitization and challenge (Figure 20).

20.2 Lung histopathology

To further assess the cells infiltration in pulmonary tissue and the degree of inflammation, left lung morphology was screened by H&E to evaluate the extent and severity of inflammation. Micrographs of lung demonstrated a greater influx inflammatory cells in the sub-epithelium of airways, in addition, it is clear the airway epithelial hyperplasia in mice treated with OVA/ALUM+OVA compared to control animals (Figure 21).

20.3 Allergic response induced by OVA impairs aorta vasorelaxation

Aortic rings from both groups showed a dose-dependent contractile response to phenylephrine (Phe). However, there was no difference in the contractile response to Phe between the OVA/ALUM+OVA and PBS/ALUM+PBS group (Figure 22A). In addition, the responses of the aortic rings to 1 μM PhE were not significantly different for each other (Figure 22B). These results indicate that ovalbumin systemic sensitization and the local nasal challenge does not affect the adrenergic contractile ability of the thoracic aorta. Aortic rings from both the groups were sub-maximally pre-contracted with 1 μM PhE and then a concentration-response curve of Ach was performed to evaluate the relaxation response. Ovalbumin sensitization leads to an impaired endothelial-dependent Ach-induced relaxation response (Figure 22C) while the presence of SNP does not affect the aortic rings indicating that the reduction in the vasorelaxant effect is only related to an impairment of endothelium functionality (Figure 22D).

20.4 Allergic response induced by OVA affect mRNA immune/inflammatory gene expression in aortic tissue

Few previous papers reported an impairment in endothelium relaxation in a mouse model of allergy [214,230]. However, the mechanism behind the aorta reduction vasorelaxant response in an allergic scenario is still unknown. By using real-time polymerase chain reaction (PCR), we sought to understand which aortic gene expression could be affected after systemic and nasally allergen administration. The mRNA expression of chemokine (C-C motif) ligand 4 (CCL4) and 5 (CCL5) tended to

be decreased without reaching statistical significance. The reduction was significative for CCL2 in the aorta of OVA/ALUM+OVA group compared to the PBS/ALUM+PBS group. The chemokine receptor 3 (CCR3) mRNA expression, where CCR3 is the main Eotaxin target [231], was significantly increased in OVA/ALUM+OVA group compared to the control group. While the mRNA expression of CCR5, which is the CCL5 target [232(p5)], was significantly reduced in OVA/ALUM+OVA group compared to the control group (Figure 23). Thus, chemokines ligands and receptors alteration could be involved in endothelium hypo-reactivity during the allergic response. Moreover, the RT-PCR also showed the over-expression of several pro-inflammatory genes; *Ptgs2*, which encodes for the main enzyme involved in cyclooxygenase 2 (COX_2) signalling, where COX_2 is actively involved in endothelium dysfunction [233(p2)]; *Smad7*, a negative regulator for the signalling of Transforming growth factor-beta (TGF-β) family members [234], *Gusb, Fibronectin-1 (Fn1)*, which is involved in cardiac remodelling [235] and *Socs2* (p=0.068), a pro-inflammatory gene which activation occurs when cytokines are released [208] (Figure 24A, B).

20.5 Allergic response affects eNOS, NOX1 and NOX4 protein expression in the aortic tissue

Ach relaxes vascular smooth muscle cells via NO, and impairment in relaxation could be the result of an alteration in eNOS signalling. Using western blotting analysis, we, therefore, evaluated the eNOS protein expression in aortic tissue. eNOS expression is significantly increased in OVA/ALUM+OVA mice compared to the control group (Figure 25). Moreover, reactive oxygen species (ROS) control NO bioavailability

through eNOS activity [236]. On this basis, we also evaluated the expression of NADPH oxidase 1 (NOX1) and NADPH oxidase 4 (NOX4), which are enzymes involved in the release of oxygen radicals and they are expressed in the smooth muscle of vascular system. NOX1 is considered deputy in detrimental O_2^- production, while to NOX4 is addressed the production of H_2O_2 which contributes to the vessel vasorelaxation [237,238]. The Western Blotting analysis reveals that NOX1 was significantly overexpressed (Figure 26A), while NOX4 expression was significantly reduced, in OVA/ALUM+OVA group compared to the control, indicating the possible involvement of oxidative stress in the reduction of endothelium vasorelaxation in the sensitized group (Figure 26B).

20.6 Allergic response results in a systemic increase in pro-inflammatory mediators

To determine systemic changes induced by an allergic response, we performed the Luminex analysis to quantify a wide spectrum of cytokines in the serum from OVA/ALUM+OVA and control mice (Figure 27). In the sensitized group, we observed a significant increase in mediators which play a pivotal role in allergic response as IL-4, IL-5, Eotaxin and IL-6, IL-17A. Moreover, also granulocyte-colony stimulating factor (G-CSF), KC (chemokine [C-X-C motif] ligand [CXCL] 1), monocyte chemoattractant protein 1 (MCP1) referred as CCL2, macrophage inflammatory protein 1-alpha (MIP-1-alpha) referred as CCL3 and macrophage inflammatory protein 1-alpha (MIP-1-beta) indicate as CCL4 were significantly increased (Figures 27). While concentrations of granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-

1α, IL-1β, IL-2, IL-3, IL-9, IL-6, IL-12, IL-13, RANTES, and TNF-α did not change significantly between groups (Figures 28).

21. FIGURES III

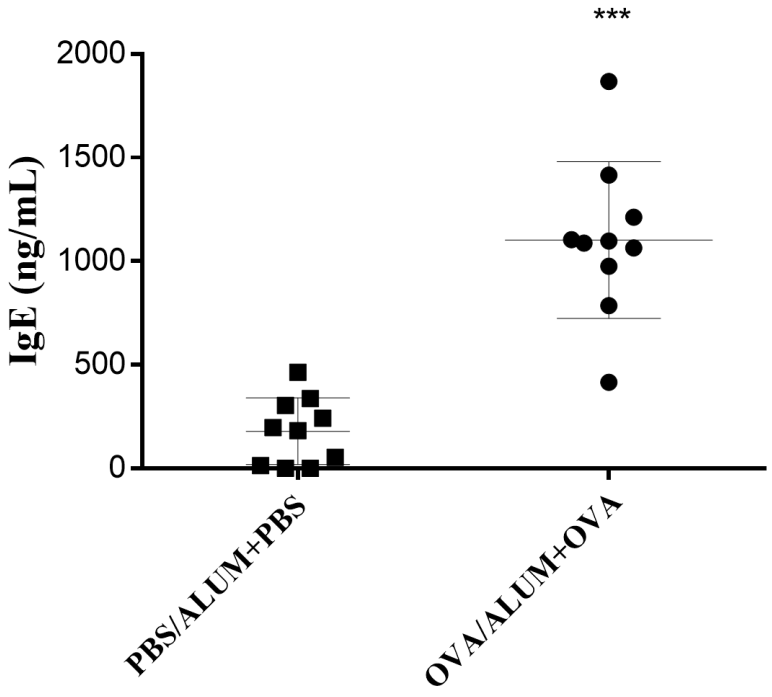

Figure 20 Serum IgE levels

Concentration is shown as nanograms (ng) of IgE per millilitre (ml) serum. Data are presented as mean ± SEM, n = 10, *** $p < 0.001$.

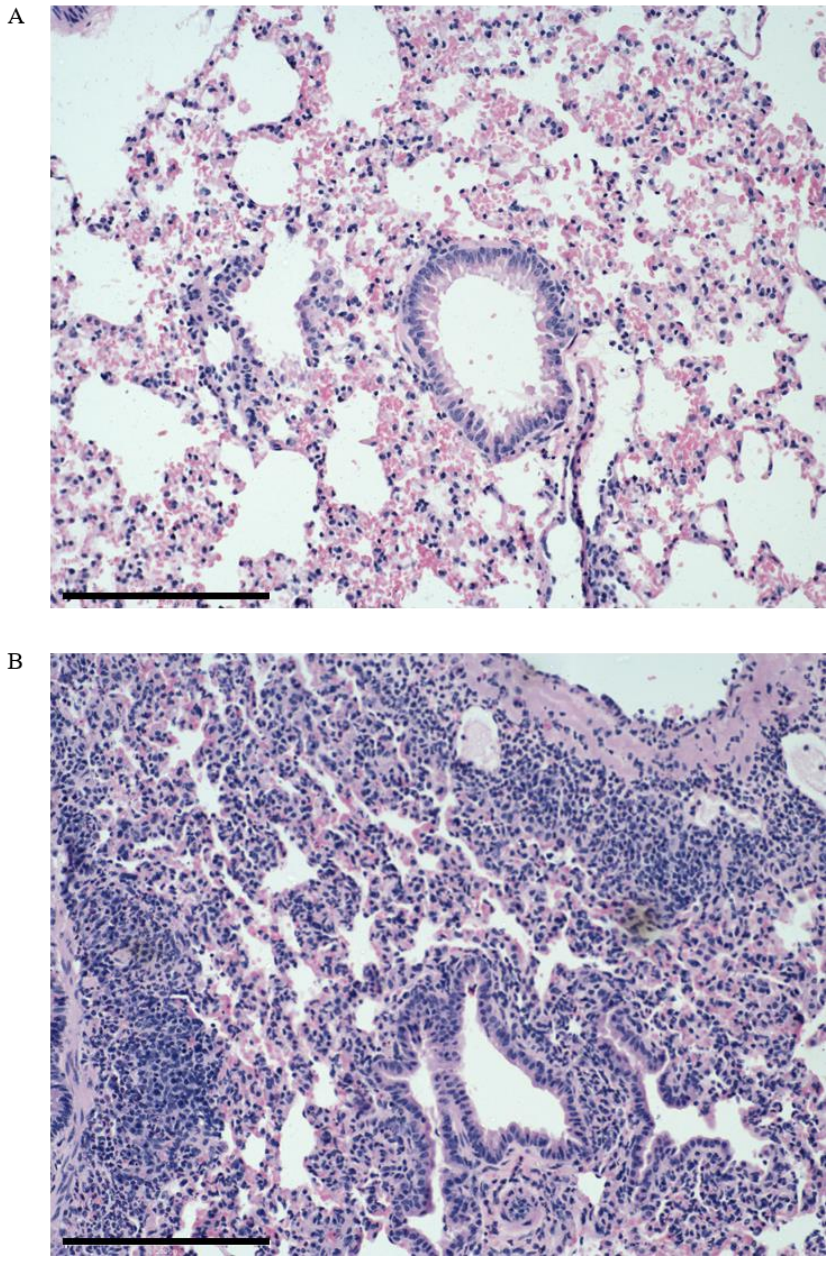

Figure 21 Lung morphology

(A) Lung morphology of the PBS/ALUM+PBS group. **(B)** Lung morphology of the OVA/ALUM+OVA group, which presents marked inflammatory infiltrates (bar = 200 μm).

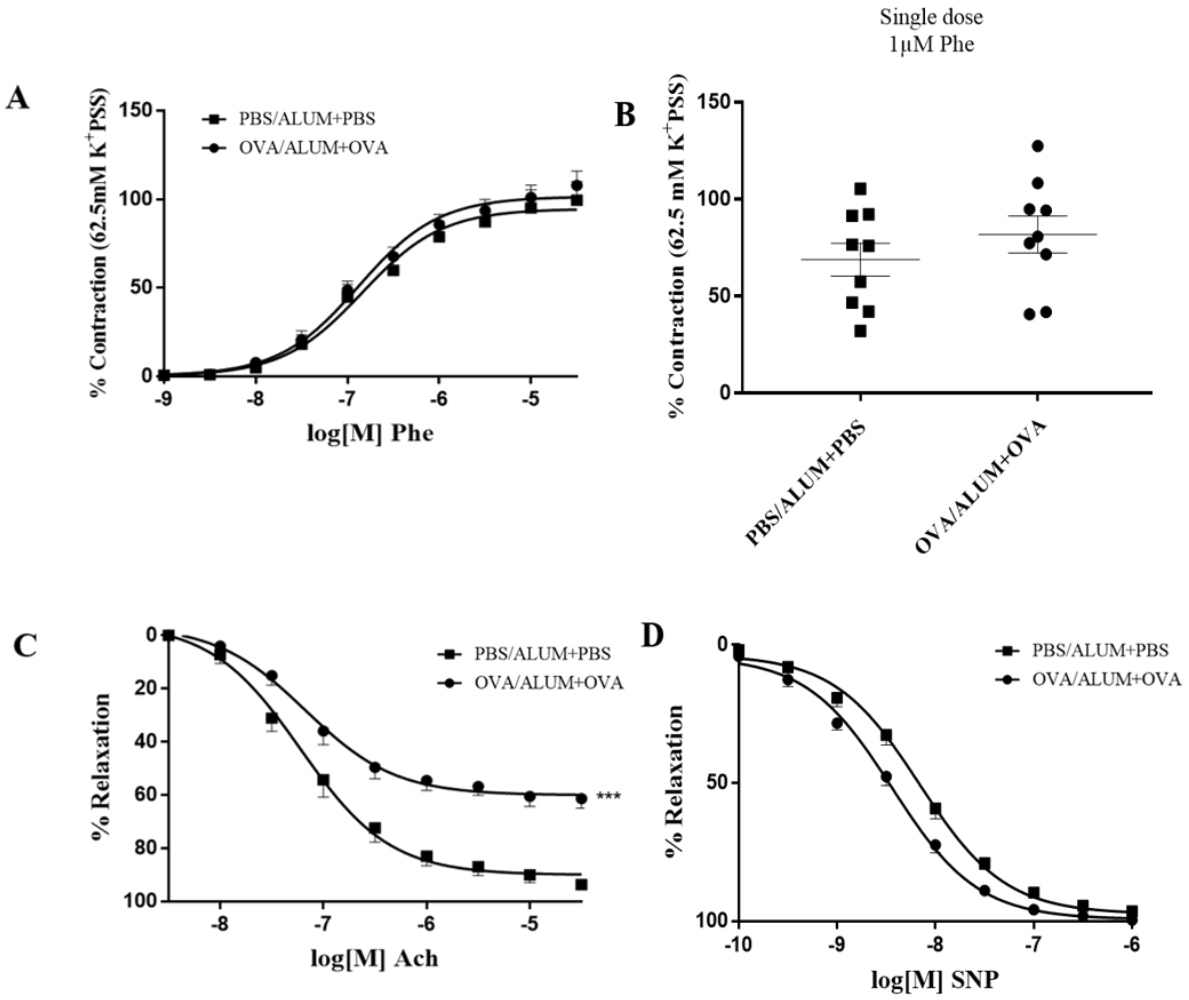

Figure 22 Vascular reactivity

(A) Cumulative dose-response of thoracic aorta to phenylephrine (Phe) **(B)** Percentage of contraction of 1 μM Phe response **(C)** Cumulative dose-response of thoracic aorta rings to acetylcholine (Ach). The relaxation induced by Ach is significantly reduced in OVA/ALUM+OVA group. ***$p < 0.001$, significantly different from PBS/ALUM+PBS (control) group. **(D)** Cumulative dose-response of thoracic aorta rings to sodium nitroprusside (SNP). Two-way ANOVA with Bonferroni's post hoc test and data points represent mean ± SEM, n = 10.

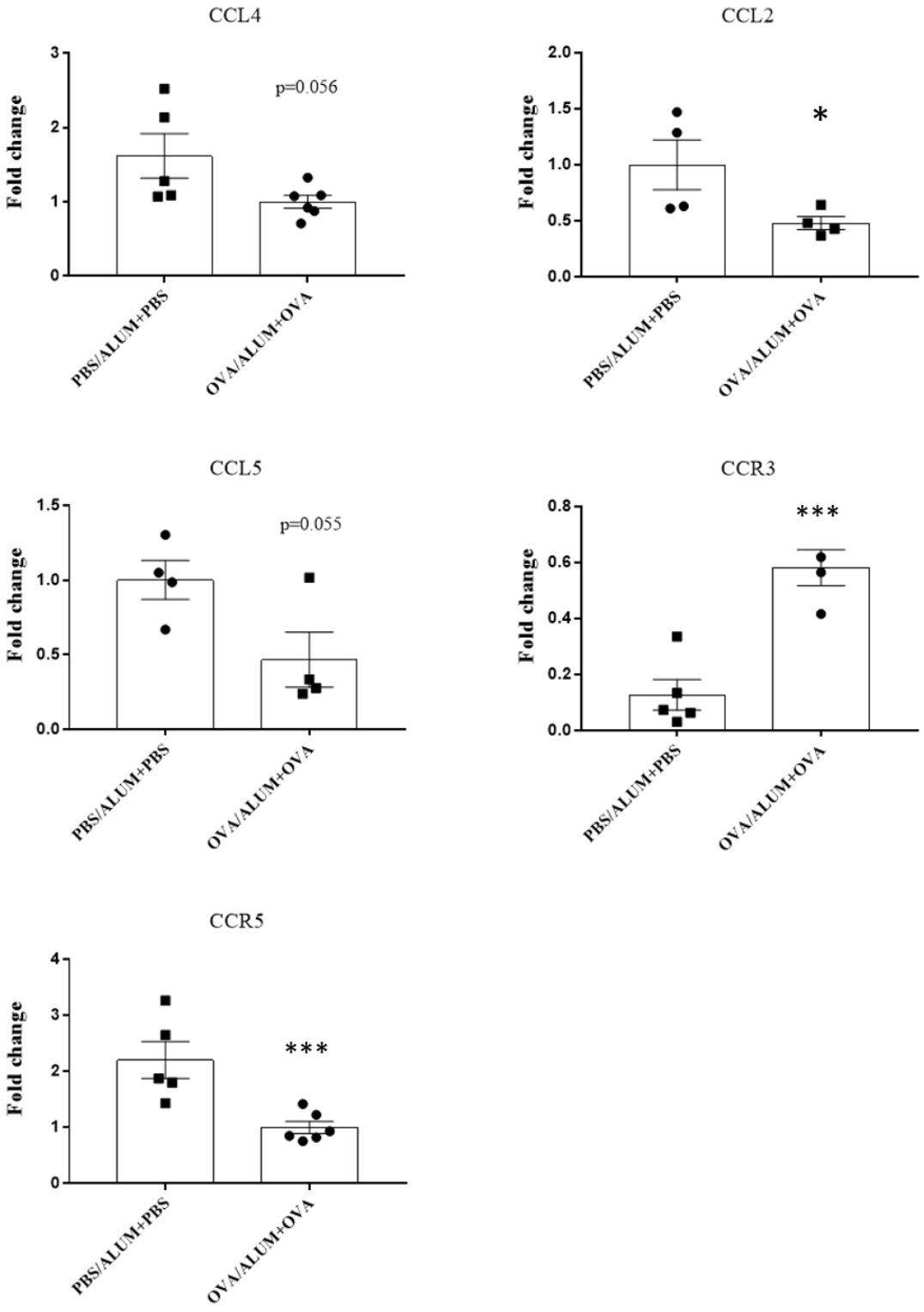

Figure 23 Aortic gene expression

Expression of aortic genes quantified using real-time polymerase chain reaction in PBS/ALUM+PBS and OVA/ALUM+OVA. Student unpaired *t*-test and data points represent mean ± SEM, n = 4-6, *p < 0.05, **p < 0.01, *** p < 0.001.

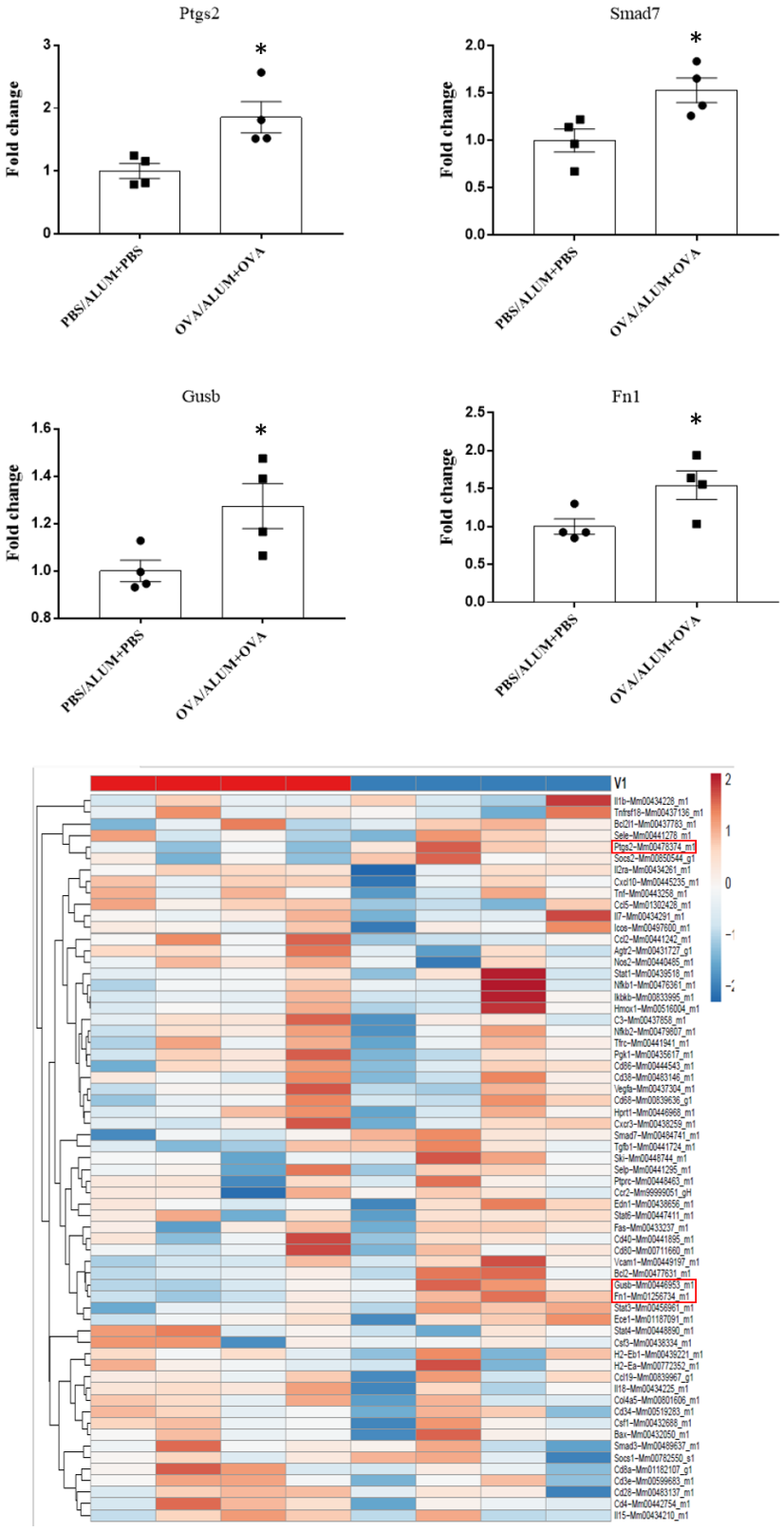

Figure 24 Aortic gene expression

(A) Expression of aortic significant pro-inflammatory genes quantified using real-time polymerase chain reaction in PBS/ALUM+PBS and OVA/ALUM+OVA. Student unpaired *t*-test and data points represent mean ± SEM, n = 4, *p < 0.05. **(B)** Heatmap of 96 genes up or downregulated in PBS/ALUM+PBS and OVA/ALUM+OVA.

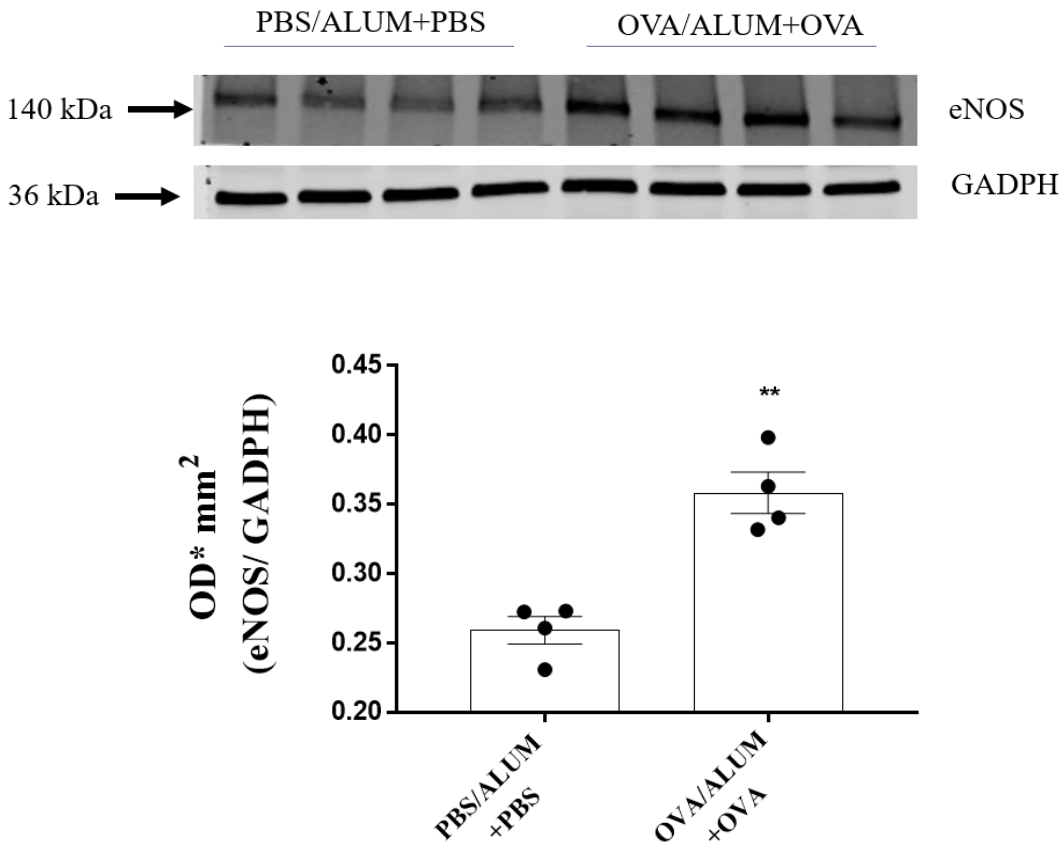

Figure 25 Aortic protein expression

Protein expression of eNOS studied by Western blotting in the aorta of PBS/ALUM+PBS and OVA/ALUM+OVA mice. Student unpaired *t*-test and data points represent mean ± SEM, n = 4, *p <0.05.

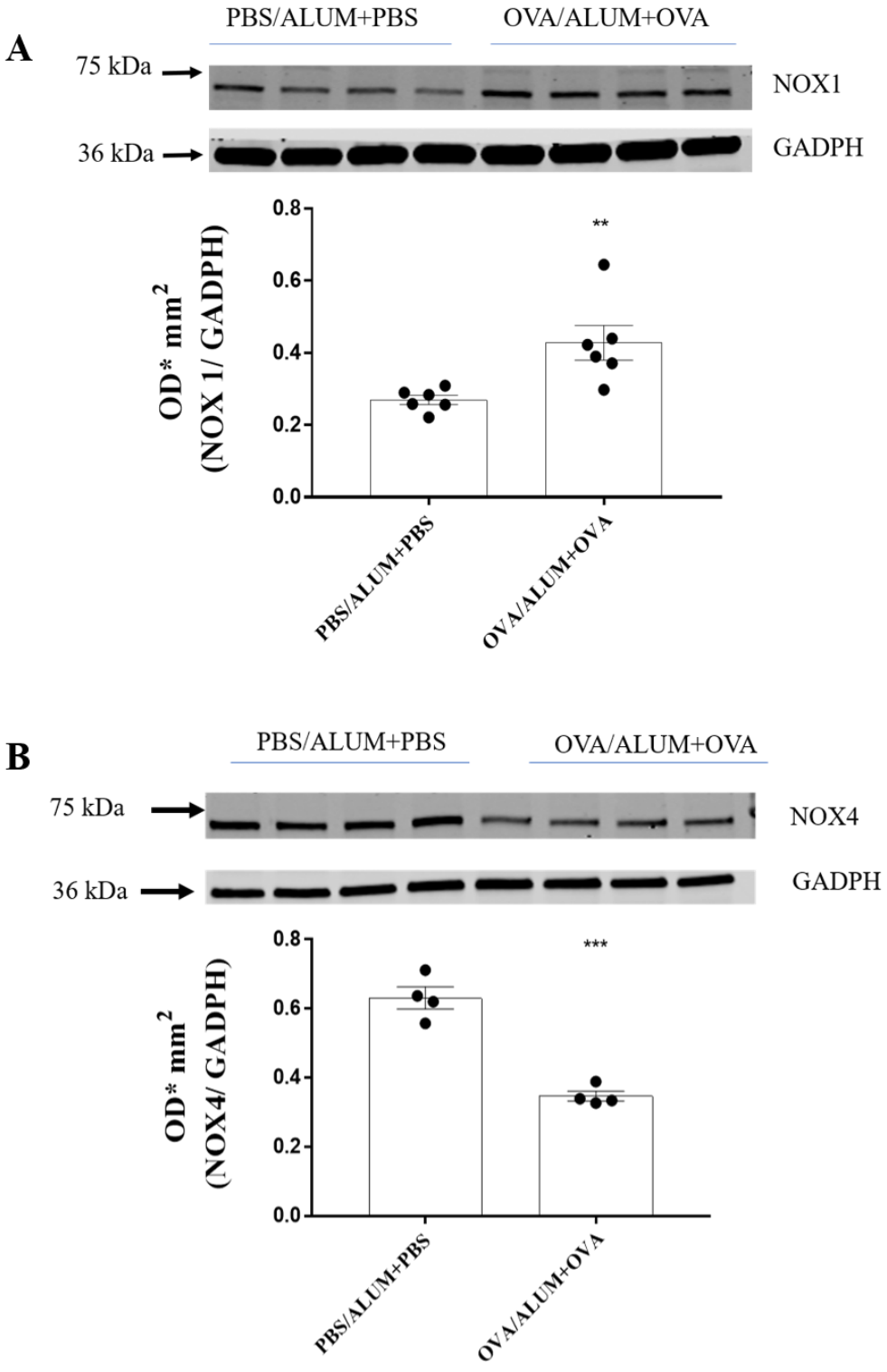

Figure 26 Aortic protein expression

Protein expression of **(A)** NOX1 and **(B)** NOX4 studied by Western blotting in the aorta of PBS/ALUM+PBS and OVA/ALUM+OVA mice. Student unpaired *t*-test and data points represent mean ± SEM, n = 4-6 **p <0.01.

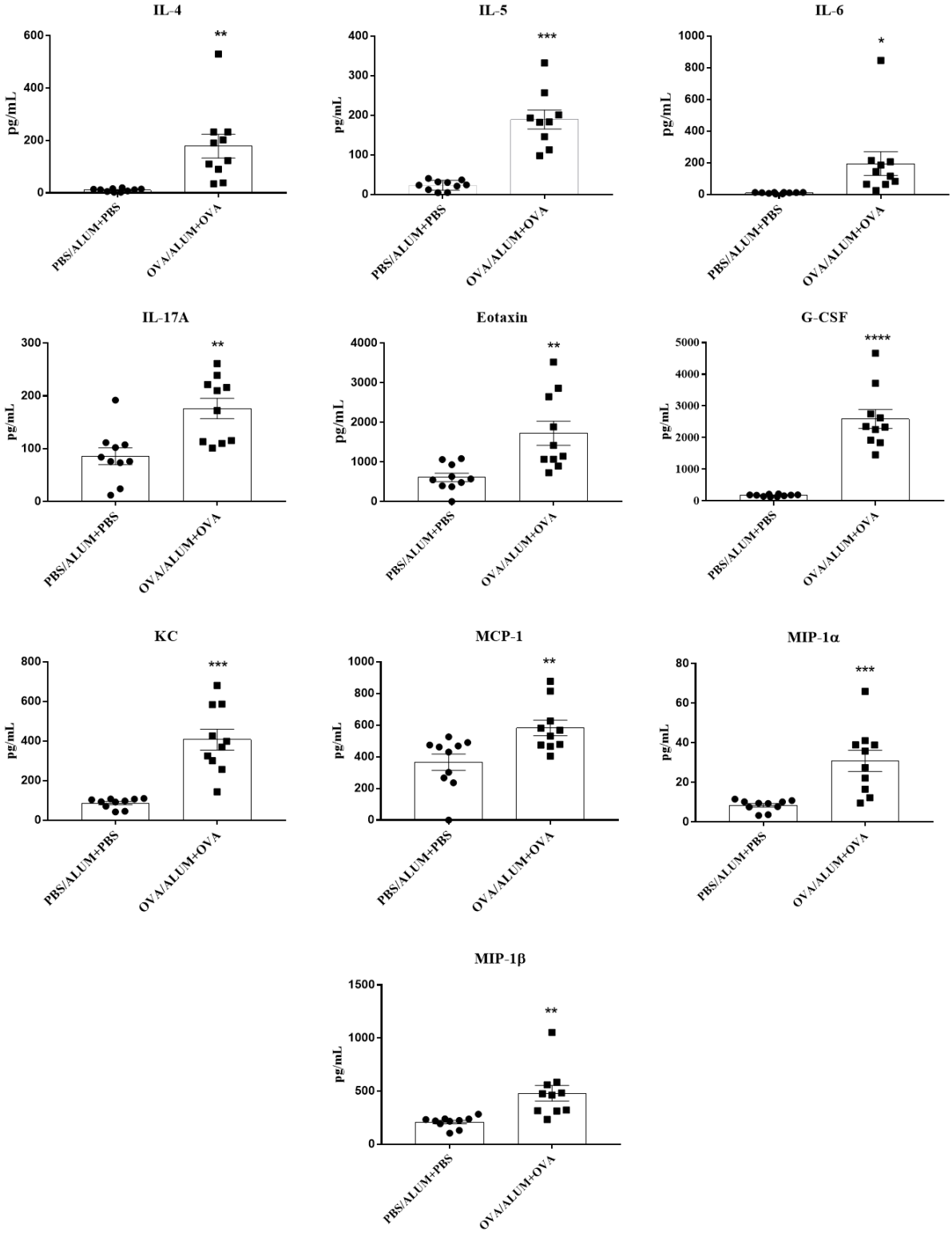

Figure 27 Serum cytokines

Serum from PBS/ALUM+PBS and OVA/ALUM+OVA mice was examined by Luminex assay. Student unpaired *t*-test and data points represent mean ± SEM, n = 10, *p <0.05, **p <0.01, *** p <0.001.

Cytokines (pg/ml)	PBS/ALUM+PBS	OVA/ALUM+OVA
IL-1α	17.02 ± 0.9648	14.86 ± 0.6686
IL-1β	10.03 ± 2.383	11.29 ± 0.7827
IL-2	12.8 ± 2.688	19.21 ± 1.625
IL-3	6.307 ± 0.6608	7.401 ± 0.4737
IL-9	120.9 ± 13.52	113.7 ± 8.708
IL-10	148.5 ± 25.99	166.1 ± 18.39
IL-12 (p70)	423.8 ± 40.89	485.9 ± 52.26
IL-13	213 ± 40.85	203.8 ± 17.56
GM-CSF	91.35 ± 14.39	100.1 ± 5.403
IFNγ	96.2 ± 13.76	95 ± 5.906
RANTES	66.59 ± 3.665	63.42 ± 4.67
TNFα	252 ± 37.07	308 ± 21.33

Figure 28 Non-significative serum cytokines

Serum from PBS/ALUM+PBS and OVA/ALUM+OVA mice was examined by Luminex assay. data points represent mean ± SEM, n = 10 per group.

22. DISCUSSION

CVD and allergic respiratory diseases are a heterogenic group of disorders which shared several features including high incidence, high socio-economic impact, chronic inflammation and uncontrolled immune response [169].

It is well established that chronic inflammation leads to CVD development [170]. However, the correlation between CVD and allergic respiratory diseases is still debatable. Endothelium dysfunction is considered the early sign of CVD development [5], nevertheless, its evaluation in allergic respiratory diseases has not been fully elucidated.

In this study, a murine model of pulmonary allergic response ovalbumin mediated was showed to induce a strong systemic inflammation. Indeed, serum Luminex assay revealed a significant increase in a plethora of pro-inflammatory mediators, supporting clinical evidence regarding the correlation between systemic inflammation and lower and upper airways allergic and not-allergic diseases [182,239]. Interestingly, we observed an altered thoracic aorta relaxation response to Ach, indicating a perturbation in eNOS/NO signalling in allergic mice. This finding corroborates a previous study performed on ragweed sensitized and challenged mice [214]. It is classically described that impairment in endothelium functionality results in a reduction in NO bioavailability. However, this is not correlated to a reduction in eNOS expression. A growing body of evidence suggests that eNOS could have a double face in the pathophysiology of CVD. Moreover, it has been reported that eNOS overexpression correlates to endothelium dysfunction [240,241]. Here we demonstrated that allergy

induces a significant increase of eNOS expression in aorta. However, further investigation is required to address its exact role. NOX1 and NOX4 are expressed in vascular smooth muscle cells and they are the predominant sources of ROS in the vasculature. However, NOX1 mainly produces ROS in the vessel while the role of NOX4 is still to clarify [242]. Indeed, evidence reported a correlation between NOX4 overexpression and CVD [243], while other authors suggested a NOX4 protective role [244]. Here we showed that in the aorta of allergic mice there was a significant increase of NOX1 expression and a significant decrease in NOX4 expression. We can hypothesize that allergic response may induce an increase in ROS production which regulates the balancing between NOX1/NOX4 activity, affecting the vascular response. However, further studies are needed to verify this hypothesis.

Vascular inflammation triggers ROS production in the vessel wall [245]. Performing PCR analysis on aortic tissue we showed an increase in several pro-inflammatory gene expression. However, it is not possible to establish if ROS can activate these genes or *vice versa.*

These findings demonstrated: (*i*) inflammation in allergic respiratory diseases causes impairment in aorta vascular reactivity, (*ii*) allergic respiratory diseases alter aorta ROS producing enzymes expression, and (*iii*) allergic respiratory diseases affect pro-inflammatory aorta gene expression. However, further studies are needed to better understand the mechanism(s) behind the link between allergic respiratory diseases and endothelium dysfunction.

23. SIGNIFICANCE AND CLINICAL PERSPECTIVES OF THE WORK

Vasodilation is a physiological event necessary for human cardiovascular wellbeing. Several biological molecules enter in the control of vasodilation. Among these, a pivotal role is played by gasotransmitters, which are small gaseous molecules able to rapidly diffuse between the vasculature wall in order to activate a wide range of secondary messengers and to exert their biological function. The major gasotransmitters involved in vascular relaxation are NO and H_2S [18,39]. Although the literature accounts for a variety of studies that tried to address the independent and the mutual mechanisms of action of these two gases [12,18,41,53,99], numerous are the gaps that represent a critical barrier in the research progress in the study of NO and H_2S in the cardiovascular system [16].

Genetic mutation of CBS, which is an H_2S-producing enzyme, leads to a severe and complex pathological scenario that strongly affect physiological vascular response [108]. L-serine is a metabolic CBS product in the H_2S release [39]. L-serine is involved in the biological production of S1P, where S1P induces vasorelaxation through NO [152].

Here we demonstrate for the first time that the L-serine/S1P/NO pathway contributes to the well-known L-cysteine/CBS/H_2S vasorelaxant effect. This finding could not only improve the scientific knowledge in the mutual physiological interplay between NO and H_2S, but it also could represent a possible pharmacological target in CBS-mutation related diseases in the future.

The vascular system runs for the entire human body. Indeed, alteration of vascular reactivity is the secondary outcome in several pathologies, such as upper and lower airways allergic inflammatory diseases. Asthma and allergic rhinitis could affect the local vascular endothelium [6,190], resulting in the migration of pro-inflammatory mediators from the site of inflammation to the system and *vice versa* [7]. Alteration in the endothelium physiology leads to the initiation of CVD [5]. However, the published clinical studies on the correlation between respiratory allergic diseases and CVD are controversial, and the literature is pauper of pre-clinical evidence on the effect of allergy on the systemic vasculature. Here we show that a condition of systemic and pulmonary inflammation induced by an allergic stimulus impairs the systemic vasculature, alters the protein expression of the major enzymes involved in the vascular functionality (eNOS, NOX1 and NOX4) and increases the expression of vascular pro-inflammatory genes. This evidence could represent the opening step for upcoming studies on the mechanisms underlying the systemic vascular dysfunction in an allergic respiratory scenario, unravelling the correlation between allergic respiratory diseases and CVD.

Schematic summary of this thesis work is reported in Figure 29.

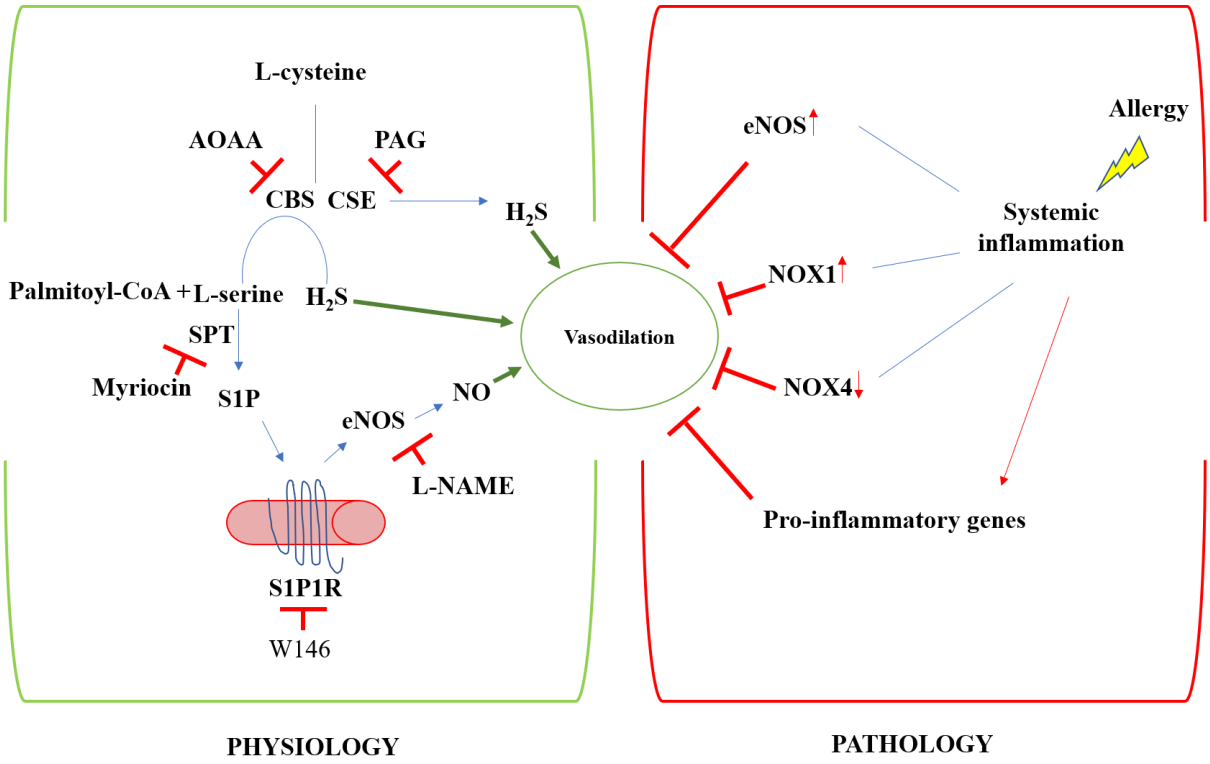

Figure 29 Vascular reactivity in physiology and pathology

Aminooxyacetic acid (AOAA), DL-propargylglycine (PAG), Cystathionine-β-synthase (CBS), cystathionine γ-lyase (CSE), Hydrogen Sulfide (H_2S), Serine palmitoyltransferase (SPT), Sphingosine 1-phosphate (S1P), Sphingosine 1-phosphate receptor 1 (S1P1R), endothelium Nitric Oxide (eNOS), N(ω)-nitro-L-arginine methyl ester (L-NAME), Nitric Oxide (NO), NADPH oxidase 1 (NOX1), NADPH oxidase 4 (NOX4).

24. REFERENCES

1. World Health Organization. *Global Status Report on Noncommunicable Diseases: 2010*. Geneva: World Health Organization; 2011.

2. Non-communicable disease. In: *Wikipedia*.; 2019. https://en.wikipedia.org/w/index.php?title=Non-communicable_disease&oldid=917265644.

3. Gheorghe A, Griffiths U, Murphy A, Legido-Quigley H, Lamptey P, Perel P. The economic burden of cardiovascular disease and hypertension in low- and middle-income countries: a systematic review. *BMC Public Health*. 2018;18. doi:10.1186/s12889-018-5806-x

4. Nurkiewicz TR, Frisbee JC, Boegehold MA. 6.08 Assessment of Vascular Reactivity. :16.

5. Widmer RJ, Lerman A. Endothelial dysfunction and cardiovascular disease. *Glob Cardiol Sci Pract*. 2014;2014(3):291-308. doi:10.5339/gcsp.2014.43

6. Green CE, Turner AM. The role of the endothelium in asthma and chronic obstructive pulmonary disease (COPD). *Respir Res*. 2017;18. doi:10.1186/s12931-017-0505-1

7. Galley HF, Webster NR. Physiology of the endothelium. *British Journal of Anaesthesia*. 2004;93(1):105-113. doi:10.1093/bja/aeh163

8. Kerekes G, Soltész P, Dér H, et al. Effects of Biologics on Vascular Function and Atherosclerosis Associated with Rheumatoid Arthritis. *Annals of the New York Academy of Sciences*. 2009;1173(1):814-821. doi:10.1111/j.1749-6632.2009.04645.x

9. Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. *Acta Cardiol*. 2000;55(4):221-232. doi:10.2143/AC.55.4.2005744

10. Mathier MA, Rose GA, Fifer MA, et al. Coronary endothelial dysfunction in patients with acute-onset idiopathic dilated cardiomyopathy. *J Am Coll Cardiol*. 1998;32(1):216-224. doi:10.1016/s0735-1097(98)00209-5

11. Bhagat K. Endothelial function and myocardial infarction. *Cardiovasc Res*. 1998;39(2):312-317. doi:10.1016/S0008-6363(98)00138-2

12. Furchgott RF. Endothelium-Derived Relaxing Factor: Discovery, Early Studies, and Identifcation as Nitric Oxide (Nobel Lecture). *Angewandte Chemie International Edition*. 1999;38(13-14):1870-1880. doi:10.1002/(SICI)1521-3773(19990712)38:13/14<1870::AID-ANIE1870>3.0.CO;2-8

13. Atochin DN, Huang PL. Endothelial nitric oxide synthase transgenic models of endothelial dysfunction. *Pflugers Arch*. 2010;460(6):965-974. doi:10.1007/s00424-010-0867-4

14. The Nobel Prize in Physiology or Medicine 1998 - NobelPrize.org. https://www.nobelprize.org/prizes/medicine/1998/summary/.

15. Otterbein LE, Foresti R, Motterlini R. Heme oxygenase-1 and carbon monoxide in the heart: The balancing act between danger signaling and pro-survival. *Circ Res*. 2016;118(12):1940-1959. doi:10.1161/CIRCRESAHA.116.306588

16. Cirino G, Vellecco V, Bucci M. Nitric oxide and hydrogen sulfide: the gasotransmitter paradigm of the vascular system. *Br J Pharmacol*. 2017;174(22):4021-4031. doi:10.1111/bph.13815

17. Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? *FASEB J*. 2002;16(13):1792-1798. doi:10.1096/fj.02-0211hyp

18. Farah C, Michel LYM, Balligand J-L. Nitric oxide signalling in cardiovascular health and disease. *Nat Rev Cardiol*. 2018;15(5):292-316. doi:10.1038/nrcardio.2017.224

19. D'Aniello G, Tolino A, Fisher G. Plasma L-arginine is markedly reduced in pregnant women affected by preeclampsia. *J Chromatogr B Biomed Sci Appl*. 2001;753(2):427-431. doi:10.1016/s0378-4347(00)00565-x

20. Tetrahydrobiopterin deficiency | Genetic and Rare Diseases Information Center (GARD) – an NCATS Program. https://rarediseases.info.nih.gov/diseases/7751/tetrahydrobiopterin-deficiency.

21. Xu S, Touyz RM. Reactive oxygen species and vascular remodelling in hypertension: Still alive. *Can J Cardiol*. 2006;22(11):947-951.

22. Takaya T, Hirata K, Yamashita T, et al. A Specific Role for eNOS-Derived Reactive Oxygen Species in Atherosclerosis Progression. *ATVB*. 2007;27(7):1632-1637. doi:10.1161/ATVBAHA.107.142182

23. Potje SR, Grando MD, Chignalia AZ, Antoniali C, Bendhack LM. Reduced caveolae density in arteries of SHR contributes to endothelial dysfunction and ROS production. *Sci Rep*. 2019;9(1):6696. doi:10.1038/s41598-019-43193-8

24. Trane AE, Pavlov D, Sharma A, et al. Deciphering the Binding of Caveolin-1 to Client Protein Endothelial Nitric-oxide Synthase (eNOS) Scaffolding Subdomain Identification, Interaction Modeling, And Biological Significance. *J Biol Chem*. 2014;289(19):13273-13283. doi:10.1074/jbc.M113.528695

25. Fridolfsson HN, Patel HH. Caveolin and caveolae in age associated cardiovascular disease. *J Geriatr Cardiol*. 2013;10(1):66-74. doi:10.3969/j.issn.1671-5411.2013.01.011

26. Bucci M, Gratton JP, Rudic RD, et al. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med*. 2000;6(12):1362-1367. doi:10.1038/82176

27. Razani B, Lisanti MP. Caveolin-deficient mice: insights into caveolar function human disease. *J Clin Invest*. 2001;108(11):1553-1561.

28. Gimbrone MA, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci*. 2000;902:230-239; discussion 239-240. doi:10.1111/j.1749-6632.2000.tb06318.x

29. Frank PG, Lee H, Park DS, Tandon NN, Scherer PE, Lisanti MP. Genetic Ablation of Caveolin-1 Confers Protection Against Atherosclerosis. *ATVB*. 2004;24(1):98-105. doi:10.1161/01.ATV.0000101182.89118.E5

30. Feron O, Dessy C, Moniotte S, Desager J-P, Balligand J-L. Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest*. 1999;103(6):897-905.

31. Bucci M, Roviezzo F, Brancaleone V, et al. Diabetic Mouse Angiopathy Is Linked to Progressive Sympathetic Receptor Deletion Coupled to an Enhanced Caveolin-1 Expression. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2004;24(4):721-726. doi:10.1161/01.ATV.0000122362.44628.09

32. Ooboshi Hiroaki, Toyoda Kazunori, Faraci Frank M., Lang Markus G., Heistad Donald D. Improvement of Relaxation in an Atherosclerotic Artery by Gene Transfer of Endothelial Nitric Oxide Synthase. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 1998;18(11):1752-1758. doi:10.1161/01.ATV.18.11.1752

33. d'Uscio Livius V., Baker Timothy A., Mantilla Carlos B., et al. Mechanism of Endothelial Dysfunction in Apolipoprotein E–Deficient Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2001;21(6):1017-1022. doi:10.1161/01.ATV.21.6.1017

34. Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, Mallat Z.Circulating microparticles from patients with myocardial infarction causeendothelial dysfunction. *Circulation*. 2001 Nov 27;104(22):2649-52.

35. Yan T-T, Li Q, Zhang X-H, et al. Homocysteine impaired endothelial function through compromised vascular endothelial growth factor/Akt/endothelial nitric

oxide synthase signalling. *Clin Exp Pharmacol Physiol*. 2010;37(11):1071-1077. doi:10.1111/j.1440-1681.2010.05438.x

36. Gerassimou C, Kotanidou A, Zhou Z, et al. Regulation of the expression of soluble guanylyl cyclase by reactive oxygen species. *Br J Pharmacol*. 2007;150(8):1084-1091. doi:10.1038/sj.bjp.0707179

37. Gheorghiade M, Greene SJ, Filippatos G, et al. Cinaciguat, a soluble guanylate cyclase activator: results from the randomized, controlled, phase IIb COMPOSE programme in acute heart failure syndromes. *Eur J Heart Fail*. 2012;14(9):1056-1066. doi:10.1093/eurjhf/hfs093

38. Korkmaz ME, Oto A, Saraclar Y, et al. Levels of IgE in the serum of patients with coronary arterial disease. *International Journal of Cardiology*. 1991;31(2):199-204. doi:10.1016/0167-5273(91)90216-C

39. Huang CW, Moore PK. H2S Synthesizing Enzymes: Biochemistry and Molecular Aspects. In: Moore PK, Whiteman M, eds. *Chemistry, Biochemistry and Pharmacology of Hydrogen Sulfide*. Handbook of Experimental Pharmacology. Cham: Springer International Publishing; 2015:3-25. doi:10.1007/978-3-319-18144-8_1

40. Yang G, Wu L, Jiang B, et al. H2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine γ-Lyase. *Science*. 2008;322(5901):587-590. doi:10.1126/science.1162667

41. Shibuya N, Tanaka M, Yoshida M, et al. 3-Mercaptopyruvate Sulfurtransferase Produces Hydrogen Sulfide and Bound Sulfane Sulfur in the Brain. *Antioxidants & Redox Signaling*. 2009;11(4):703-714. doi:10.1089/ars.2008.2253

42. Stipanuk MH. Metabolism of Sulfur-Containing Amino Acids. *Annual Review of Nutrition*. 1986;6(1):179-209. doi:10.1146/annurev.nu.06.070186.001143

43. Chen X, Jhee K-H, Kruger WD. Production of the Neuromodulator H2S by Cystathionine β-Synthase via the Condensation of Cysteine and Homocysteine. *J Biol Chem*. 2004;279(50):52082-52086. doi:10.1074/jbc.C400481200

44. Miles EW, Kraus JP. Cystathionine β-Synthase: Structure, Function, Regulation, and Location of Homocystinuria-causing Mutations. *J Biol Chem*. 2004;279(29):29871-29874. doi:10.1074/jbc.R400005200

45. Koutmos M, Kabil O, Smith JL, Banerjee R. Structural basis for substrate activation and regulation by cystathionine beta-synthase (CBS) domains in cystathionine {beta}-synthase. *Proc Natl Acad Sci USA*. 2010;107(49):20958-20963. doi:10.1073/pnas.1011448107

46. Meier M, Janosik M, Kery V, Kraus JP, Burkhard P. Structure of human cystathionine beta-synthase: a unique pyridoxal 5'-phosphate-dependent heme protein. *EMBO J*. 2001;20(15):3910-3916. doi:10.1093/emboj/20.15.3910

47. Taoka S, Lepore BW, Kabil O, Ojha S, Ringe D, Banerjee R. Human cystathionine beta-synthase is a heme sensor protein. Evidence that the redox sensor is heme and not the vicinal cysteines in the CXXC motif seen in the crystal structure of the truncated enzyme. *Biochemistry*. 2002;41(33):10454-10461. doi:10.1021/bi026052d

48. Puranik M, Weeks CL, Lahaye D, et al. Dynamics of Carbon Monoxide Binding to Cystathionine β-Synthase. *J Biol Chem*. 2006;281(19):13433-13438. doi:10.1074/jbc.M600246200

49. Taoka S, Banerjee R. Characterization of NO binding to human cystathionine β-synthase:: Possible implications of the effects of CO and NO binding to the human enzyme. *Journal of Inorganic Biochemistry*. 2001;87(4):245-251. doi:10.1016/S0162-0134(01)00335-X

50. Banerjee R, Zou C-G. Redox regulation and reaction mechanism of human cystathionine-beta-synthase: a PLP-dependent hemesensor protein. *Arch Biochem Biophys*. 2005;433(1):144-156. doi:10.1016/j.abb.2004.08.037

51. Finkelstein JD, Kyle WE, Martin JL, Pick AM. Activation of cystathionine synthase by adenosylmethionine and adenosylethionine. *Biochem Biophys Res Commun*. 1975;66(1):81-87. doi:10.1016/s0006-291x(75)80297-x

52. Sun Q, Collins R, Huang S, et al. Structural Basis for the Inhibition Mechanism of Human Cystathionine γ-Lyase, an Enzyme Responsible for the Production of H_2S. *J Biol Chem*. 2009;284(5):3076-3085. doi:10.1074/jbc.M805459200

53. Wang R. The gasotransmitter role of hydrogen sulfide. *Antioxid Redox Signal*. 2003;5(4):493-501. doi:10.1089/152308603768295249

54. Wang Y, Zhao X, Jin H, et al. Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol*. 2009;29(2):173-179. doi:10.1161/ATVBAHA.108.179333

55. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J*. 2001;20(21):6008-6016. doi:10.1093/emboj/20.21.6008

56. Fu M, Zhang W, Wu L, Yang G, Li H, Wang R. Hydrogen sulfide (H2S) metabolism in mitochondria and its regulatory role in energy production. *Proc Natl Acad Sci U S A*. 2012;109(8):2943-2948. doi:10.1073/pnas.1115634109

57. Mikami Y, Shibuya N, Ogasawara Y, Kimura H. Hydrogen sulfide is produced by cystathionine γ-lyase at the steady-state low intracellular Ca2+ concentrations. *Biochemical and Biophysical Research Communications*. 2013;431(2):131-135. doi:10.1016/j.bbrc.2013.01.010

58. Wu D, Hu Q, Tan B, Rose P, Zhu D, Zhu YZ. Amelioration of mitochondrial dysfunction in heart failure through S-sulfhydration of Ca2+/calmodulin-dependent protein kinase II. *Redox Biol*. 2018;19:250-262. doi:10.1016/j.redox.2018.08.008

59. Mikami Y, Shibuya N, Kimura Y, Nagahara N, Ogasawara Y, Kimura H. Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochem J*. 2011;439(3):479-485. doi:10.1042/BJ20110841

60. Nagahara N. Regulation of Mercaptopyruvate Sulfurtransferase Activity Via Intrasubunit and Intersubunit Redox-Sensing Switches. *Antioxidants & Redox Signaling*. 2012;19(15):1792-1802. doi:10.1089/ars.2012.5031

61. Yadav PK, Yamada K, Chiku T, Koutmos M, Banerjee R. Structure and Kinetic Analysis of H2S Production by Human Mercaptopyruvate Sulfurtransferase. *J Biol Chem*. 2013;288(27):20002-20013. doi:10.1074/jbc.M113.466177

62. Kimura H. Production and Physiological Effects of Hydrogen Sulfide. *Antioxid Redox Signal*. 2014;20(5):783-793. doi:10.1089/ars.2013.5309

63. Hartle MD, Pluth MD. A practical guide to working with H2S at the interface of chemistry and biology. *Chem Soc Rev*. 2016;45(22):6108-6117. doi:10.1039/C6CS00212A

64. Beauchamp RO, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA, Leber P. A Critical Review of the Literature on Hydrogen Sulfide Toxicity. *CRC Critical Reviews in Toxicology*. 1984;13(1):25-97. doi:10.3109/10408448409029321

65. Searcy DG. HS−:O2Oxidoreductase Activity of Cu,Zn Superoxide Dismutase. *Archives of Biochemistry and Biophysics*. 1996;334(1):50-58. doi:10.1006/abbi.1996.0428

66. Pitcher MCL, Beatty ER, Harris RM, Waring RH, Cummings JH. Sulfur Metabolism in Ulcerative Colitis (Investigation of Detoxification Enzymes in Peripheral Blood). *Dig Dis Sci*. 1998;43(9):2080-2085. doi:10.1023/A:1018867516575

67. Roediger WE, Babidge W, Millard S. Methionine derivatives diminish sulphide damage to colonocytes--implications for ulcerative colitis. *Gut*. 1996;39(1):77-81. doi:10.1136/gut.39.1.77

68. Roediger WEW, Moore J, Babidge W. Colonic Sulfide in Pathogenesis and Treatment of Ulcerative Colitis. *Dig Dis Sci*. 1997;42(8):1571-1579. doi:10.1023/A:1018851723920

69. Weisiger RA, Pinkus LM, Jakoby WB. Thiol S-methyltransferase: suggested role in detoxication of intestinal hydrogen sulfide. *Biochemical Pharmacology*. 1980;29(20):2885-2887. doi:10.1016/0006-2952(80)90029-5

70. Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *J Inherit Metab Dis*. 2011;34(1):75-81. doi:10.1007/s10545-010-9177-4

71. Kim-Shapiro DB, Gladwin MT. Chapter 6 - Heme Protein Metabolism of NO and Nitrite. In: Ignarro LJ, Freeman BA, eds. *Nitric Oxide (Third Edition)*. Academic Press; 2017:85-96. doi:10.1016/B978-0-12-804273-1.00006-5

72. Wang R. Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. 1998;76:15.

73. Whiteman M, Armstrong JS, Chu SH, et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite "scavenger"? *J Neurochem*. 2004;90(3):765-768. doi:10.1111/j.1471-4159.2004.02617.x

74. Whitfield NL, Kreimier EL, Verdial FC, Skovgaard N, Olson KR. Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(6):R1930-1937. doi:10.1152/ajpregu.00025.2008

75. Olson KR. Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2009;1787(7):856-863. doi:10.1016/j.bbabio.2009.03.019

76. Ahn YJ, Lee YJ, Lee J, Lee D, Park H-K, Lee G-J. Colorimetric detection of endogenous hydrogen sulfide production in living cells. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 2017;177:118-124. doi:10.1016/j.saa.2017.01.040

77. Heshka NE, Hager DB. A multidimensional gas chromatography method for the analysis of hydrogen sulfide in crude oil and crude oil headspace. *Journal of Separation Science*. 2014;37(24):3649-3655. doi:10.1002/jssc.201400727

78. Jarosz AP, Yep T, Mutus B. Microplate-Based Colorimetric Detection of Free Hydrogen Sulfide. *Anal Chem*. 2013;85(7):3638-3643. doi:10.1021/ac303543r

79. Moest RR. Hydrogen sulfide determination by the methylene blue method. *Anal Chem*. 1975;47(7):1204-1205. doi:10.1021/ac60357a008

80. Ni P, Sun Y, Dai H, et al. Colorimetric detection of sulfide ions in water samples based on the in situ formation of Ag2S nanoparticles. *Sensors and Actuators B: Chemical*. 2015;220:210-215. doi:10.1016/j.snb.2015.05.066

81. Rath HJ, Wimmer J. Gas chromatographic analysis of traces of hydrogen sulfide in hydrogen. *Chromatographia*. 1980;13(8):513-514. doi:10.1007/BF02261623

82. Karunya R, Jayaprakash KS, Gaikwad R, et al. Rapid measurement of hydrogen sulphide in human blood plasma using a microfluidic method. *Sci Rep*. 2019;9(1):3258. doi:10.1038/s41598-019-39389-7

83. Kolluru GK, Shen X, Bir SC, Kevil CG. Hydrogen sulfide chemical biology: Pathophysiological roles and detection. *Nitric Oxide*. 2013;35:5-20. doi:10.1016/j.niox.2013.07.002

84. Jeroschewski P, Steuckart C, Kühl M. An Amperometric Microsensor for the Determination of H2S in Aquatic Environments. *Anal Chem*. 1996;68(24):4351-4357. doi:10.1021/ac960091b

85. Hall JR, Schoenfisch MH. Direct Electrochemical Sensing of Hydrogen Sulfide without Sulfur Poisoning. *Anal Chem*. 2018;90(8):5194-5200. doi:10.1021/acs.analchem.7b05421

86. Gu K, Cowie CC, Harris MI. Diabetes and Decline in Heart Disease Mortality in US Adults. *JAMA*. 1999;281(14):1291-1297. doi:10.1001/jama.281.14.1291

87. Jain SK, Bull R, Rains JL, et al. Low Levels of Hydrogen Sulfide in the Blood of Diabetes Patients and Streptozotocin-Treated Rats Causes Vascular Inflammation? *Antioxid Redox Signal*. 2010;12(11):1333-1337. doi:10.1089/ars.2009.2956

88. Whiteman M, Gooding KM, Whatmore JL, et al. Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulphide. *Diabetologia*. 2010;53(8):1722-1726. doi:10.1007/s00125-010-1761-5

89. Peter EA, Shen X, Shah SH, et al. Plasma Free H2S Levels are Elevated in Patients With Cardiovascular Disease. *J Am Heart Assoc*. 2013;2(5). doi:10.1161/JAHA.113.000387

90. Hosoki R, Matsuki N, Kimura H. The Possible Role of Hydrogen Sulfide as an Endogenous Smooth Muscle Relaxant in Synergy with Nitric Oxide. *Biochemical and Biophysical Research Communications*. 1997;237(3):527-531. doi:10.1006/bbrc.1997.6878

91. Mustafa AK, Sikka G, Gazi SK, et al. Hydrogen Sulfide as Endothelial Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circ Res*. 2011;109(11):1259-1268. doi:10.1161/CIRCRESAHA.111.240242

92. Ozkor MA, Quyyumi AA. Endothelium-Derived Hyperpolarizing Factor and Vascular Function. *Cardiol Res Pract*. 2011;2011. doi:10.4061/2011/156146

93. Tang G, Yang G, Jiang B, Ju Y, Wu L, Wang R. H_2S is an endothelium-derived hyperpolarizing factor. *Antioxid Redox Signal*. 2013;19(14):1634-1646. doi:10.1089/ars.2012.4805

94. Bao L, Vlček Č, Pačes V, Kraus JP. Identification and Tissue Distribution of Human Cystathionine β-Synthase mRNA Isoforms. *Archives of Biochemistry and Biophysics*. 1998;350(1):95-103. doi:10.1006/abbi.1997.0486

95. Hellmich MR, Coletta C, Chao C, Szabo C. The Therapeutic Potential of Cystathionine β-Synthetase/Hydrogen Sulfide Inhibition in Cancer. *Antioxid Redox Signal*. 2015;22(5):424-448. doi:10.1089/ars.2014.5933

96. Asimakopoulou A, Panopoulos P, Chasapis CT, et al. Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). *Br J Pharmacol*. 2013;169(4):922-932. doi:10.1111/bph.12171

97. Saha S, Chakraborty PK, Xiong X, et al. Cystathionine β-synthase regulates endothelial function via protein S-sulfhydration. *FASEB J*. 2016;30(1):441-456. doi:10.1096/fj.15-278648

98. Shibuya N, Mikami Y, Kimura Y, Nagahara N, Kimura H. Vascular Endothelium Expresses 3-Mercaptopyruvate Sulfurtransferase and Produces Hydrogen Sulfide. *J Biochem*. 2009;146(5):623-626. doi:10.1093/jb/mvp111

99. Kimura H. Hydrogen sulfide: its production, release and functions. *Amino Acids*. 2011;41(1):113-121. doi:10.1007/s00726-010-0510-x

100. Coletta C, Módis K, Szczesny B, et al. Regulation of Vascular Tone, Angiogenesis and Cellular Bioenergetics by the 3-Mercaptopyruvate Sulfurtransferase/H2S Pathway: Functional Impairment by Hyperglycemia and Restoration by dl-α-Lipoic Acid. *Mol Med*. 2015;21(1):1-14. doi:10.2119/molmed.2015.00035

101. Mitidieri E, Tramontano T, Gurgone D, et al. Mercaptopyruvate acts as endogenous vasodilator independently of 3-mercaptopyruvate sulfurtransferase activity. *Nitric Oxide*. 2018;75:53-59. doi:10.1016/j.niox.2018.02.003

102. Holmes MV, Newcombe P, Hubacek JA, et al. Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. *Lancet*. 2011;378(9791):584-594. doi:10.1016/S0140-6736(11)60872-6

103. Moll Stephan, Varga Elizabeth A. Homocysteine and MTHFR Mutations. *Circulation*. 2015;132(1):e6-e9. doi:10.1161/CIRCULATIONAHA.114.013311

104. Dean L, Pratt V, McLeod H, Rubinstein W, Dean L, Kattman B, Malheiro A, Methylenetetrahydrofolate Reductase Deficiency eds. *Medical Genetics Summaries*. Bethesda (MD): National Center for Biotechnology Information (US); 2012.

105. Reference GH. CBS gene. Genetics Home Reference. https://ghr.nlm.nih.gov/gene/CBS. Accessed December 15, 2019.

106. Faeh D, Chiolero A, Paccaud F. Homocysteine as a risk factor for cardiovascular disease: should we (still) worry about? *Swiss Med Wkly*. 2006;136(47-48):745-756. doi:2006/47/smw-11283

107. Ansari R, Mahta A, Mallack E, Luo JJ. Hyperhomocysteinemia and Neurologic Disorders: a Review. *J Clin Neurol*. 2014;10(4):281-288. doi:10.3988/jcn.2014.10.4.281

108. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol*. 1969;56(1):111-128.

109. Ye Z, Zhang Q, Li Y, et al. High Prevalence of Hyperhomocysteinemia and Its Association with Target Organ Damage in Chinese Patients with Chronic Kidney Disease. *Nutrients*. 2016;8(10). doi:10.3390/nu8100645

110. Wierzbicki AS. Homocysteine and cardiovascular disease: a review of the evidence. *Diab Vasc Dis Res*. 2007;4(2):143-150. doi:10.3132/dvdr.2007.033

111. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ*. 2002;325(7374):1202. doi:10.1136/bmj.325.7374.1202

112. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. *Nutrition Journal*. 2015;14(1):6. doi:10.1186/1475-2891-14-6

113. Undas A, Brożek J, Szczeklik A. Homocysteine and thrombosis: from basic science to clinical evidence. *Thromb Haemost*. 2005;94(11):907-915. doi:10.1160/TH05-05-0313

114. Leoncini G, Pascale R, Signorello MG. Effects of homocysteine on l-arginine transport and nitric oxide formation in human platelets. *Eur J Clin Invest*. 2003;33(8):713-719. doi:10.1046/j.1365-2362.2003.01203.x

115. Makris M. Hyperhomocysteinemia and thrombosis. *Clinical & Laboratory Haematology*. 2000;22(3):133-143. doi:10.1046/j.1365-2257.2000.00301.x

116. Zagli G, Patacchini R, Trevisani M, et al. Hydrogen sulfide inhibits human platelet aggregation. *European Journal of Pharmacology*. 2007;559(1):65-68. doi:10.1016/j.ejphar.2006.12.011

117. d'Emmanuele di Villa Bianca R, Mitidieri E, Di Minno MND, et al. Hydrogen sulphide pathway contributes to the enhanced human platelet aggregation in hyperhomocysteinemia. *Proceedings of the National Academy of Sciences*. 2013;110(39):15812-15817. doi:10.1073/pnas.1309049110

118. Williams SR, Yang Q, Chen F, et al. Genome-Wide Meta-Analysis of Homocysteine and Methionine Metabolism Identifies Five One Carbon Metabolism Loci and a Novel Association of ALDH1L1 with Ischemic Stroke. Scheet P, ed. *PLoS Genet*. 2014;10(3):e1004214. doi:10.1371/journal.pgen.1004214

119. Eberhardt RT, Forgione MA, Cap A, et al. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. doi:10.1172/JCI8342

120. Beard RS, Bearden SE. Vascular complications of cystathionine β-synthase deficiency: future directions for homocysteine-to-hydrogen sulfide research. *Am J Physiol Heart Circ Physiol*. 2011;300(1):H13-H26. doi:10.1152/ajpheart.00598.2010

121. Tranzer JP, Baumgartner HR. Filling Gaps in the Vascular Endothelium with Blood Platelets. *Nature*. 1967;216(5120):1126-1128. doi:10.1038/2161126a0

122. Stühlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. *Circulation*. 2001;104(21):2569-2575. doi:10.1161/hc4601.098514

123. Austin RC, Lentz SR, Werstuck GH. Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. *Cell Death Differ*. 2004;11(1):S56-S64. doi:10.1038/sj.cdd.4401451

124. Kruger WD. Cystathionine β-synthase Deficiency: Of Mice and Men. *Mol Genet Metab*. 2017;121(3):199-205. doi:10.1016/j.ymgme.2017.05.011

125. Jiang X, Yang F, Tan H, et al. Hyperhomocystinemia Impairs Endothelial Function and eNOS Activity via PKC Activation. *Arterioscler Thromb Vasc Biol*. 2005;25(12):2515-2521. doi:10.1161/01.ATV.0000189559.87328.e4

126. Pushpakumar S, Kundu S, Sen U. Hydrogen Sulfide Protects Hyperhomocysteinemia-Induced Renal Damage by Modulation of Caveolin and eNOS Interaction. *Sci Rep*. 2019;9(1):1-13. doi:10.1038/s41598-018-38467-6

127. Lo Faro ML, Fox B, Whatmore JL, Winyard PG, Whiteman M. Hydrogen sulfide and nitric oxide interactions in inflammation. *Nitric Oxide*. 2014;41:38-47. doi:10.1016/j.niox.2014.05.014

128. Kubo S, Doe I, Kurokawa Y, Nishikawa H, Kawabata A. Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: Contribution to dual modulation of vascular tension. *Toxicology*. 2007;232(1):138-146. doi:10.1016/j.tox.2006.12.023

129. Bertova A, Cacanyiova S, Kristek F, Krizanova O, Ondrias K, Tomaskova Z. The hypothesis of the main role of H2S in coupled sulphide-nitroso signalling pathway. *Gen Physiol Biophys*. 2010;29(4):402-410. doi:10.4149/gpb_2010_04_402

130. Wu D, Hu Q, Zhu D. An Update on Hydrogen Sulfide and Nitric Oxide Interactions in the Cardiovascular System. *Oxid Med Cell Longev*. 2018;2018. doi:10.1155/2018/4579140

131. Coletta C, Papapetropoulos A, Erdelyi K, et al. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci U S A*. 2012;109(23):9161-9166. doi:10.1073/pnas.1202916109

132. Bucci M, Papapetropoulos A, Vellecco V, et al. Hydrogen Sulfide Is an Endogenous Inhibitor of Phosphodiesterase Activity. *ATVB*. 2010;30(10):1998-2004. doi:10.1161/ATVBAHA.110.209783

133. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399(6736):601-605. doi:10.1038/21224

134. Zhang Y, Tang Z-H, Ren Z, et al. Hydrogen Sulfide, the Next Potent Preventive and Therapeutic Agent in Aging and Age-Associated Diseases. *Mol Cell Biol*. 2013;33(6):1104-1113. doi:10.1128/MCB.01215-12

135. Arumugam TV, Kennedy BK. H2S to Mitigate Vascular Aging: A SIRT1 Connection. *Cell*. 2018;173(1):8-10. doi:10.1016/j.cell.2018.03.011

136. Xiong Y, Chang L, Tran B, et al. ZYZ-803, a novel hydrogen sulfide-nitric oxide conjugated donor, promotes angiogenesis via cross-talk between STAT3 and CaMKII. *Acta Pharmacol Sin*. July 2019:1-11. doi:10.1038/s41401-019-0255-3

137. Mattagajasingh I, Kim C-S, Naqvi A, et al. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*. 2007;104(37):14855-14860. doi:10.1073/pnas.0704329104

138. Das A, Huang GX, Bonkowski MS, et al. Impairment of an Endothelial NAD+-H2S Signaling Network Is a Reversible Cause of Vascular Aging. *Cell*. 2018;173(1):74-89.e20. doi:10.1016/j.cell.2018.02.008

139. Whiteman M, Li L, Kostetski I, et al. Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. *Biochemical and Biophysical Research Communications*. 2006;343(1):303-310. doi:10.1016/j.bbrc.2006.02.154

140. Liu T, Schroeder HJ, Wilson SM, et al. Local and Systemic Vasodilatory Effects of Low Molecular Weight S-nitrosothiols. *Free Radic Biol Med*. 2016;91:215-223. doi:10.1016/j.freeradbiomed.2015.12.009

141. Wang Y, Mainali P, Tang C, et al. Effects of nitric oxide and hydrogen sulfide on the relaxation of pulmonary arteries in rats. *Chin Med J*. 2008;121(5):420-423.

142. Yang G, Wu L, Jiang B, et al. H2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine g-Lyase. 2008;322:5.

143. Ishii I, Akahoshi N, Yamada H, Nakano S, Izumi T, Suematsu M. Cystathionine gamma-Lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury. *J Biol Chem*. 2010;285(34):26358-26368. doi:10.1074/jbc.M110.147439

144. HuQingxun, WuDan, MaFenfen, et al. Novel Angiogenic Activity and Molecular Mechanisms of ZYZ-803, a Slow-Releasing Hydrogen Sulfide–Nitric Oxide Hybrid Molecule. *Antioxidants & Redox Signaling*. September 2016. doi:10.1089/ars.2015.6607

145. Wu D, Hu Q, Ma F, Zhu YZ. Vasorelaxant Effect of a New Hydrogen Sulfide-Nitric Oxide Conjugated Donor in Isolated Rat Aortic Rings through cGMP Pathway. *Oxid Med Cell Longev*. 2016;2016. doi:10.1155/2016/7075682

146. Zhang Y, Huang Y, Cantalupo A, et al. Endothelial Nogo-B regulates sphingolipid biosynthesis to promote pathological cardiac hypertrophy during chronic pressure overload. *JCI Insight*. 1(5). doi:10.1172/jci.insight.85484

147. Sun J, Zhang X, Broderick M, Fein H. Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. *Sensors*. 2003;3(8):276-284. doi:10.3390/s30800276

148. Kanagy NL, Szabo C, Papapetropoulos A. Vascular biology of hydrogen sulfide. *Am J Physiol Cell Physiol*. 2017;312(5):C537-C549. doi:10.1152/ajpcell.00329.2016

149. Szabo C. Hydrogen sulfide, an enhancer of vascular nitric oxide signaling: mechanisms and implications. *American Journal of Physiology-Cell Physiology*. 2016;312(1):C3-C15. doi:10.1152/ajpcell.00282.2016

150. Pan LL, Liu XH, Gong QH, Yang HB, Zhu YZ. Role of Cystathionine γ-Lyase/Hydrogen Sulfide Pathway in Cardiovascular Disease: A Novel Therapeutic Strategy? *Antioxid Redox Signal*. 2012;17(1):106-118. doi:10.1089/ars.2011.4349

151. Jiang X-C, Goldberg IJ, Park T-S. Sphingolipids and Cardiovascular Diseases: Lipoprotein Metabolism, Atherosclerosis and Cardiomyopathy. In: Cowart LA, ed. *Sphingolipids and Metabolic Disease*. Advances in Experimental Medicine and Biology. New York, NY: Springer; 2011:19-39. doi:10.1007/978-1-4614-0650-1_2

152. Cantalupo A, Zhang Y, Kothiya M, et al. Nogo-B regulates endothelial sphingolipid homeostasis to control vascular function and blood pressure. *Nat Med*. 2015;21(9):1028-1037. doi:10.1038/nm.3934

153. Singleton PA, Dudek SM, Ma S-F, Garcia JGN. Transactivation of Sphingosine 1-Phosphate Receptors Is Essential for Vascular Barrier Regulation Novel Role For Hyaluronan And Cd44 Receptor Family. *J Biol Chem*. 2006;281(45):34381-34393. doi:10.1074/jbc.M603680200

154. Mishra RC, Tripathy S, Desai KM, et al. Nitric Oxide Synthase Inhibition Promotes Endothelium-Dependent Vasodilatation and the Antihypertensive Effect of l-Serine. *Hypertension*. 2008;51(3):791-6. doi: 10.1161/HYPERTENSIONAHA.107.099598.

155. Maralani MN, Movahedian A, Javanmard ShH. Antioxidant and cytoprotective effects of L-Serine on human endothelial cells. *Res Pharm Sci*. 2012;7(4):209-215.

156. Jafurulla M, Chattopadhyay A. Sphingolipids in the function of G protein-coupled receptors. *European Journal of Pharmacology*. 2015;763:241-246. doi:10.1016/j.ejphar.2015.07.019

157. Sheibani L, Lechuga TJ, Zhang H, et al. Augmented H2S production via cystathionine-beta-synthase upregulation plays a role in pregnancy-associated uterine vasodilation. *Biol Reprod*. 2017;96(3):664-672. doi:10.1095/biolreprod.116.143834

158. Bucci M, Papapetropoulos A, Vellecco V, et al. cGMP-Dependent Protein Kinase Contributes to Hydrogen Sulfide-Stimulated Vasorelaxation. *PLOS ONE*. 2012;7(12):e53319. doi:10.1371/journal.pone.0053319

159. Pushpakumar S, Kundu S, Sen U. Endothelial Dysfunction: The Link Between Homocysteine and Hydrogen Sulfide. *Curr Med Chem*. 2014;21(32):3662-3672.

160. Types of Allergic Diseases. https://web.archive.org/web/20150617123632/http://www.niaid.nih.gov/topics/allergicdiseases/Pages/allergic-diseases-types.aspx.

161. Valero A, Quirce S, Dávila I, Delgado J, Domínguez-Ortega J. Allergic respiratory disease: Different allergens, different symptoms. *Allergy*. 2017;72(9):1306-1316. doi:10.1111/all.13141

162. Immunoglobulin E (IgE) Defined | AAAAI. https://www.aaaai.org/conditions-and-treatments/conditions-dictionary/immunoglobulin-e-(ige).

163. Seifert R, Strasser A, Schneider EH, Neumann D, Dove S, Buschauer A. Molecular and cellular analysis of human histamine receptor subtypes.*Trends Pharmacol Sci*. 2013;34(1):33–58. doi:10.1016/j.tips.2012.11.001

164. Environmental Allergies: Symptoms. https://web.archive.org/web/20150618023408/http://www.niaid.nih.gov/topics/environmental-allergies/Pages/symptoms.aspx. Accessed January 23, 2020.

165. Delgado J, Dávila I, Domínguez-Ortega J, Quirce S, Martí-Guadaño E, Valero A. Quality of Life in Patients With Respiratory Allergy Is InÁuenced by the Causative Allergen. *J Investig Allergol Clin Immunol*. 2013;23:6.

166. Eifan AO, Keles S, Bahceciler NN, Barlan IB. Anaphylaxis to multiple pollen allergen sublingual immunotherapy. *Allergy*. 2007 May;62(5):567-8.0.

167. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454(7203):445–454. doi:10.1038/nature07204

168. Bahadori K, Doyle-Waters MM, Marra C, et al. Economic burden of asthma: a systematic review. *BMC Pulm Med*. 2009;9(1):24. doi:10.1186/1471-2466-9-24

169. Bergmann K, Sypniewska G. Is there an association of allergy and cardiovascular disease? *Biochem Med*. 2011;21(3):210-218. doi:10.11613/BM.2011.030

170. Mason JC, Libby P. Cardiovascular disease in patients with chronic inflammation: mechanisms underlying premature cardiovascular events in rheumatologic conditions. *European Heart Journal*. 2015;36(8):482-489. doi:10.1093/eurheartj/ehu403

171. Schroeder EB. Lung Function and Incident Coronary Heart Disease: The Atherosclerosis Risk in Communities Study. *American Journal of Epidemiology*. 2003;158(12):1171-1181. doi:10.1093/aje/kwg276

172. Engström G, Lind P, Hedblad B, et al. Lung Function and Cardiovascular Risk: Relationship With Inflammation-Sensitive Plasma Proteins. *Circulation*. 2002;106(20):2555-2560. doi:10.1161/01.CIR.0000037220.00065.0D

173. Knuiman MW, James AL, Divitini ML. Lung Function, Respiratory Symptoms, and Mortality: Results from the Busselton Health Study. 9(5):10.

174. James AL, Knuiman M w, Divitini M l, Musk AW, Ryan G, Bartholomew H c. Associations between white blood cell count, lung function, respiratory illness and mortality: the Busselton Health Study. *Eur Respir J*. 1999;13(5):1115. doi:10.1034/j.1399-3003.1999.13e29.x

175. Schu¨nemann HJ, Dorn J, Grant BJB, Winkelstein W, Trevisan M. Pulmonary Function Is a Long-term Predictor of Mortality in the General Population. *Chest*. 2000;118(3):656-664. doi:10.1378/chest.118.3.656

176. Hole DJ, Watt GCM, Davey-Smith G, Hart CL, Gillis CR, Hawthorne VM. Impaired lung function and mortality risk in men and women: findings from the Renfrew and Paisley prospective population study. *BMJ*. 1996;313(7059):711-715. doi:10.1136/bmj.313.7059.711

177. Song Y, Hou J, Huang X, et al. The Wuhan-Zhuhai (WHZH) cohort study of environmental air particulate matter and the pathogenesis of cardiopulmonary diseases: study design, methods and baseline characteristics of the cohort. *BMC Public Health*. 2014;14(1):994. doi:10.1186/1471-2458-14-994

178. Baines KJ, Backer V, Gibson PG, Powel H, Porsbjerg CM. Impaired lung function is associated with systemic inflammation and macrophage activation. *Eur Respir J*. 2015;45(2):557-559. doi:10.1183/09031936.00187514

179. van Schayck CP, Chavannes NH. Detection of asthma and chronic obstructive pulmonary disease in primary care. *European Respiratory Journal*. 2003;21(Supplement 39):16S - 22s. doi:10.1183/09031936.03.00040403

180. Goldie RG, Pedersen KE. Mechanisms Of Increased Airway Microvascular Permeability: Role In Airway Inflammation And Obstruction. *Clin Exp*

Pharmacol Physiol. 1995;22(6-7):387-396. doi:10.1111/j.1440-1681.1995.tb02028.x

181. Kido T, Tamagawa E, Bai N, et al. Particulate Matter Induces Translocation of IL-6 from the Lung to the Systemic Circulation. *Am J Respir Cell Mol Biol*. 2011;44(2):197-204. doi:10.1165/rcmb.2009-0427OC

182. Borish L. Allergic rhinitis: Systemic inflammation and implications for management. *J ALLERGY CLIN IMMUNOL*. 2003;112(6):11.

183. Agusti À, Soriano JB. COPD as a Systemic Disease. *COPD: Journal of Chronic Obstructive Pulmonary Disease*. 2008;5(2):133-138. doi:10.1080/15412550801941349

184. Polverino F, Celli BR, Owen CA. COPD as an endothelial disorder: endothelial injury linking lesions in the lungs and other organs? (2017 Grover Conference Series). *Pulm Circ*. 2018;8(1):204589401875852. doi:10.1177/2045894018758528

185. Clarenbach CF, Senn O, Sievi NA, et al. Determinants of endothelial function in patients with COPD. *Eur Respir J*. 2013;42(5):1194-1204. doi:10.1183/09031936.00144612

186. Clarenbach CF, Sievi NA, Kohler M. Annual progression of endothelial dysfunction in patients with COPD. *Respiratory Medicine*. 2017;132:15-20. doi:10.1016/j.rmed.2017.09.005

187. Makieieva N, Odinets Y, Vasylchenko Y, Biriukova M. Prognosing a severe course of asthma in children following the study of endothelial function. *Pneumologia*. 2019;68(2):68-74. doi:10.2478/pneum-2019-0014

188. Augusto LS, Silva GC, Pinho JF, et al. Vascular function in asthmatic children and adolescents. *Respir Res*. 2017;18(1):17. doi:10.1186/s12931-016-0488-3

189. Yildiz P, Oflaz H, Cine N, et al. Endothelial Dysfunction in Patients with Asthma: The Role of Polymorphisms of ACE and Endothelial NOS Genes. *Journal of Asthma*. 2004;41(2):159-166. doi:10.1081/JAS-120026073

190. G B, R C, I D. Endothelial Functionality and Echocardiographic Parameters in Patients with Rhinitis and Nasal Polyps. *Arch Med*. 2016;08(06). doi:10.21767/1989-5216.1000179

191. Strand LB, Tsai MK, Wen CP, Chang S-S, Brumpton BM. Is having asthma associated with an increased risk of dying from cardiovascular disease? A prospective cohort study of 446 346 Taiwanese adults. *BMJ Open*. 2018;8(5):e019992. doi:10.1136/bmjopen-2017-019992

192. Onufrak S, Abramson J, Vaccarino V. Adult-onset asthma is associated with increased carotid atherosclerosis among women in the atherosclerosis risk in communities (ARIC) study. *Atherosclerosis*. 2007;195(1):129-137. doi:10.1016/j.atherosclerosis.2006.09.004

193. Onufrak SJ, Abramson JL, Austin HD, Holguin F, McClellan WM, Vaccarino LV. Relation of Adult-Onset Asthma to Coronary Heart Disease and Stroke. *The American Journal of Cardiology*. 2008;101(9):1247-1252. doi:10.1016/j.amjcard.2007.12.024

194. Lee HM, Truong ST, Wong ND. Association of adult-onset asthma with specific cardiovascular conditions. *Respiratory Medicine*. 2012;106(7):948-953. doi:10.1016/j.rmed.2012.02.017

195. Tattersall MC, Guo M, Korcarz CE, et al. Asthma Predicts Cardiovascular Disease Events: The Multi-Ethnic Study of Atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2015;35(6):1520-1525. doi:10.1161/ATVBAHA.115.305452

196. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med*. 2012;18(5):716-725. doi:10.1038/nm.2678

197. Iribarren C. Are patients with asthma at increased risk of coronary heart disease? *International Journal of Epidemiology*. 2004;33(4):743-748. doi:10.1093/ije/dyh081

198. Iribarren C, Tolstykh IV, Miller MK, Sobel E, Eisner MD. Adult Asthma and Risk of Coronary Heart Disease, Cerebrovascular Disease, and Heart Failure: A Prospective Study of 2 Matched Cohorts. *American Journal of Epidemiology*. 2012;176(11):1014-1024. doi:10.1093/aje/kws181

199. Kim J, Purushottam B, Chae YK, Chebrolu L, Amanullah A. Relation Between Common Allergic Symptoms and Coronary Heart Disease Among NHANES III Participants. *The American Journal of Cardiology*. 2010;106(7):984-987. doi:10.1016/j.amjcard.2010.05.029

200. Vijayakumar J, Subramanian S, Singh P, et al. Arterial inflammation in bronchial asthma. *J Nucl Cardiol*. 2013;20(3):385-395. doi:10.1007/s12350-013-9697-z

201. Elcioglu OC, Afsar B, Bakan A, et al. Chronic Rhinosinusitis, Endothelial Dysfunction, and Atherosclerosis. *Am J Rhinol Allergy*. 2016;30(3):e58-e61. doi:10.2500/ajra.2016.30.4325

202. Knoflach M, Kiechl S, Mayr A, Willeit J, Poewe W, Wick G. Allergic Rhinitis, Asthma, and Atherosclerosis in the Bruneck and ARMY Studies. *Arch Intern Med*. 2005;165(21):2521. doi:10.1001/archinte.165.21.2521

203. Schanen JG. Asthma and incident cardiovascular disease: the Atherosclerosis Risk in Communities Study. *Thorax*. 2005;60(8):633-638. doi:10.1136/thx.2004.026484

204. Yoon AMC, Chiu V, Rana JS, Sheikh J. Association of allergic rhinitis, coronary heart disease, cerebrovascular disease, and all-cause mortality. *Annals of Allergy, Asthma & Immunology*. 2016;117(4):359-364.e1. doi:10.1016/j.anai.2016.08.021

205. Tseng C-H, Chen J-H, Lin C-L, Kao C-H. Risk relation between rhinitis and acute ischemic stroke. *Allergy Asthma Proc*. 2015;36(6):e106-112. doi:10.2500/aap.2015.36.3891

206. Wu D, Zeng Y, Fan Y, et al. Reverse-migrated neutrophils regulated by JAM-C are involved in acute pancreatitis-associated lung injury. *Sci Rep*. 2016;6. doi:10.1038/srep20545

207. Kanazawa H, Nomura S, Asai K. Roles of Angiopoietin-1 and Angiopoietin-2 on Airway Microvascular Permeability in Asthmatic Patients. *Chest*. 2007;131(4):1035-1041. doi:10.1378/chest.06-2758

208. Dauphinee S, Karsan A. *Endothelial Dysfunction and Inflammation*. Springer; 2010.

209. Peinado VI, Barberà JA, Ramírez J, et al. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 1998;274(6):L908-L913. doi:10.1152/ajplung.1998.274.6.L908

210. Dinh-Xuan AT, Pepke-Zaba J, Butt AY, Cremona G, Higenbottam TW. Impairment of pulmonary-artery endothelium-dependent relaxation in chronic obstructive lung disease is not due to dysfunction of endothelial cell membrane receptors nor to L-arginine deficiency. *Br J Pharmacol*. 1993;109(2):587-591.

211. Wanner A, Mendes ES. Airway Endothelial Dysfunction in Asthma and Chronic Obstructive Pulmonary Disease: A Challenge for Future Research. *Am J Respir Crit Care Med*. 2010;182(11):1344-1351. doi:10.1164/rccm.201001-0038PP

212. Togias A. Systemic effects of local allergic disease. *Journal of Allergy and Clinical Immunology*. 2004;113(1):S8-S14. doi:10.1016/j.jaci.2003.09.051

213. Togias A. Rhinitis and asthma: Evidence for respiratory system integration. *Journal of Allergy and Clinical Immunology*. 2003;111(6):1171-1183. doi:10.1067/mai.2003.1592

214. Hazarika S, Van Scott MR, Lust RM, Wingard CJ. Pulmonary allergic reactions impair systemic vascular relaxation in ragweed sensitive mice. *Vascular Pharmacology*. 2010;53(5-6):258-263. doi:10.1016/j.vph.2010.09.005

215. Sahiner UM, Birben E, Erzurum S, Sackesen C, Kalayci O. Oxidative Stress in Asthma. *World Allergy Organ J*. 2011;4(10):151-158. doi:10.1097/WOX.0b013e318232389e

216. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med*. 1996;154(4):1055-1060. doi:10.1164/ajrccm.154.4.8887607

217. Rupa P, Mine Y. Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in Escherichia coli. *Biotechnology Letters*. 2003;25(22):1917-1924. doi:10.1023/B:BILE.0000003987.60659.ea

218. Al-Harbi NO, Nadeem A, Al-Harbi MM, et al. Oxidative airway inflammation leads to systemic and vascular oxidative stress in a murine model of allergic asthma. *International Immunopharmacology*. 2015;26(1):237-245. doi:10.1016/j.intimp.2015.03.032

219. Ricciardolo FLM. Multiple roles of nitric oxide in the airways. *Thorax*. 2003;58(2):175-182. doi:10.1136/thorax.58.2.175

220. Lubos E, Handy DE, Loscalzo J. Role of oxidative stress and nitric oxide in atherothrombosis. *Front Biosci*. 2008;13:5323-5344.

221. Ricciardolo FLM, Timmersa MC, Geppetti P, et al. Allergen-induced impairment of bronchoprotective nitric oxide synthesis in asthma. *Journal of Allergy and Clinical Immunology*. 2001;108(2):198-204. doi:10.1067/mai.2001.116572

222. Gueders MM, Paulissen G, Crahay C, et al. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res*. 2009;58(12):845-854. doi:10.1007/s00011-009-0054-2

223. Kumar R, Herbert C, Foster P. The Classical Ovalbumin Challenge Model of Asthma in Mice. *CDT*. 2008;9(6):485-494. doi:10.2174/138945008784533561

224. Baldacci S, Omenaas E, Oryszczyn MP. Allergy markers in respiratory epidemiology. *European Respiratory Journal*. 2001;17(4):773-790.

225. Castañeda AR, Bein KJ, Smiley-Jewell S, Pinkerton KE. Fine particulate matter ($PM_{2.5}$) enhances allergic sensitization in BALB/ *c* mice. *Journal of Toxicology and Environmental Health, Part A*. 2017;80(4):197-207. doi:10.1080/15287394.2016.1222920

226. del Campo L, Ferrer M. Wire Myography to Study Vascular Tone and Vascular Structure of Isolated Mouse Arteries. *Methods Mol Biol*. 2015;1339:255-276. doi:10.1007/978-1-4939-2929-0_18

227. Friederich JA, Butterworth JF. Sodium Nitroprusside: Twenty Years and Counting. *Anesthesia & Analgesia*. 1995;81(1):152-162.

228. Zetterström O, Johansson SGO. IgE Concentrations Measured by PRIST® in Serum of Healthy Adults and in Patients with Respiratory Allergy. *Allergy*. 1981;36(8):537-547. doi:10.1111/j.1398-9995.1981.tb01871.x

229. Brunekreef B, Hoek G, Fischer P, Spieksma FTM. Relation between airborne pollen concentrations and daily cardiovascular and respiratory-disease mortality. *The Lancet*. 2000;355(9214):1517-1518. doi:10.1016/S0140-6736(00)02168-1

230. Ponnoth DS, Nadeem A, Mustafa SJ. Adenosine-mediated alteration of vascular reactivity and inflammation in a murine model of asthma. *American Journal of Physiology-Heart and Circulatory Physiology*. 2008;294(5):H2158-H2165. doi:10.1152/ajpheart.01224.2007

231. Kodali Ravindra B., Kim William J.H., Galaria Irfan I., et al. CCL11 (Eotaxin) Induces CCR3-Dependent Smooth Muscle Cell Migration. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2004;24(7):1211-1216. doi:10.1161/01.ATV.0000131654.90788.f5

232. Braunersreuther Vincent, Zernecke Alma, Arnaud Claire, et al. Ccr5 But Not Ccr1 Deficiency Reduces Development of Diet-Induced Atherosclerosis in Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2007;27(2):373-379. doi:10.1161/01.ATV.0000253886.44609.ae

233. Félétou M, Huang Y, Vanhoutte PM. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *Br J Pharmacol*. 2011;164(3):894-912. doi:10.1111/j.1476-5381.2011.01276.x

234. Chen Q, Chen H, Zheng D, et al. Smad7 Is Required for the Development and Function of the Heart. *J Biol Chem*. 2009;284(1):292-300. doi:10.1074/jbc.M807233200

235. Zou L, Cao S, Kang N, Huebert RC, Shah VH. Fibronectin Induces Endothelial Cell Migration through β1 Integrin and Src-dependent Phosphorylation of Fibroblast Growth Factor Receptor-1 at Tyrosines 653/654 and 766. *J Biol Chem*. 2012;287(10):7190-7202. doi:10.1074/jbc.M111.304972

236. Förstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch - Eur J Physiol*. 2010;459(6):923-939. doi:10.1007/s00424-010-0808-2

237. Gavazzi Gaetan, Deffert Christine, Trocme Candice, Schäppi Michela, Herrmann François R., Krause Karl-Heinz. NOX1 Deficiency Protects From Aortic Dissection in Response to Angiotensin II. *Hypertension*. 2007;50(1):189-196. doi:10.1161/HYPERTENSIONAHA.107.089706

238. Craige SM, Kant S, Reif M, et al. Endothelial NADPH oxidase 4 protects ApoE-/- mice from atherosclerotic lesions. *Free Radical Biology and Medicine*. 2015;89:1-7. doi:10.1016/j.freeradbiomed.2015.07.004

239. Wouters EFM, Reynaert NL, Dentener MA, Vernooy JHJ. Systemic and Local Inflammation in Asthma and Chronic Obstructive Pulmonary Disease: Is There a Connection? *Proceedings of the American Thoracic Society*. 2009;6(8):638-647. doi:10.1513/pats.200907-073DP

240. Heiss C, Rodriguez-Mateos A, Kelm M. Central Role of eNOS in the Maintenance of Endothelial Homeostasis. *Antioxidants & Redox Signaling*. 2015;22(14):1230-1242. doi:10.1089/ars.2014.6158

241. Kawashima S, Yokoyama M. Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis. *ATVB*. 2004;24(6):998-1005. doi:10.1161/01.ATV.0000125114.88079.96

242. Lassègue B, Sorescu D, Szöcs K, et al. Novel gp91 ^{phox} Homologues in Vascular Smooth Muscle Cells: nox1 Mediates Angiotensin II–Induced Superoxide Formation and Redox-Sensitive Signaling Pathways. *Circulation Research*. 2001;88(9):888-894. doi:10.1161/hh0901.090299

243. Paravicini TM, Chrissobolis S, Drummond GR, Sobey CG. Increased NADPH-Oxidase Activity and Nox4 Expression During Chronic Hypertension Is Associated With Enhanced Cerebral Vasodilatation to NADPH In Vivo. *Stroke*. 2004;35(2):584-589. doi:10.1161/01.STR.0000112974.37028.58

244. Schröder K, Zhang M, Benkhoff S, et al. Nox4 Is a Protective Reactive Oxygen Species Generating Vascular NADPH Oxidase. *Circ Res*. 2012;110(9):1217-1225. doi:10.1161/CIRCRESAHA.112.267054

245. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling*. 2014;20(7):1126-1167. doi:10.1089/ars.2012.5149